MOLECULAR ANALYSIS OF THE BCR-ABL TRANSLOCATION IN CHRONIC MYELOID LEUKAEMIA

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

by

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DECLARATION OF ORIGINALITY

This thesis is the result of my own work. The work was carried out between January 1992 and May 1996 in the LRF Centre for Adult Leukaemia at the Royal Postgraduate Medical School in London. Apart from the karyotypic analyses described in Chapter 7, which were performed by Mr. Andrew Chase and the RT-PCR for analysis of minimal residual disease described in Chapter 7, which was performed by Mr. Feng Lin, all other technical work was performed by myself.

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ABSTRACT

Chronic myeloid leukaemia (CML) is characterised by a reciprocal t(9;22) translocation, that results in the formation of the Philadelphia chromosome. At the molecular level, a chimaeric BCR-ABL fusion gene is generated that encodes a p210 BCR-ABL tyrosine kinase protein that is thought to be primarily involved in the pathogenesis of the disease. CML can be cured by allogeneic bone marrow transplantation (BMT) in the majority of cases. Cure is not achieved solely by high dose chemoradiotherapy but also involves elimination or lifelong suppression of the leukaemic clone by a graft versus leukaemia effect. However, relapse after transplantation is still a problem.

In order to understand better the mechanism underlying the translocation between the BCR and ABL genes in CML, I have exploited a bubble polymerase chain reaction (PCR) technique to clone genomic breakpoints. BCR-ABL junction fragments were successfully amplified and sequenced in 17/42 (40%) patients tested. Breakpoints were dispersed throughout the major breakpoint cluster region (M-BCR) of the BCR gene without any subclustering or hot spots. In three cases Alu sequences were found at or near the breakpoint on the ABL side of the translocation but no other obvious sequence homologies were found either in BCR or ABL. The translocation event was characterised further in three patients by amplifying the reciprocal ABL-BCR junction on the 9q+ chromosome and also normal ABL sequences around the breakpoints. In two of these patients a few nucleotides of BCR and ABL were either duplicated or deleted on translocation suggesting that staggered cuts had been made in the DNA strands prior to recombination. In the third patient 50 bp of ABL was deleted and 159 bp of M-BCR including exon b3 was duplicated, indicating either that the single stranded cuts may span a larger distance than previously thought or that another mechanism, perhaps involving gene conversion, may be involved.

Analysis of all known BCR-ABL breakpoints indicated a significant clustering in the vicinity of ABL exon a2. Of 24 breakpoints that could be localised within the ABL gene, 13 (54%) fell within an 31 kb region. To determine if there reproducible was a subclustering of breakpoints in this region, I performed Southern blot analysis on 64 CML patients using two novel probes. The results did not indicate a clustering of breakpoints in the vicinity of ABL exon 2 and possible reasons for the discrepancy between the sequence and Southern analyses are discussed.

The vast majority of patients in remission after allogeneic BMT have no evidence of residual CML cells when their blood or bone marrow is studied by reverse transcription PCR (RT-PCR). However, it has been suggested that some patients might still harbour a pool of CML cells that do not express BCR-ABL mRNA and could not therefore be detected by RT-PCR. I therefore compared the results and sensitivity of amplification of BCR-ABL from genomic DNA with the results from conventional RT-PCR and cytogenetics. Bubble PCR was used to amplify the genomic BCR-ABL translocation breakpoints from chronic phase DNA from 10 CML patients who subsequently underwent BMT. After cloning and sequencing of the amplification products, patient-specific ABL primers were synthesized and tested for both specificity and sensitivity in nested, or hemi-nested, combinations with a variety of primers derived from the major breakpoint cluster region of the BCR gene. Patterns of residual disease obtained by serial RT-PCR and DNA-PCR analysis of blood and bone marrow samples obtained after BMT were similar for most patients, including one treated for relapse by infusion of donor leukocytes. Of the 24 samples for direct comparison of RT-PCR and DNA-PCR, results were concordant in 19 (79%) of cases. Since the great majority of samples (79%) gave concordant results with the two assays, I concluded that patients in remission do not generally harbour a substantial pool of CML cells that do not express BCR-ABL mRNA.

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ABBREVIATION

ALL	Acute lymphoid leukaemia
AML	Acute myeloid leukaemia
A-MuLV	Abelson murine leukaemia virus
APL	Acute promyelocytic leukemia
ATP	Adenosine 5'-triphosphate
dATP	Deoxyadenosine 5'-triphosphate
b2a2	BCR-ABL mRNA with a junction where the M-BCR exon 2 is spliced to ABL exon 2
b3a2	BCR-ABL mRNA with a junction between the M-BCR exon 3 and ABL exon 2
BC	Blast crisis
BM	Bone marrow
BMT	Bone marrow transplantation
BSA	Bovine serum albumin
BV173	Human leukemia cell line
cDNA	Complementary DNA
CML	Chronic myeloid leukaemia
CMML	Chronic myelomonocytic Leukaemia
°C	Degree centigrade
CFU-GM	Granulocyte macrophage colony forming unit
CIAP	Calf intestinal alkaline phosphatase
CP	Chronic phase
CTP	Cytidine 5'-triphosphate
dCTP	Deoxycytidine 5'-triphosphate
DEPC	Diethyl pyrocarbonate
dGTP	Deoxyguanosine 5'-triphosphate
DNA	Deoxyribonucleic acid

dGTP	Deoxyguanosine triphosphate
dNTP	Deoxynucleoside 5'-triphosphate
DSB	Double-strand breaks
DTT	Dithiothreitol
dTTP	Deoxythymidine 5'-triphosphate
EDTA	Ethylenediaminetetra-acetic acid
ERK	Extracellular signal-regulated kinase
FISH	Fluorescence *in-situ* hybridisation
FNR	False negative rate
FPR	False positive rate
g	gram
GAP	GTPase-activating protein
GTP	Guanosine triphosphate
GVL	Graft versus leukemia
Gy	Grays (unit of radiation)
JAK	Janus family kinases
JNK	Jun N-terminal kinase
K562	Human leukemia cell line
kb	Kilobase
kD	Kilodalton
MAPK	Mitogen-activated protein kinase
M-BCR	Major breakpoint cluster region in BCR gene
m-BCR	Minor breakpoint cluster region in BCR gene
mbr	Major breakpoint cluster region in BCL2 gene
MDS	Myelodysplastic syndrome
MER elements	Medium reiteration frequency interspersed repetitive elements
mg	Milligram
µg	Microgram
min	Minute

ml	Millilitre
MLL	Mixed lineage leukaemia
µl	Microlitre
mM	Millimolar
M-MuLV	Moloney murine leukaemia virus
MRD	Minimal residual disease
mRNA	Messenger RNA
ng	Nanogram
NIH3T3	Mouse fibroblast cell line
Oligo	Oligonucleotide
^{32}P	An isotope of phosphate
PB	Peripheral blood
PEG	polyethylene glycol
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
Ph	Philadelphia
PSR	Putative subcluster region
RARA	Retinoic acid receptor alpha
RNA	Ribonucleic acid
RT-PCR	Reverse transcription PCR
TEMED	N,N,N', N'-tetramethylethylenediamine
SAR	Scaffold attachment region
SDS	Sodium dodecyl sulphate
SSC	150 mM NaCl and 15 mM citric acid
STAT	Signal transducers and activators of transcription
t(9;22)	Reciprocal translocation between chromosomes 9 and 22
TAE	Tris-acetate-EDTA
tAML	Therapy related AML
TBE	890 mM Tris-borate

TdT	Terminal deoxynucleotidyl transferase
TE	1 mM EDTA and 10 mM Tris
TFO	Triplex-forming oligonucleotide
TTP	Thymidine 5'-triphosphate
UV	Ultraviolet
v-abl	Transforming sequences of mouse origin in A-MuLV
WBC	White blood cell

CHAPTER ONE CHRONIC MYELOID LEUKAEMIA

1.1. Epidemiology

Chronic myeloid leukaemia (CML) accounts for about 20-30% of adult leukaemias (Koeffler & Golde, 1981) but only 1 - 5% of childhood cases (Fioretos *et al* 1992; Altman, 1988). CML has an incidence of approximately one per 100,000 persons per year (Koeffler & Golde, 1981). This incidence is consistent world-wide with very little international variation. Although CML can occur at any age, the incidence rises linearly with age. There is a slight preponderance of males amongst CML cases (Groves *et al* 1995).

1.2. Aetiology

CML was first recognised as a clinical entity in the last century (Goldman & Gordon, 1996). However, the aetiology of the disease is not fully understood. Some evidence has suggested that environmental factors play a role in human leukaemogenesis. Exposure to magnetic fields of the type generated by high-voltage power lines increases the incidence of leukaemia, including chronic myeloid leukaemia (Feychting & Ahlbom, 1994). There is clear evidence linking exposure to ionising radiation and development of all subtypes of leukaemia, except perhaps adult T-cell leukaemia (Preston *et al* 1994). Toxic exposure to chemicals such as benzene has been predominantly linked to cases of acute myeloid leukaemias rather than CML or Ph positive ALL (Infante *et al* 1977; Rinsky *et al*, 1981; Ciccone *et al* 1993).

Some reports (Rubin *et al* 1990; Nandakumar *et al* 1991; Haubenstock *et al* 1985; Curtis *et al* 1989; Jackson *et al* 1994; and Aguiar *et al* 1994) have indicated that patients treated with chemotherapy for ovarian and breast cancer or lymphomas or other

unrelated cases can develop a wide spectrum of leukaemias, including CML. This is particularly true when alkylating agents, together with radiation or steroid therapy are used. Therefore, chemotherapy, radiotherapy and chronic immuno-suppressive therapy, alone or in combination, disrupt the system of normal haemopoiesis, and can lead to the development of secondary CML.

The presence and expression of human endogenous retrovirus from patients with CML has been reported previously (Brodsky *et al* 1993). Nevertheless, no direct evidence of a viral aetiology of CML has been found so far.

In most cases of CML there are no clear predisposing factors. There is no evidence to suggest that there is a genetic predisposition. However, a report was presented that BCR-ABL mRNA, usually found in CML patients, exists in a proportion of healthy individuals (Biernaux *et al* 1995).

1.3. Clinical and haematological features

CML is a paradigm of tumour evolution (Goldman & Lu, 1982). The disease begins with a chronic phase characterised by marked hyperplasia of myeloid cells which maintain their maturation capacity and is usually easily controlled with therapy (Champlin *et al* 1985). The chronic phase can last for several years, but eventually either changes suddenly to the acute or blastic phase or may evolves more gradually via an accelerated phase. In accelerated phase, the myeloid cells gradually lose their capacity for terminal differentiation, frequently accompanied by basophilia, thrombocytosis, and cytogenetic clonal evolution. Blastic crisis is characterised by a high proportion of blasts in the bone marrow and peripheral blood: this phase is usually highly resistant to therapy (Fialkow *et al* 1981).

The clinical onset of the chronic phase is generally insidious. Accordingly, some patients are diagnosed while still asymptomatic during health screening tests, Other patients present with fatigue, malaise, and weight loss, night sweats, abdominal fullness, or have symptoms resulting from splenic enlargement (Koeffler & Golde, 1981). Less common are features related to granlocyte or platelet dysfunction, such as infections, thrombosis, or bleeding. Occasionally patients present with leukostatic manifestations due to severe leukocytosis (Hild & Meyers, 1980) or thrombocytosis (Schafer, 1984) such as vaso-occlusive disease, cerebrovascular accidents, infarction, venous thrombosis, priapism, visual disturbances and pulmonary insufficiency (Suri *et al* 1980).

Elevated white blood cell count (WBCs) with various degrees of immaturity of the granulocytic series are observed in chronic phase. The peripheral blood leukocyte count is typically elevated to between 25 and 500 x 10^9/litre. If untreated, the white blood cell count tends to rise over the course of time. However, cyclic variations have also been reported (Morley *et al* 1967). Such cycles are usually 50 to 70 days long, probably reflecting a periodicity in granulocyte production. Usually there are less than 5% peripheral blasts. Myeloblasts and promyelocytes generally account for less than 10% of the cells. The neutrophils are characterised by a low cytoplasmic alkaline phosphatase content (Bennett & Rutenburg, 1966). A modest absolute monocytosis is common at diagnosis (Solal Celigny *et al* 1984). Basophilia and eosinophilia are present (Spiers *et al* 1977; Koide & Watanabe, 1979). The degree of basophilia is inversely related to prognosis: if the percentage of basophils in the peripheral differential exceeds 20%, a blast crisis is imminent (Denburg *et al* 1982). A progressive increase in eosinophils may also be a poor prognostic sign, heralding the emergence of an accelerated course (Theologides, 1972). Circulating normoblasts may be present. Thrombocytosis (platelet count greater than 450 x 10^9/L) occurs in 50% of patients at presentation, with an additional 15% developing elevated platelet counts during the course of the disease (Mason *et al* 1974). Levels greater than 100,000 x 10^9/L are noted

in about 25% of patients with CML (Mason *et al* 1974). Thrombocytosis, probably reflective of an increased disease burden, is also inversely related to prognosis (Sokal *et al* 1984). Serum level of vitamin B12 and vitamin B12 binding proteins are generally elevated (Gilbert *et al*, 1969). Increased histamine production secondary to basophilia occurs in later stages, causing diarrhoea and flushing (Rosenthal *et al* 1977; Youman *et al* 1973; Denburg *et al* 1982).

The accelerated phase of CML may occur at any time during the natural history of the disease, although this phenomena generally is seen within 30 to 36 months after the initial diagnosis (Marks *et al* 1978). This phase, which develops in about one-half of patients, may be heralded by progressive splenomegaly, leukostasis, and a demonstration that patient has become refractory to previously effective chemotherapy. The peripheral blood may show erythroid abnormalities such as marked aniso- and poikilocytosis with circulating myeloblasts and a leukoerythroblastic blood picture. Basophils and eosinophils may increase and thrombocytosis may worsen. A bone marrow biopsy performed at this time is likely to show myelofibrosis; cytogenetic studies may reveal chromosomal abnormalities and aneuploidy (Kantarjian *et al* 1988). Features that have suggested entry into an accelerated phase (associated with a median survival of less than 18 months) have included peripheral blood or bone marrow blasts exceeding 15% of the nucleated differential, blasts and promyelocytes greater than 30% of the dfferential, or platelets less than 100 x 10^9/L; unexplained decrease in haemoglobin (less than 7 g/dl); and extramedullary disease (Kantarjian *et al* 1988).

Transformation to blast crisis is accompanied by typically fever, bone and joint pains, significant weight loss, bleeding, thrombosis, infections, anaemia and lack of responsiveness to chemotherapy. Blast crisis is defined by the presence of 30% or more blasts in the peripheral blood or bone marrow (Kantarjian *et al* 1987; Karanas & Silver, 1968). Blast cells can be classified as myeloid, lymphoid or undifferentiated, based on morphological, cytochemical and immunological features (Polli *et al* 1985; San Miguel

et al 1985). Approximately 70% of patients have blast cells with a myeloid phenotype (Peterson *et al* 1976). In 20 - 30% of patients blast cells are lymphoid, expressing B-lymphoblast or pre-B markers and in rare cases blast cells express markers associated with T-lymphocytes (Kantarjian *et al* 1987; Jacobs & Greaves, 1984; Greaves, 1982). Occasionally megakaryoblastic membrane markers are expressed on the blast cells (Bain *et al* 1977). Rarely, patients develop a localised extramedullary transformation of CML also known as chloroma or granulocytic sarcoma with deposits of acute leukaemia cells in the lymphnodes, skin, subcutaneous tissue, liver, spleen, bone, meninges or pleural cavity (Woodson *et al* 1974; Inverardi *et al* 1990; Ohyashiki *et al* 1985; Beguin *et al* 1989; Carlsen *et al* 1990). At blast crisis, progressive anaemia and thrombocytopenia occur, with death usually due to bleeding or infection. Leukaemic involvement of most organs is evident at autopsy. The median survival of those who develop blast crisis is only about 6 months.

1.4. The Philadelphia chromosome

In 1960 Nowell and Hungerford found an abnormally small chromosome in all the metaphase cells in the bone marrow of patients with CML and regarded this as a marker of the tumour. This chromosome was later named the Philadelphia (Ph) chromosome since it was found in Philadelphia. In 1973 the Ph chromosome was shown to result from a reciprocal exchange of chromosome material between the long arms of chromosome 9 and chromosome 22, i.e. t(9;22)(q34;q11) (Figure 1-1) (Rowley, 1973). This cytogenetic 'hallmark' of CML was found in 90 percent of the CML cases (Champlin & Golde, 1985). The Philadelphia chromosome was also found in 25 - 30% of adult and 2 - 10% of childhood acute lymphoblastic leukaemia (ALL) (Priest *et al* 1980; Sandberg, 1990), and in 2% of acute myelogenous leukaemia (AML) (Sandberg, 1990). In addition, some patients with CML may have complex translocations involving three, four, or five chromosomes (including chromosomes 9 and 22). However, the molecular consequences of these chromosomal changes appear similar to those resulting from the typical Ph translocation.

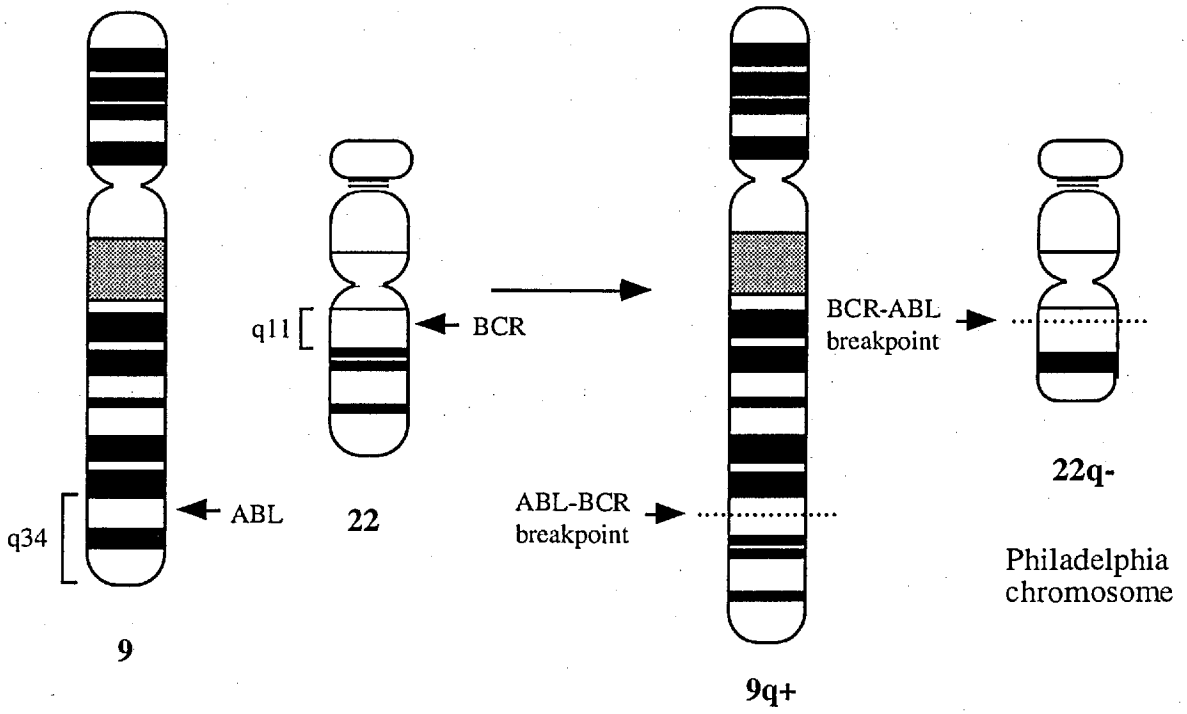


Figure 1-1. The t(9;22)(q34;q11) reciprocal translocation in CML

Short arrows indicate the breakpoints in the ABL gene on chromosome 9, the BCR gene on chromosome 22, the position of ABL-BCR on chromosome 9q+ and BCR-ABL on chromosome 22q-. Brackets indicate the chromosome bands involved in the translocation.

1.5. Genes involved in the translocation

1.5.1. The ABL gene

The ABL gene was originally defined as part of a transforming retrovirus, the Abelson murine leukaemia virus (A-MuLV). Abelson and Rabstein in 1970 isolated A-MuLV from a thymectomized mouse inoculated with M-MuLV. One mouse developed an acute B-cell leukaemia (Abelson & Rabstein, 1970). The virus isolated from this mouse was shown to differ from M-MuLV in that it was replication-defective and could transform murine NIH3T3 fibroblasts and lymphoid cells *in vitro* (Scher and Siegler, 1975; Rosenberg *et al* 1975). The non-M-MuLV component (v-abl) was found to be derived from a normal cellular gene in mouse, designated c-abl. Molecular cloning and sequencing confirmed the sequence derived from mouse was the normal mouse ABL

gene (Goff *et al* 1980; Reddy *et al* 1983; Wang et *al* 1984). The human ABL gene was isolated and localised to the long arm of chromosome 9 using somatic cell hybrids and *in situ* hybridization (Heisterkamp *et al* 1982; de Klein *et al* 1982; Bartram *et al* 1983). It was noted that the location of ABL was similar to the chromosome 9 breakpoint in the t(9;22)(q34;q11). Southern blot analysis of patient and cell line material subsequently demonstrated that the bulk of the ABL gene is translocated to the Ph chromosome (de Klein *et al* 1982) and that this gene is in fact disrupted by the translocation (Heisterkamp *et al* 1983).

ABL gene is highly conserved in evolution and is present not only in human and other vertebrates but even in *Drosophila melanogaster* (Hoffman *et al* 1983). This phylogenetic conservation suggests that the normal ABL gene may participate in essential cellular processes. Cloning of ABL has revealed that it spans at least 230 kb contains at least 11 exons, and is oriented with its 5' end toward the centromere (Bernards *et al* 1987; Shtivelman *et al* 1985; Shtivelman *et al* 1986). Two alternative first exons exist, exons Ia and Ib. Exon Ia is 12 kb proximal to exon a2 (Genbank, U07563), exon Ib is located ≥ 200 kb proximal to exon a2. As a result, two major ABL messages are transcribed as 6 kb and 7 kb mRNA molecules. The former consists of exon Ia to 11; the latter begins with exon Ib, skips the 200 kb distance to exon a2 without exon Ia, and joins to exons two to eleven (Westin *et al* 1982; Shitivelman *et al* 1986; Bernards *et al* 1987) (Figure 1-2).

ABL is ubiquitously expressed in all mammalian cell types, although its expression in the haematopoietic system decreases with myeloid maturation. The functional domains of ABL include a myristylation site at the N-terminal end of ABL Ib which is believed to target tyrosine kinase activity to the cell membrane (Daley *et al* 1992). This is followed by a kinase regulatory domain which includes two regions of homology to Src tyrosine kinase (SH2 and SH3 domains) (Koch *et al* 1991; Zhu *et al* 1993) and the SH1 or tyrosine kinase domain. The SH2 domain appears to positively regulate the kinase

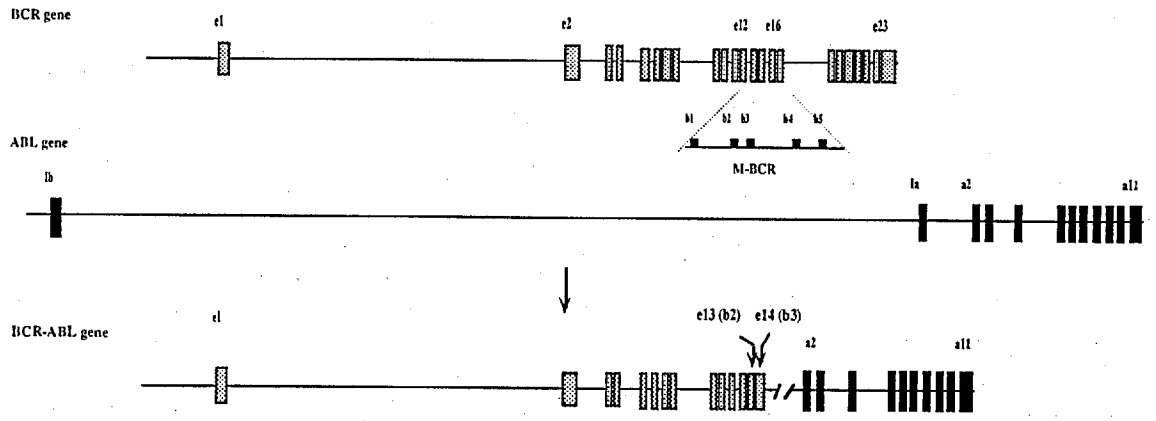


Figure 1-2. Genomic structure of the BCR, ABL and BCR-ABL fusion genes

and transforming activity of ABL (Maru & Witte, 1990); the SH3 domain plays a negative regulatory role (Shore *et al* 1990). The C-terminal end of ABL appears to be important in determining the subcellular localisation of ABL protein since it has domains which can bind to DNA (Kipreos & Wang, 1992; Arcinas *et al* 1994) and to actin (Van Etten *et al* 1994) (Figure 1-3). The ABL protein is localised both in the nucleus where it is bound to chromatin, and in the cytoplasm in association with F-actin (Wetzler *et al* 1993; Laneuville, 1995). Nuclear ABL is regulated in a cell cycle dependent manner probably by binding to the retinoblastoma (RB) protein (Welch & Wang, 1995). The tyrosine kinase activity of ABL is quiescent in G0/G1 and becomes activated when cells progress through the G1/S phase and RB disassociates from ABL. ABL has been implicated in the regulation of gene transcription and overexpression of ABL inhibits cellular growth (Sawyers *et al* 1994). Homozygous disruption of the tyrosine kinase activity of ABL in mice results in increased antenatal mortality and abnormalities of the head and eye. In addition there is a marked reduction in the number of pre-B cells in the marrow, splenic hyotrophy and T-lymphocytopenia suggesting that ABL plays an essential role in early lymphogenesis (Schwartzberg *et al* 1991). More recently it has been shown that ABL is necessary for G1 arrest in response to DNA damage (Yuan *et al* 1996) and that it may be a component of integrin signal transduction pathway (Lewis *et al* 1996).

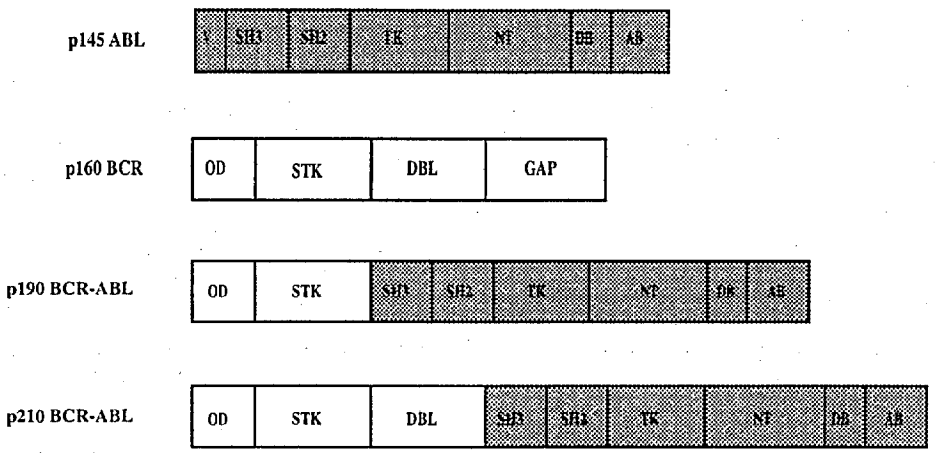


Figure 1-3. The structure of the ABL, BCR and BCR-ABL proteins

p145 ABL: V, variable first exon encoded domain; SH3 and SH2, src homologous domains 3 and 2; TK, tyrosine kinase domain; NT, nuclear translocation signal; DB, DNA-binding domain; AB, acting binding domain. p160 BCR: OD, oligomerisation domain; STK, serine/threonine kinase domain; DBL, Dbl homologous domain; GAP, GTPase activating protein function.

1.5.2. The BCR gene

Using an ABL probe, a t(9;22) junction fragment was cloned from a genomic library constructed from a patient with CML (Groffen *et al* 1984). Hybridisation of the chromosome 22 specific portion of this clone to DNA isolated from leukaemic spleen cells of a second CML patient revealed the presence of abnormal restriction fragments (Groffen *et al* 1984). This finding indicated that a breakpoint had occurred in both patients within a limited stretch of chromosome 22 DNA. Analysis of further patients indicated that all, or virtually all Ph positive CML patients had a breakpoint in a very small region (approximately 5.8 kb) of chromosome 22 (Ganesan *et al* 1986; Leibowitz *et al* 1985; Groffen *et al* 1984). The name major breakpoint cluster region or M-BCR was proposed for this region (Gale & Goldman, 1988). To determine whether the M-BCR was a part of gene, probes from this region were tested for their ability to hybridise to cDNA sequences in a human fibroblast cDNA library. One (a 0.6 kb Hind III/BamH I BCR restriction enzyme fragment) of the probes did indeed result in a

positive signal and was used to isolate several BCR cDNA clones (Heisterkamp *et al*, 1985).

The BCR gene spans about 135 kb on chromosome 22 (Groffen *et al* 1984; Stam *et al* 1985; Heisterkamp *et al* 1985; Grosveld *et al* 1986; Zhu *et al* 1990; Feinstein *et al* 1992) and is transcribed into 4.5 and 6.7 kb mRNAs (Stam *et al* 1985; Shtivelman *et al* 1985; Heisterkamp *et al,* 1985; Grosveld *et al* 1986; Hariharan & Adams, 1987). The BCR protein is 160 kDa (Timmons & Witte, 1989) and is widely expressed.

The role of BCR has not been fully elucidated, but some domains have been identified that suggest a role in intracellular signalling pathways involving phosphorylation and guanosine triphosphate (GTP) binding (Figure 1-3). Studies of BCR null mutant mice indicate that BCR acts a negative regulator of the respiratory burst in both cells with phagocytic capacity such as neutrophils and macrophages, and also in B lymphocytes (Voncken *et al* 1995; Diekmann *et al* 1995). These cell types have in common an active NADPH associated oxidative respiratory complex. BCR null mice are fully viable and fertile.

The BCR gene product has recently been shown to be a multifunctional protein. Sequences encoded by exon 1 of BCR have the capacity to bind with high affinity to the ABL SH2 domain (Pendergast *et al* 1991) and to SH2 domains from other proteins (Muller *et al* 1992). Two domains in BCR have regulatory activity toward at least some members of the rac and rho family (Diekmann *et al* 1991; Diekmann *et al* 1995). In common with Ras, these proteins are active when bound to GTP, but inactive when bound to GDP. The C-terminal region of BCR has GTPase activating protein (GAP) activity and the central region (Dbl homology domain) has GTP/GDP exchange factor activity toward these proteins (Chuang *et al* 1995). Rac and rho are implicated in the regulation of cytoskeletal organisation, cell shape and NADPH superoxide production,

therefore the putative function of BCR as a regulator of these proteins is at least partially consistent with the findings in the BCR knockout mouse.

1.6. Structure of BCR-ABL fusion genes and their products

On the Ph chromosome, 5' BCR sequences are juxtaposed to 3' ABL sequences derived from chromosome 9. The breakpoints in ABL occur either in the first, second or third introns or upstream of ABL exon Ib. The total size of the region within which ABL breakpoints may occur is very large, >200 kb (Bernards *et al* 1987). The great majority of breakpoints are upstream of ABL exon a2.

Conventionally, the BCR exons within the M-BCR are referred to as exons b1 - b5 (Gale & Goldman, 1988); it is now known that these correspond to exons 12 - 16 of the complete gene. Breakpoints in the 5.8 kb M-BCR usually occur either between exons b2 and b3 or alternatively between b3 and b4. After splicing of the primary transcripts, the great majority of CML patients express BCR-ABL transcripts in which BCR b2 or b3 is fused to ABL exon a2 (Figure 1-4). These transcripts are referred to as b2a2 or b3a2 respectively and differ in size by only 75 nucleotides. Both transcripts are translated to yield a p210 kDa protein. ABL exons Ia and Ib are excluded from the BCR-ABL transcript since they do not have splice acceptor sites and cannot fuse with donor sites in BCR. The reciprocal ABL-BCR gene on the 9q+ chromosome is also expressed in about 70% of CML cases, but its role, if any, in the pathogenesis of the disease is unclear (Melo *et al* 1993a).

In addition to CML, the Ph chromosome is also found in 2-5% of paediatric acute lymphoblastic leukaemia (ALL) (Bloomfield *et al* 1986; Dreazen *et al* 1987; Crist *et al* 1990; Fletcher *et al* 1992) and 17-35% in adult ALL (Bloomfield *et al* 1986; Dreazen *et al* 1987; Westbrook *et al* 1992). In a large proportion of ALL patients, no rearrangements were identified in the M-BCR region as in CML (Erikson *et al* 1986;

Hermans *et al* 1987) but subsequently BCR breakpoints were found further 5', in the first intron of BCR (m-BCR) (Rubin *et al* 1988; Chen *et al* 1988). This alternative BCR-ABL chimaeric gene was considered to be a distinct oncogene in ALL, giving rise to a smaller p190 BCR-ABL protein (Clark *et al* 1988). Although the breakpoints in 30% of ALL patients are found within the M-BCR region giving rise to a p210 BCR-ABL protein indistinguishable from that found in CML (Craig *et al* 1990), breaks in the m-BCR region are almost exclusively found in ALL (Rassool *et al* 1988; Heisterkamp *et al* 1988; Chen *et al* 1988). However, rare cases of p190 CML have been described (Melo *et al* 1994b).

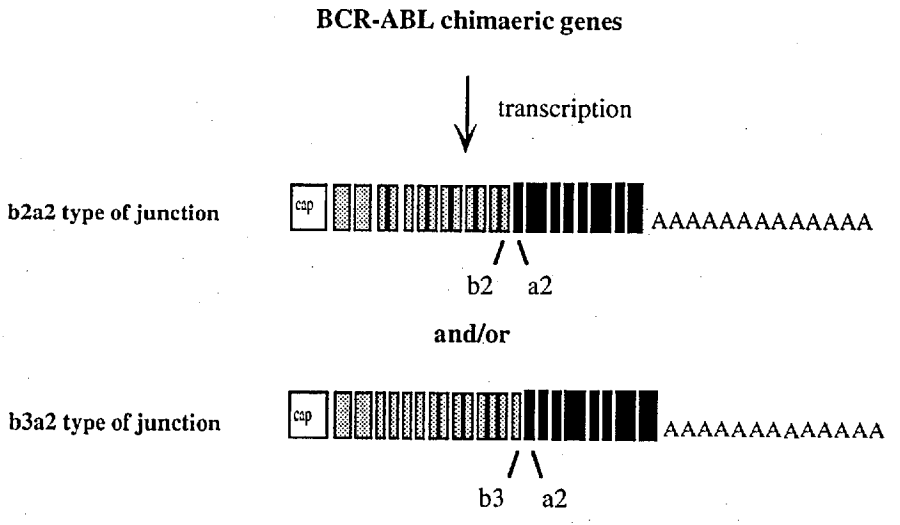


Figure 1-4. BCR-ABL transcripts

These observations raise the question if the leukaemia phenotype is determined by intrinsically different transforming properties of p210 and p190 BCR-ABL or alternatively by the target cell transformed regardless of whether p210 or p190 BCR-ABL is expressed. The latter hypothesis implies that p210 ALL could be a variant of CML arising in a pluripotent stem cell and presenting as lymphoid blast crisis after a silent or undiagnosed chronic phase while p190 ALL represents a true *de novo* ALL due to transformation of an early committed B-lymphoid progenitor cell. If this hypothesis would be true, than one might expect that the molecular heterogeneity of Ph

positive ALL results in different clinical and pathological manifestations and a different response to treatment.

1.7. Ph-negative CML

There have been a number of reports concerning Ph negative CML (reviewed by Kantarjian *et al* 1986). Those patients, accounting for about 10% of CML cases, are heterogenous both at molecular and clinical levels. Some patients display features indistinguishable from typical CML. Others, which resemble CML more than other well-defined haematological disorders, are more atypical. Molecular investigation of Ph negative patients has shown that approximately half of the cases have BCR-ABL rearrangements (van der Plas *et al* 1991; Kantarjian *et al* 1988; Dobrovic *et al* 1991) and are referred to as BCR, or BCR-ABL positive, Ph negative CML.

Ph negative BCR positive patients include those with complex, variant or masked Ph chromosomes (Bartram *et al* 1985; Kurzrock *et al* 1986; Koduru *et al* 1993; and Morris *et al* 1996). Some cases had an insertion of ABL from chromosome 9 into chromosome 22 (Mohamed *et al* 1995; Lazaridou *et al* 1994; and Morris *et al* 1990). Clinically those patients with Ph negative, BCR positive CML have indistinguishable characteristics and clinical course compared to patients with typical Ph-positive CML.

Patients with CML, who have neither a discernible BCR rearrangement nor a BCR-ABL message (Dreazen *et al* 1987), have a distinct clinical course (Kurzrock *et al* 1990). In contrast to the blastic transformation that inevitably complicates BCR-ABL positive CML, the natural history of the Ph-negative, BCR-negative CML patients is frequently characterized by increasing leukaemia burden with leukocytosis, pronounced organomegaly, extramedullary infiltrates, and eventual bone marrow failure (anaemia and thrombocytopenia) without marked increases in blast cells. Some patients may be

an atypical CML (aCML) or chronic myelomonocytic Leukaemia (CMML) or other myelodysplastic syndromes (MDS) (van der Plas *et al* 1991).

1.8. BCR-ABL transforming activity

Several research groups have investigated the oncogenic activity of the p210 BCR-ABL protein. The v-abl oncogene of the Abelson murine leukemia virus (A-MuLV) is known to efficiently transform NIH3T3 fibroblasts *in vitro* and to cause an acute lymphosarcoma in susceptible murine hosts. However, *in vitro* experiments indicated that p210 generated from BCR-ABL chimaeric gene did not transform NIH3T3 fibroblasts (Daley *et al* 1987).

A retroviral gene-transfer system was established to express p210 BCR-ABL in mouse bone marrow cells. When infected bone marrow were plated under conditions for long-term culture of cells of the B-lymphoid lineage, cells expressing high amounts of p210 BCR-ABL tyrosine kinase dominated the culture and rapidly lead to clonal outgrowths of immature lymphoid cells. Expression of p210 BCR-ABL was growth-stimulatory but not sufficient for full oncogenic behaviour. Some clonal lines progressed toward a fully malignant phenotype as judged by increased cloning efficiency in agar suspension and frequency and rapidity of tumor induction in syngeneic mice (McLaughlin *et al* 1987).

However, the presence of BCR-ABL alone in multipotent progenitor cells does not cause growth factor independence, although it initiates a stepwise process (Gishizky & Witte, 1992). Other results were concordant with those above, suggesting that BCR-ABL confers a proliferative advantage on diverse haemopoietic cells but complete transformation probably involves additional genetic changes (Elefanty *et al* 1990).

To determine whether p210 BCR-ABL can induce chronic myelogenous leukemia *in vivo*, murine bone marrow was infected with a retrovirus encoding p210 BCR-ABL and

transplanted into irradiated syngeneic recipients. Transplant recipients developed several haematologic malignancies; prominent among them was a myeloproliferative syndrome closely resembling the chronic phase of human chronic myelogenous leukemia. Tumour tissue from diseased mice harboured the provirus encoding p210 BCR-ABL. These results demonstrate that p210 BCR-ABL expression can induce chronic myelogenous leukemia like disorders (Daley *et al* 1990; Kelliher *et al* 1990), and is the most compelling evidence that this fusion protein play a primary role in the pathogenesis of CML.

The BCR-ABL mRNAs in CML are translated to give rise to the p210 BCR-ABL, which has constitutive tyrosine kinase activity (Figure 1-3). The fused BCR portion activates the tyrosine kinase and transforming activities of ABL. In addition, BCR also changes the subcellular location of ABL by blocking ABL nuclear translocation signals and activating the ABL F-actin binding domain. Two domains encoded by BCR exon 1 have been identified which are essential to the transforming potential of BCR-ABL. The first domain has an alpha-helical coiled structure which induces the formation of BCR-ABL tetramer thereby promoting intermolecular cross-phosphorylation by the tyrosine kinase. Furthermore, tetramerisation leads to clustering of actin-binding domains, resulting in the ability to bind to, and cross-link, actin filaments (McWhirter *et al* 1993). It has been proposed that the localisation of BCR-ABL to the cytoskeletal and interaction with actin, paxillin and talin may allow for the interaction with focal adhesion proteins or receptors that link the process of cellular adhesion to the cytoskeleton (Salgia *et al* 1995; Salgia *et al* 1996).

Recent studies indicate that BCR-ABL can increase tyrosine phosphorylation of focal adhesion proteins which may be involved in regulating integrin function (Verfaillie *et al* 1997). In haemopoiesis, it is well established that bone marrow stromal cells negatively regulate primitive progenitors by mechanisms that involve cellular adhesion. In CML, this negative regulation is disrupted (Gordon *et al* 1987; Gordon *et al* 1991)

and it is possible, but not yet proved, that the interactions of BCR-ABL with the cytoskeleton are relevant to this defect.

BCR exon 1 encodes for a serine/threonine kinase domain which has high affinity to the phosphotyrosine binding SH2 domain of ABL and is at least partially responsible for activating ABL, probably by interfering with the negative regulatory function of the SH3 domain. The BCR Dbl homology domain is present in p210 BCR-ABL but lost in p190 BCR-ABL suggesting that it is not essential to the transforming activity of BCR-ABL, but it may be relevant to the phenotype differences between CML and ALL.

A complex containing p160 BCR protein and p210 BCR-ABL protein was found both *in vitro* and *in vivo* (Liu *et al* 1993; Lu *et al* 1993). Phosphorylation of p160 BCR in BCR-ABL complexes was related to the kinase activity of BCR-ABL (Li *et al* 1989; Campbell *et al* 1993; Lu *et al* 1993). These findings suggested that the activated ABL protein kinase of BCR-ABL proteins modulates the putative signal transduction activities of p160 BCR by tyrosine phosphorylation of exon 1 sequences. These alterations and the stable physical interaction in the complex may perturb normal BCR functions leading to the pathologic processes found in Ph chromosome-associated leukemias.

1.9. Signal transduction in CML cells

The connection between the BCR-ABL tyrosine kinase signal and downstream signal transduction pathways is provided by multiple functional domains (Muller *et al* 1991; McWhirter & Wang, 1991; Pendergast *et al* 1993a, Pendergast *et al* 1993b; Afar *et al* 1994) (Figure 1-5). Mutations of a major autophosphorylation site (Y793) within the catalytic domain (Pendergast *et al* 1993b; Afar *et al* 1994), the Grb2-binding site (Y177F) within BCR (Pendergast *et al* 1993a; Puil *et al* 1994), or the SH2 domain (R552L) (Afar *et al* 1994) block the ability of BCR-ABL to stimulate growth of rodent

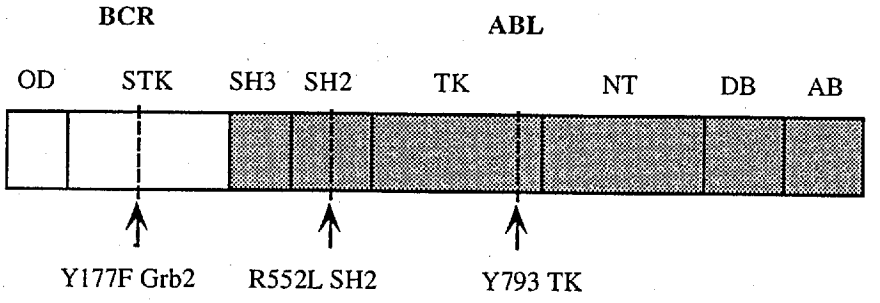


Figure 1-5. Diagram of point mutations generated in the 190 BCR-ABL gene

Arrows and dashed lines indicate the positions of mutations. Open boxes are BCR and stippled boxes are ABL. The domains of p190 BCR-ABL are indicated: OD, oligomerisation domain; STK, serine/ threonine kinase domain; SH3 and SH2, src homologous domains 3 and 2; TK, tyrosine kinase domain; NT, nuclear translocation signal; DB, DNA-binding domain; AB, acting binding domain.

fibroblast cells in soft agar without significantly altering the activity of the kinase. Autophosphorylation of Y793 in p190 may provide a docking site for SH2-containing cellular proteins (Pendergast *et al* 1993b; Afar *et al* 1994). Mutation of the arginine residue within the conserved FLVRES motif of the ABL SH2 domain (R552L) blocks binding to phosphotyrosine-containing proteins (Mayer *et al* 1992). Mutation of Y177F blocks the direct interaction between the Grb2 adaptor molecule and BCR-ABL (Pendergast *et al* 1993a; Puil *et al* 1994). This mutation has previously been reported to impair BCR-ABL activation of the Ras signal transduction pathway in fibroblast cells and to block haematopoietic cell transformation (Pendergast *et al* 1993a). Furthermore, it has been found recently that tetramerization domain of BCR-ABL is essential for interactions of these downstream molecules (Tauchi *et al* 1997).

There is an alternative signalling pathway to Ras in haematopoietic cells when the domain for BCR-ABL signalling to Grb2 adapter molecule is experimentally blocked. Activation of Shc adapter protein provides an alternative signal from BCR-ABL to Ras in haematopoietic cells. Increased Shc dosage can complement both fibroblast and haematopoietic transformation by BCR-ABL (Goga *et al* 1995).

Ras is situated in a very important central position in transformation by oncogenes, including v-abl or BCR-ABL. A genetic approach was used to define the effect of impaired Ras activity on the ability of BCR-ABL or v-abl to transform cells. Expression of the catalytic domain of the GTPase activating protein for Ras (Gap C terminus) impaired soft agar colony formation by fibroblast expressing v-abl or BCR-ABL by 70 - 80%. In a mouse model that more closely resembles clinical diseases involving BCR-ABL, double gene retroviruses expressing BCR-ABL paired with the Gap C-terminus, or dominant negative Ras were introduced into naive mouse bone marrow cells. Transformation by BCR-ABL was completely blocked in both situations. Coexpression of normal H-Ras accelerated the transforming activity of BCR-ABL. These findings show that Ras activation is essential for the leukaemogenic activity of ABL oncogenes in two distinct model systems. The results genetically define a connection between the BCR-ABL cytoplasmic tyrosine kinase and Ras and add to the accumulating evidence that deregulation of Ras is a central event in the genesis of myeloid leukaemia (Sawyers *et al* 1995; Skorski *et al* 1994). Ras receives signals transduced from BCR-ABL via multiple signal chains and passes them down to mitogen-activated protein kinase (MAPK), the extracellular signal-regulated kinase (ERK) and the Jun N-terminal kinase (JNK), subsequently activating transcription from Jun responsive promoters (Raitano *et al* 1995).

Oncogenic signals induce cellular proliferation by deregulating the cell division cycle. Cyclin D1, a regulator of G1-phase progression, acts synergistically with ABL oncogenes in transforming fibroblasts and haematopoietic cells in culture. Synergy with v-abl depended on a motif in cyclin D1 that mediates its binding to the retinoblastoma protein, suggesting that ABL oncogenes in part mediate their mitogenic effects via a retinoblastoma protein-dependent pathway. Overexpression of cyclin D1, but not cyclin E, rescued a signalling-defective SH2 domain mutant of BCR-ABL for transformation of cells in culture and malignant tumor formation *in vivo*. These results demonstrated

that cyclin D1 can provide essential signals for malignant transformation in concert with an activated tyrosine kinase (Afar *et al* 1995).

It has been suggested that p210 BCR-ABL and the IL-3 receptor may activate a common signal transduction pathways. An important pathway for IL-3 signalling involves activation of the Janus family kinases (JAKs) and subsequent tyrosyl phosphorylation of signal transducers and activators of transcription (STAT) proteins. This pathway directly links growth factor receptors to gene transcription. Ligation of cytokine receptors results in tyrosine phosphorylation and activation of receptor-associated Jak protein tyrosine kinases and cytoplasmic STAT transcription factors. These then translocate to the nucleus, leading to the formation of specific DNA-binding complexes containing STAT proteins. STAT1 and STAT5 were found to be constitutively phosphorylated in the murine cell lines transformed by BCR-ABL, but not in the untransformed parental cell lines in the absence of IL-3 (Carlesso *et al* 1996). Phosphorylation of STAT1 and STAT5 was also observed in the human leukaemia cell lines K562 and BV173, which express the BCR-ABL oncogene, but not in several BCR-ABL-negative leukemia cell lines. Phosphorylation of STAT1 and STAT5 was directly due to the tyrosine kinase activity of BCR-ABL since it could be activated or deactivated by temperature shifting of cells expressing a BCR-ABL temperature sensitive mutant. DNA-STAT complexes were detected in all BCR-ABL transformed cell lines and they were supershifted by antibodies against STAT1 and STAT5. DNA-STAT complexes in 32D p210 BCR-ABL cells were similar, but not identical, to those formed after IL-3 stimulation. It is interesting to note that JAK kinases (JAK1, JAK2, JAK3, and Tyk2) were not consistently activated in BCR-ABL positive cells. These data suggest that STATs can be activated directly by BCR-ABL, possibly by passing JAK family kinase activation. This could be a novel mechanism that contributes to some of the major biological effects of BCR-ABL transformation (Carlesso *et al* 1996; Shuai *et al* 1996).

BCR-ABL activation of multiple signalling pathways also involves c-Myc. Ectopic expression of this gene efficiently restored transformation activity when SH2 domain of BCR-ABL was mutated (Afar *et al* 1994).

1.10. The mechanism underlying the reciprocal translocation between chromosomes 9 and 22 in CML

How the translocation t(9;22) occurs is one of the important outstanding questions in our understanding of the biology of Ph positive leukaemias (Goldman *et al* 1989). In addition, several groups have suggested that the location of the breakpoints are associated with type and progression of the diseases. Therefore, characterisation of the breakpoints in the patients with CML would benefit both basic scientific research and clinical applications (Hagemeijer, 1987; Groffen & Heisterkamp, 1987; Clark *et al*, 1989; Goldman, 1989; Morgan & Wiedemann, 1989; Cannistra, 1990; Heisterkamp & Groffen, 1991; Kurzrock & Talpaz, 1991; Goldman, 1993).

The genomic DNA sequence flanking the BCR-ABL and/or reciprocal ABL-BCR translocation has been determined for several patients with CML, AML or ALL (Groffen *et al* 1984; Heisterkamp *et al* 1985; Grosveld *et al* 1986; de Klein *et a*l 1986; Chen *et al* 1989a; Chen *et al* 1989b; van der Feltz *et al* 1989; Papadopoulos *et al* 1990; Soekarman *et al* 1990; Mills *et al* 1992; Litz *et al* 1993; Sowerby *et al* 1993). No common sequence has been detected at or near the breakpoints, although some groups have suggested that neighbouring Alu elements may play a role in the translocation. However, the mechanism by which the t(9;22) is formed remains unclear.

1.11. Transplantation and minimal residual disease

CML can be cured by allogeneic bone marrow transplantation (BMT) if a suitable donor can be identified but some patients will relapse (Goldman *et al* 1986; Thomas *et*

al 1986). Cure is not achieved solely by high dose chemoradiotherapy but also involves elimination or suppression of the leukaemic clone by a graft versus leukemia (GVL) effect (Horowitz *et al* 1990). There are various observations that suggest that in the majority of patients the Ph-positive clone is suppressed rather than totally eradicated after BMT. First, evidence of residual disease has been found by RT-PCR in some long term survivors after BMT (van Rhee *et al* 1994; Radich *et al* 1995). Second, BCR-ABL positive myeloid progenitor cells have been identified in some patients who remain in sustained remission (Xu *et al* 1994; Pichert *et al* 1994). Third, two female patients have been recently described who relapsed during or shortly after pregnancy despite having been in remission for 4.25 and 6.5 years following allogeneic BMT for CML (Atkinson *et al* 1994; Rio *et al* 1994). It is possible that the immunosuppression associated with pregnancy (Hunt, 1992) abrogated GVL, enabling a dormant or suppressed leukaemia clone to re-emerge.

There has been considerable interest in the use of the polymerase chain reaction (PCR) to detect residual disease in CML patients after BMT. Several groups have demonstrated that BCR-ABL mRNA may be specifically and efficiently detected by reverse transcription of leukocyte RNA followed by amplification of reverse transcription of mRNA, i.e., reverse transcription PCR (RT-PCR). Whilst RT-PCR can identify groups of patients with an increased risk of relapse, many patients are PCR positive for several months or longer after BMT without subsequent relapse. Thus this qualitative technique is of limited value in the clinical management of individual patients (Radich *et al* 1995; Sawyers *et al* 1990; Delage *et al* 1991; Roth *et al* 1992; Cross *et al* 1993b; Miyamura *et al* 1993; reviewed in Miyamura *et al* 1994). The Hammersmith group, and others, have therefore developed PCR assays to quantitate BCR-ABL transcripts and have shown that rising levels of the fusion gene mRNA can be observed prior to detection of relapse at the cytogenetic level (Cross *et al* 1993a; Lion *et al* 1993).

It has recently been suggested that some Ph chromosome positive CML cells may not actively express the BCR-ABL fusion gene (Keating *et al* 1994; Bedi *et al* 1993). Such cells would escape detection by RT-PCR but could be detectable by methods that recognise the rearrangement in genomic DNA. It is conceivable that such transcriptionally 'silent' CML cells are present in patients after BMT and, after reactivation, could contribute to relapse.

1.12. Principal objectives of this work

1.12.1. Establishment of a rapid method to characterise the translocation breakpoints

To overcome the difficulty in cloning BCR-ABL translocation breakpoints, I developed a more rapid approach. A one sided PCR technique, Bubble PCR, was established to amplify, clone and sequence the BCR-ABL and ABL-BCR breakpoints from individual patients with CML.

1.12.2. Characterisation of BCR-ABL breakpoints

In order to gain a better understanding of the BCR-ABL translocation in CML, I have used Bubble PCR to amplify BCR-ABL breaks in 17 CML cases. For 3 of these patients the translocation was completely characterised by deriving sequences from the 9q+ break and the corresponding regions of the normal chromosomes 9 and 22.

1.12.3. Detection of minimal residual disease for patients after BMT at the genomic DNA level

In order to test the hypothesis that CML patients after BMT possess a pool of leukaemia cells that do not express BCR-ABL mRNA, a set of patient specific PCR

primers, which were derived from the information obtained by Bubble PCR, were used to amplify the genomic breakpoint as a tumour cell marker. I then compared results of residual disease detected by RT-PCR with results obtained by amplification of BCR-ABL breakpoints from genomic DNA.

CHAPTER TWO MATERIALS AND METHODS

2.1. Sample preparation.

20 ml peripheral blood (PB) or 5 ml bone marrow (BM) samples were obtained from the clinic. Sample were spun at 1,500 rpm for 10 minutes at room temperature. The buffy coat including about 0.5 cm of red cells was transferred to a 50 ml Falcon tube. The cells were suspended in red cell lysis buffer (RCLB: 0.155 M NH_4Cl, 10 mM $KHCO_3$, 100 mM EDTA (pH 7.4) at 4°C) (Roos & Loos, 1970), mixed well and incubated on ice for 5 minutes with intermittent shaking. The RCLB was then removed by centrifugation at 1,500 rpm for 10 minutes. The pellet was washed with 20 ml phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer solution, pH7.4 at 25°C). After centrifugation again, cells were resuspended in a small volume of PBS and counted by a Sysmex F-520 Cell Counter. Usually processed samples were either suspended in 2.5 ml of 4 M guanidinium thiocyanate solution (GTC: 4 M guanidinium thiocyanate, 5 mM EDTA, 25 mM citrate pH 7.0, 0.5% sarcosyl, 0.1M β-mercaptoethanol and 2.2 M CsCl) for subsequent RNA extraction or frozen as a pellet at -70°C for subsequent DNA extraction (see below).

2.2. DNA extraction

High molecular weight DNA was extracted from leukocytes or buffy coat cells. First cells were washed twice in PBS with 0.5% nonidet P40 (BDH). The pellet was lysed with 5 - 10 mls of lysis buffer (7 M urea, 300 mM NaCl, 20 mM EDTA and 10 mM Tris-HCl, pH 7.5). After mixing well, sodium dodecyl sulfate (SDS) was added to a final concentration of 0.05% and the lysate was incubated at 37°C for 10 minutes. Then an equal volume of neutralised phenol (Sambrook *et al*, 1989) and a mixture of chloroform:isoamyl alcohol (24:1) was added to the lysate. The solution was vigorously

mixed and then spun at 1,500 rpm for 10 minutes. The top layer of solution was removed to a new tube and DNA precipitated by adding 2 volumes of ethanol at room temperature. The precipitated DNA was 'hooked out' using a sterile loop and washed twice with 70% ethanol. After air drying briefly at room temperature, the DNA was resuspended in 200 µl - 1 ml of sterilised distilled water overnight to dissolve before measuring the OD_{260} and OD_{280} values (Sykes, 1983).

2.3. RNA extraction

Samples for RNA analysis were lysed in 2.5 ml of GTC and stored at -20°C. The samples from frozen cells were prepared by thawing the cells in 2.5 ml GTC (adding GTC solution straight into the tube containing cells) after removal from the -70°C freezer or liquid nitrogen.

All steps were performed in a laminar air flow hood. Solutions for RNA extraction were treated with DEPC (diethyl pyrocarbonate, SIGMA) to remove traces of RNase by adding 0.1% of DEPC into the solutions, shaking vigorously for 1 minute, incubating at 37°C overnight and autoclaving the next day (Sambrook *et al,* 1989). Plugged pipettes and pipette tips were used at all stages to prevent contamination. Where possible reagents were mixed and stored as single-use aliquots. Samples were usually processed in batches of 5 plus a negative control, usually 5 x 10^6 HL60 cells or 50 µg *E. coli* tRNA (SIGMA), which was included each time RNA extraction was performed. Samples were layered onto a 1 ml cushion of 5.7 M CsCl, 1 mM EDTA, 25 mM citrate (pH 7.0) and centrifuged in a SW55 swing-out rotor at 36,000 rpm for 16 hours. After carefully taking off liquid and drying the inside wall of the centrifuge tube with a tissue, the pellet was resuspended in 250 µl of TES (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.1% SDS). After ethanol precipitation by adding 25 µl of NaAc pH 5.6 and 750 µl of ethanol, the solution was mixed gently and kept in -20°C for at least 2 hours; the RNA was then pelleted by spinning at 14,000 rpm for 30 minutes and washed with 80%

ethanol. The RNA was then dissolved in 48 µl sterile dH_2O (Hammersmith Hospital Pharmacy). A 10 µl aliquot was removed from the RNA solution for the spectrophotometric determination of concentration and the remainder was stored at -70°C. Usually 10 - 40 µg of RNA was recovered from the leukocytes extracted from 20 ml peripheral blood. This method was used in almost all the samples for localisation of the BCR-ABL breakpoint by reverse transcription polymerase chain reaction (RT-PCR).

2.4. Reverse transcription

Reverse transcription (RT) was carried out by heating the RNA samples to 65°C for 5 minutes, cooling on ice for 2 minutes and incubating in a volume of 40 µl with a final concentration of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 1 mM each of dATP, dCTP, dTTP and dGTP (dNTP set, Pharmacia); together with 200 µg/ml of random hexamers $pd(N)_6$ (Pharmacia), 750 units/ml of M-MLV reverse transcriptase (Gibco-BRL) and 750 units/ml of RNasin (Promega) at 37°C for 2 hours. The reaction was terminated by heating to 65°C for 10 minutes and the cDNAs were stored at -20°C.

2.5. Polymerase chain reaction

2.5.1. DNA-PCR

2.5.1.1. DNA-PCR conditions

Typically, 200 ng to 1 µg genomic DNA template was amplified with a pair of primers at a final concentration of 0.5 µM. The incubation buffer contained 0.2 mM each of dATP, dCTP, dGTP, dTTP, 25 mM Tris-HCl, pH 8.4, 50 mM KCl, 1 - 2.5 mM $MgCl_2$, 1 mM dithiothreitol at pH 8.0 (20°C), and 30 units/ml Taq polymerase. The incubation temperatures in the first cycle were: 100°C, 10 seconds; 97°C, 50 seconds; 56° - 68°C,

3 minutes; 72°C, 2 minutes. The temperatures in the following 30 cycles were: 97°C, 20 seconds (denaturation); 56°-68°C, 25 seconds (annealing); 72°C, 50 seconds (extension) followed by 72°C for 10 minutes and then held at 4°C. All PCRs were performed on a MJ Research Inc. programmable heating block (Cross *et al,* 1993b). The magnesium concentration in the buffer and annealing temperature for PCR cycling were optimised for each target.

2.5.1.2. Oligonucleotide primers used for DNA-PCR

Oligonucleotide PCR primers were used in these studies for a number of different purposes: 1) Amplification of the breakpoint region by Bubble PCR and characterisation of the reciprocal translocation between chromosomes 9 and 22, 2) Detection of minimal residual disease in the patients post BMT and evaluation of DNA quality by competitive PCR, and 3) Generation of probes for Southern blot. The sequences of the primers utilised in the studies are listed in Table 2-1.

2.5.2. RT-PCR

2.5.2.1. RT-PCR conditions

Reverse transcription polymerase chain reaction (RT-PCR) amplifies multiple copies from a cDNA template derived from mRNA. One to 5 µl of cDNA was mixed with 19 µl of BCR-ABL mix (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 µM primers B2A and CA3, 0.25 mM each of dATP, dCTP, dTTP and dGTP, 30 units/ml Taq polymerase). Amplification was performed for 30 cycles of 97°C 30 seconds; 64°C 50 seconds; 72°C 1 minute on a MJ Research Inc. programmable heating block. The reaction products were electrophoresed on a 1.8% agarose gel in a separate room using dedicated pipettes. To ensure that cDNA was of suitable quality part of the ABL gene was amplified from any sample which proved to be negative for BCR-ABL. 1 µl of cDNA was mixed with 24 µl of ABL mix (the ABL mix was the same as BCR-ABL

Table 2-1. Primers for DNA-PCR

Name	Sequence	Application
B2A	5'-TTCAGAAGCTTCTCCCTGACAT-3'	Bubble PCR
B2B	5'-ACAGaATTCCGCTGACCATCAATAAG-3'	Bubble PCR
JGZ1	5'-CAGGGCACCTGCAGGGAGGGCA-3'	Bubble PCR
JGZ2	5'-CCTGAAttCTGATCCCCCCTTC-3'	Bubble PCR
B3A	5'-TGAGTCTCCGGGGCTCTAT-3'	Bubble PCR
B3B	5'-TTTCTGAATtcCATCGTCCACTCAGCC-3'	Bubble PCR
JGZ3	5'-GTTTGGGGAGGAGGGTTGCAGC-3'	Bubble PCR
JGZ4	5'-AGGaatTCCACCCAGGAAGGAC-3'	Bubble PCR
Z4A	5'-GTGGAGACAGAAAGCTTACCA-3'	Bubble PCR
Z4B	5'-GGGAATtcGGTTGGGAGAGAGGA-3'	Bubble PCR
BALABL2	5'-GGACATAAGAGGAACAAGTTTG-3'	Bubble PCR
Z4BW-C	5'-TTTGaaTTcGGGATGCAAGGTTGAGTG-3'	Bubble PCR
Z4BW3	5'-GCAACTCATGTGTTACCAGCCTT-3'	Bubble PCR
Z4BW4	5'-TTCAgaaTTCTGACAGCTCCAGTAG-3'	Bubble PCR
Z3BW3	5'-GGAAACACCAGCGTTTATGAGGC-3'	Bubble PCR
Z3BW4	5'-CAGAGAattcGGCCCAGGAGAAAGA-3'	Bubble PCR
NCB3-3	5'-CTTACTTGAACTCTGCTTAAATCC-3'	Bubble PCR
NCB3-2	5'-TGgaATTCAGAAACCCATAGAGC-3'	Bubble PCR
Vecamp-1	5'-GGCAGCAATTACACCCGGAT-3'	Bubble PCR
Vecamp-2	5'-CAGCAATTACACCCGGATCCT-3'	Bubble PCR
Vecamp-3	5'-GGTGTAATTGCTGCCGAGCTG-3'	Bubble PCR
Nvamp-1	5'-TGCTCGTAGTAATCGTTCGCAC-3'	Bubble PCR
Nvamp-2	5'-GTTCGCACGAGAATCGCAAGAT-3'	Bubble PCR
BCR-B	5'-CCCCCGGAGTTTTGAGGATTGC-3'	Competitive PCR
BCR-D	5'-ATGGAAGGCGCCCTCGTCATC-3'	Competitive PCR
1489ABL	5'-TTTTTACTTTTTCTGATTCTGCAA-3'	Residual disease
1620BA	5'-TGGCgAAtTCACTTAAATGCCCTTC-3'	Residual disease
1535ABL	5'-TCTGAAttcCTAATCCCAAATCATGTCA-3'	Residual disease
1572BA	5'-ACAGggaTCcTACCATGGTTGGATCAA-3'	Residual disease
1546ABL	5'-GGACgAAtTCTGAACTGCCTTAGA-3'	Confirmation
1797BA	5'-TACAggatCcATTTGTGCCCCTTTCTGT-3'	Residual disease
1818BA	5'-CCCAggaTCCACCAAATATCTCCAAAA-3'	Residual disease
1638ABL	5'-TGCTATGTTGTCCAGGCCACT-3'	Comfirmation
1854ABL1	5'-TGGGgAaTTcAGGGGAAAGCCAGGA-3'	Residual disease
1854ABL2	5'-AAGGGGAAAGCCAGGATTTAA-3'	Residual disease
GLMNABL	5'-TTGAAttCAACAGAGAAGTCCAA-3'	Bubble PCR
MaisABL	5'-TGGgaaTTCAAAAGTGCCTCTCACTCCA-3'	Residual disease
1472ABL1	5'-CCAGaaTTcTGCCATGTGATACCAC-3'	Residual disease
1472ABL2	5'-CCCTCACTATTCTTTCTTGCAT-3'	Residual disease
1732BA1	5'-GGTGTGAGTTCAGAGTGAGAG-3'	Residual disease
1732BA2	5'-TTCCggaTCCTTTGGCCGAGTGCCA-3'	Residual disease
1569ABL	5'-TGGCgaatTCTCGGCTCACTGCAG-3'	Comfirmation
1489AB	5'-TACACggaTccTACAGATCCCAATAG-3'	Bubble PCR
1620AB	5'-TGCCggaTcCTCTTTGCACCCTGAT-3'	Bubble PCR
ABLGLMN	5'-TTGgGaTCcTTCTACCTACTC-3'	Bubble PCR
1854AB	5'-TGACggAtccTTGAAGAGACTGGTAGG-3'	Bubble PCR

Note: Letters in lower case indicate bases that were changed in order to introduce restriction enzyme sites for cloning.

mix except that primer A2 replaced primer B2A) and amplified on the same program as for the BCR-ABL above. The size of the amplified fragments for b2a2, b3a2 and ABL control is expected to be 385 bp, 456 bp and 277 bp respectively (Cross *et al* 1993b).

2.5.2.2. Primers used for RT-PCR

RT-PCR for identification of breakpoint junction type of BCR-ABL mRNA. The annealing positions for the primers used for RT-PCR are indicated in Figure 4-1 (see Chapter 4). Primer sequence is listed below in Table 2-2:

Table 2-2. PCR primers for RT-PCR

B2A	5'-TTCAGAAGCTTCTCCCTGACAT-3'
CA3-A2	5'-TGTTGACTGGCGTGATGTAGTTGCTTGG-3' 5'-TTCAGCGGCCAGTAGCATCTGACTT-3'

2.6. Gel electrophoresis

Electrophoresis is a method whereby charged DNA molecules in solution migrate in response to an electrical field. Their rate of migration or mobility through the electrical field depends on the strength of the field, on the net charge, size, and shape of the molecules, and on the ionic strength, viscosity, and temperature of the medium in which the molecules are moving. Electrophoresis is used to study the properties of a single charged species, and as a technique to separate different molecules.

2.6.1. Agarose gel

Agarose powder was dissolved in running buffer 0.5 x TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0) or 1 x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) by heating in a microwave oven. When the gel solution had cooled to about 60°C, it was poured into a tank that had been sealed with a comb and two spacers. The gel was allowed to set for

at least 1 hour at room temperature. The concentration of agarose solution was adjusted according to the size of DNA fragments which are going to be run on the gel. DNA samples were then loaded with loading buffer [0.04% bromophenol blue, 0.04% xylene cyanol FF, 2.5% Ficoll (Type 400; Pharmacia) in water]. DNA samples were usually run at 80 V in a minigel tank (Cambridge Electrophoresis Ltd: 7.5 cm x 10 cm) when the DNA size was smaller than 3 kb. For Southern blots, a larger tank was used.

2.6.2. Denaturing polyacrylamide gel

6% denaturing polyacrylamide gels were made from 400 mM acrylamide, 9.7 mM N,N'-methylenebisacrylamide, 7.7 M urea, 1.5 mM ammonium persulfate, and 9.4 mM N,N,N', N'-tetramethylethylenediamine (TEMED) in 0.5 x TBE buffer. The gel solution was mixed well and poured into a 'sandwiched' glass plate set taking care to avoid air bubbles. The gel was sealed to allow polymerisation and left at room temperature for at least 40 minutes. The top of the gel was flushed with running TBE buffer and a 'Sharkstooth' comb was inserted. The DNA samples were heated at 95°C for 2 minutes before loading (Sambrook *et al* 1989). Electrophoresis was performed at constant wattage are with an ATTO Constapower 3500 power pack (Japan). After running, the gel was fixed in a solution with 10% methanol and 10% glacial acetic acid for 40 - 60 minutes and dried on a gel dryer (Drygel SR Hoefer Scientific Instruments, San Francisco) for 40 minutes under vacuum at 80°C. Then the gel was directly exposed to a photographic film for 16 - 72 hours.

2.7. DNA gel purification

DNA fragments were gel purified prior to cloning or use as probes. DNA was run on an ultrapure agarose gel. The target fragment of DNA was sliced out of the gel with a razor blade and transferred into an eppendorf tube. The gel slice was incubated in 500 mM sodium acetate (pH 7.0), 1 mM EDTA for 30 minutes at room temperature. The slice

was transferred into a 500 µl tube that had hole in the bottom and in the lid; the bottom hole was plugged with a small amount of siliconised Whatman glass microfibre filter (3MM GFC, Whatman). The gel in the tube then was frozen in liquid nitrogen for 5 minutes, and the frozen tube was quickly put into a 1.5 ml microcentrifuge tube without a lid and spun at 14,000 rpm for 30 minutes. The solution collected in the 1.5 ml tube was extracted with phenol/chloroform, precipitated overnight, washed with 80% ethanol twice, dried and resuspended in sterilised distilled water. The concentration of DNA was estimated by running a portion of DNA on an agarose gel next to a known amount of marker DNA (Thuring *et al,* 1975; Tautz & Renz, 1983).

2.8. Southern blot

200 ng genomic DNA was first run on a small agarose gel of 0.75% along with λ DNA marker digested by Hind III to check its integrity. Then 10 µg of DNA was digested with 50 - 100 units of a restriction enzyme at 37°C overnight in the appropriate buffer supplied by the manufacturers (GIBCO BRL or New England Biolabs). The digested DNA was fractionated on a 0.75% agarose gel after having checked on a minigel that the digestion was complete. Following electrophoresis, DNA samples in the gel were visualized with UV light and photographed. Then the DNA was denatured in a solution of 1.5 M NaCl, 0.5 M NaOH for 30 minutes, neutralised in 1 M Tris [hydroxymethy aminomethane] (pH7.4) and 1.5 M NaCl and transferred onto a Hybond-N nylon membrane (Amersham) with 20 x SSC (3 M NaCl, 300 mM tri-sodium citrate, pH 7.0) for 14 hours. The filter was washed briefly in 6 x SSC, air-dried and then baked at 80°C for 2 hours. The filter was prehybridised in 6 x SSC, 5 x Denhardt's solution [0.5 g Ficoll (Type 400, Pharmacia), 0.5 g polyvinylpyrrolidone, 0.5g bovine serum albumin (Fraction V; Sigma), and H_2O up to 500 ml], 0.5% (w/v) SDS, 20 µg/ml sonicated denatured salmon sperm DNA at 65°C for 2 hours. The required volume (20 mls) of hybridization solution was preheated to 65°C. The blot was hybridized with a denatured probe at 65°C in the Hybridiser (Techne HB-1, UK) overnight. After hybridization the

blot was washed by incubating with 2 x SSC, 0.1% SDS at 65°C for 10 minutes. The further wash was carried out in 1 x SSC, 0.1% SDS for 15 minutes at 65°C, followed by 0.1 x SSC, 0.1% SDS for 30 minutes at 65°C. Finally the filter was removed from the final stringency wash, wrapped in Saran Wrap and exposed to X-ray film (Sambrook *et al* 1989).

2.9. DNA cloning in M13 phage vector

In order to produce pure single stranded-DNA templates for sequencing, DNA fragments were cloned into M13 phage vector and multiplied by propagation of phage in *E. coli*. The single stranded DNA with the target region was extracted and then sequenced by the dideoxy chain termination method (Sambrook *et al* 1989).

2.9.1. Preparation of DNA fragments to be cloned

To facilitate cloning, target DNA fragments were amplified by PCR with one primer including a BamH I recognition site and second primer including an EcoR I site. The top layer of mineral oil was removed from the PCR product. The Taq polymerase was inactivated and removed by phenol:chloroform extraction. DNA was precipitated overnight, washed, dried, resuspended in sterilised distilled water and then digested with BamH I and EcoR I overnight. The digested DNA was run on an ultrapure agarose gel. The target fragment of DNA was then gel purified as described above.

2.9.2. Preparation of M13 vector

The JM101 host strain (supE thi Δ(lac-proAB), F'[traD36 proAB$^+$ lacI^q LacZ ΔM15]) was constructed for use in conjunction with M13-derived vectors as an indicator for β-galactosidase α-complementation. 10 μg of replicative form (RF) M13mp18 and M13mp19 were digested by both BamH I and EcoR I at 37°C for 1 hour and heated at

65°C for 10 minutes to denature the restriction endonucleases. The phosphate of the 5' termini of vector DNA was removed by incubation with 2 units of calf intestinal alkaline phosphatase (CIAP) at 37°C for 30 minutes to prevent self-ligation. Then the DNA was then extracted with neutralised phenol and chloroform. The small DNA fragments of the polylinker region derived from the digestion were removed by Sephadex G50 column (Sambrook *et al,* 1989). The digested and dephosphorylated vector was then stored at a concentration of 10 µg/ml at -20°C until use.

2.9.3. Ligation of DNA fragments

To generate two different orientations of the insert DNA, a DNA fragment for insertion was ligated to both the M13mp18 and M13mp19 vectors. The two vectors differ only in the orientation of the nonsymmetrical polycloning region within the lac Z region. Typically the reaction mixture was 6 µl of sterile distilled water, 1 µl of insert DNA (50 - 100 ng), 2 µl of 5 x ligation buffer (GIBCO BRL, supplied with enzyme T4 DNA ligase), 1 µl of digested M13 RF DNA (10 ng/µl) and 0.5 µl T4 DNA ligase (1 unit), incubated at 16°C overnight (Sambrook *et al,* 1989).

2.9.4. Preparation of competent cells

A plate of YT medium [16 g bacto-tryptone, 10 g bacto-yeast extract, 5 g NaCl in deionized H_2O per litre plus 1.5% agar (DIFCO)] was streaked with strain JM101 and incubated at 37°C overnight. Master stocks of JM101 were maintained on M9 minimal medium plates (0.4% glucose, 90 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl, 15 g bacto-agar/litre). A single colony was dispersed from the plate into 5 ml of YT medium in a tube for a starter culture and incubated at 37°C overnight. 100 µl of overnight culture was transferred to 200 ml of fresh prewarmed YT medium and incubated by shaking at 37°C until the absorbence at 600 nm reached 0.3 - 0.4. For efficient transformation, it is essential that the number of viable cells did not exceed 10^8

cells/ml. The cells were collected in a 200-ml Sorvall RC-5B centrifuge tube and placed on ice for 10 minutes. All subsequent steps in this procedure were carried out aseptically. After incubation on ice the medium was removed by centrifugation at 4,000 rpm for 10 minutes at 4°C in a Sorvall GS3 rotor. The tube was placed in an inverted position for 1 minute to allow the last traces of medium to drain away. The pellet was resuspended by gentle swirling in approximately 80 ml of ice-cold transformation buffer FSB (10 mM potassium acetate, pH7.5, 45 mM $MnCl_2.4H_2O$, 10 mM $CaCl_2.2H_2O$, 100 mM KCl, 3 mM hexamminecobalt chloride, 10% glycerol). The resuspended cells were incubated on ice for further 10 minutes. The cells were recovered by centrifugation at 4,000 rpm for 10 minutes at 4°C in a Sorvall GS3 rotor. The buffer was decanted completely. The pellet was gently resuspended in 16 ml of ice-cold FSB. 560 µl of dimethyl sulfoxide (DMSO) was added to 16 ml of resuspended cells and mixed gently by swirling, and then returned to an ice bath. 500 µl aliquots of the suspension were dispensed into chilled, sterile 1.5 ml eppendorf tubes with holes in their lids. The competent cells were immediately snap-frozen by immersing the tightly closed tubes in liquid nitrogen and finally stored at -196°C until use (Sambrook *et al,* 1989).

2.9.5. Transformation

A tube of competent cells was removed from the -196°C liquid nitrogen. When the cells just started to thaw, they were transferred to an ice bath and incubated on ice for 10 minutes. Then 100 µl of the cell solution were transferred to chilled, sterile polypropylene tubes with 10 µl of the ligated DNA using a chilled, sterile pipette tip. The cells were mixed with DNA by gentle pipetting and left on ice for further 30 minutes. One positive and two negative controls, 1 µl (10 ng) of an intact M13 vector mixed with the competent cells, 10 µl (100 ng) of a digested and unligated M13 vector without an insert fragment mixed with the cells and 100 µl of the competent cells without any DNA for transformation, were always included. The tubes were transferred

to a rack placed in a circulating water bath that had been preheated to 42°C. The cells were heat-shocked at 42°C for exactly 90 seconds and rapidly transferred to an ice bath without any shaking. The cells were left on ice for 1 - 2 minutes (Sambrook *et al*, 1989).

2.9.6. Preparation of plating bacteria

A master culture of a bacterial strain carrying an F' episome, i.e. JM101, was streaked onto a M9 minimal medium agar plate and incubated for 36 hour at 37°C. 5 ml of YT medium in a tube was inoculated with a single, well-isolated colony picked from the minimal medium agar plate. The liquid culture was agitated for 6 - 8 hours at 37°C in a rotary shaker (Sambrook *et al* 1989).

2.9.7. Plating bacteriophage M13

A series of labelled, sterile tubes containing 5 ml of melted top agar (YT medium containing 0.7% Bacto-agar) were prepared and stored in 47°C waterbath. 100 µl of plating bacteria was added the eppendorf tubes with the heat-shocked, transformed cells, and then put into a tube containing the melted top agar. 16 µl of a solution of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (50 mg/ml in dimethylformamide, Promega) and 4 µl of a solution of 200 mg/ml isopropyl-β-D-thiogalactopyranoside (IPTG, Promega) were immediately added to the tube. The mixture was mixed by gentle vortexing for 3 seconds and then poured onto a labelled plate containing 30 - 35 ml of hardened YT agar medium, equilibrated to room temperature. The plate was swirled gently to ensure an even distribution of bacteria and top agar, that covered the entire surface of the medium before it set. The plates were then covered and the top agar left to harden for 5 minutes at room temperature. Finally the plates were inverted and incubated at 37°C overnight (Sambrook *et al*, 1989).

2.9.8. Preparation of single-stranded bacteriophage M13 DNA

5 ml of YT medium was dispensed into a sterile tube. An isolated white plaque on the plate was picked with a sterile toothpick. The toothpick was transferred to the YT medium and incubated at 37°C with constant agitation for 5 - 6 hours. 1 ml of the culture was transferred to a sterile microtube and centrifuged at 14,000 rpm for 10 minutes at room temperature. The supernatant was transferred to a fresh, sterile microtube. 200 µl of a solution 20% polyethylene glycol (PEG 8000) in 2.5 M NaCl was added. The tube was vortexed to mix the infected culture with PEG 8000 and then left at room temperature for 15 minutes. The precipitated bacteriophage particles were recovered by centrifugation at 14,000 rpm for 10 minutes at room temperature. The supernatant was carefully removed. The tiny pellet of precipitated bacteriophage particles was resuspended in 200 µl H_2O by vigorous vortexing. An equal volume of neutralised phenol was added and the mixture was vortexed for 30 seconds. After standing for 1 minute at room temperature, the vortexing was repeated. After centrifugation for 1 minute, the top layer aqueous phase was transferred to a fresh tube containing 600 µl of a 25:1 mixture of ethanol:3M sodium acetate (pH 5.2). After 15 minutes at room temperature the precipitated single-stranded bacteriophage DNA was recovered by centrifugation at 14,000 rpm for 10 minutes at 4°C in the tube. The supernatant was removed gently. The pellet was washed with 70% ethanol briefly, air-dried and finally resuspended in 30 µl of H_2O (Sambrook *et al,* 1989).

2.10. Sequencing

2.10.1. Standard dideoxy chain termination method

Dideoxy-nucleotide chain termination sequencing was performed using a Sequenase™ kit (USB or Amersham). For each sample a single annealing reaction was performed. In

a capped centrifuge tube 1 µl (0.5 pmole) of M13 primer (-40) (5'-GTTTTCCCAGTCACGAC-3'), 2 µl of reaction buffer (200 mM Tris-HCl, pH7.5, 100 mM $MgCl_2$ and 250 mM NaCl) and 7 µl (1 µg) of M13 DNA were combined, heated to 65°C for 2 minutes; the tube was then allowed to cool slowly to room temperature over a period of about 30 minutes. The annealed DNA mixture for each sample was labelled with 0.5 µl (5 µCi) of α-^{35}S-dATP with 7 mM DTT, 40 nM of each nucleotide, dGTP, dCTP, dTTP, and 3.25 units Sequenase™ (modified T7 DNA polymerase) at room temperature for 2 - 5 minutes. 3.5 µl of labelling reaction was transferred to each termination well (T, C, G and A) with 2.5 µl of each termination mixture (containing 80 µM dGTP, 80 µM dCTP, 80 µM dATP, 80 µM dTTP, 80 µM dCTP, 50 mM NaCl. In additional, the "G" mixture contained 8 µM dideoxy-dGTP, the "A" mix, 8 µM ddATP, the "T" mix, 8 µM ddTTP, and the "C" mix, 8 µM ddCTP) on a 96 well Techne plate, mixed and incubated at 37°C for 5 minutes. The reactions were stopped by adding 4 µl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF). A sample of the four reactions was loaded into 6% polyacrylamide gel with 1 - 3 µl in each lane (depending upon type of comb) after heating at 95°C for 2 minutes (Sambrook *et al,* 1989). Electrophoresis was performed at constant 60 W but below 2000 V for 2 hours with ATTO Constapower 3500 powerpack (Japan). Gel was then fixed in a solution with 10% methanol and 10% glacial acid for 40 - 60 minutes and dried on Drygel SR gel dryer set (Hoefer Scientific Instruments, San Francisco) for 40 minutes under vacuum at 80°C. Then the gel was directly exposed to a film.

2.10.2. Direct cycle-sequencing

Some PCR products were sequenced directly with a direct cycle sequencing kit (USB). The enzyme utilised in this protocol was derived from *Thermus aquaticus* DNA polymerase but was genetically modified. Taq DNA polymerase can tolerate a higher temperature and will synthesise DNA at elevated temperatures from single-stranded templates in the presence of a primer. Thus this protocol is slightly different from the

one described above. The radioactive labelling reaction was carried out in an initial extension phase and termination of DNA chain synthesis takes place during the subsequent phase.

Primer labelling was achieved by extending the specific primer just a few bases by leaving out one of the nucleotides from the labelling mixture. Either α-^{35}S-dATP or dCTP was incorporated into the extended primer. This method works only if the sequence for at least 10 bp downstream of the primer is known. The labelling reaction was carried out with 28.6 nM sequencing primer for 10 ng of gel purified PCR product with annealing buffer (29.7 mM Tris-HCl, pH 9.5, 7.4 mM $MgCl_2$) and the other two nucleotides (85.7 nM each) and 5 µCi (1000 Ci/mmol) of a radioactive nucleotide and 5 - 25 units of Taq polymerase on Programmable Thermal Controller (M J Research, Inc). The program was set for 30 cycles of 100°C, 10 seconds; 96°C, 15 seconds; 40° - 60°C, 30 seconds. The top mineral oil was removed from the microtubes for each sample. 3.5 µl labelled product from each reaction was aliquoted into 4 wells of a 96 well plate. 4 µl of each termination nucleotide mixture (each mixture contained 15 µM dGTP, 15 µM dATP, 15 µM dTTP, 15 µM dCTP and, in addition, the "G" mixture contained 22.5 µM dideoxy-dGTP, the "A" mix, 300 µM ddATP, the "T", 450 µM ddTTP, and the "C" 225 µM ddCTP) was added to each aliquot of labelled product on a 96 well plate. A drop of mineral oil was layered on the top of each well filled with reaction reagents. The reaction for termination was performed in a 96 well plate on Thermal cycler (Techne PHC-3) by 30 cycles of 95°C, 20 seconds; 50° - 70°C, 20 seconds; 72°C 1 minute. The plate was covered with a lid during incubation. The new strand labelled with [α-^{35}S] dATP or dCTP was continuously synthesised until either ddATP, ddCTP, ddGTP or ddTTP was randomly integrated into the strand. 4 µl of stop solution was added to each well and the sequencing product was stored at -20°C or denatured and then loaded to 6% polyacrylamide gel. The remaining steps were as described above.

2.11. Subcloning DNA fragments into a plasmid vector

2.11.1. Preparation of pEMBL cloning vector

pEMBL vector was digested with BamH I and EcoR I at 37°C for 6 hours. The phosphate of 5' termini of vector DNA was removed by incubation with CIAP to prevent self-ligation at 37°C for 60 minutes. The reaction was stopped by adding 0.5 M EDTA. The remaining CIAP in the solution would continue to eliminate the phosphate on the 5' termini of the insert if the cloning target fragment was added directly to the subsequent ligation reaction, so the phosphatase was removed by two extractions with phenol:chloroform and chloroform:isoamyl alcohol (24:1). The upper aqueous phase was precipitated with 0.5 volume of 7.5 M ammonium acetate and 2 volumes of ethanol at -20°C for 30 minutes. The precipitated DNA was then washed with 70% ethanol and resuspended in H_2O (Sambrook *et al,* 1989).

2.11.2. Ligation of DNA fragments to pEMBL vector

To obtain the optimal ratio of vector to insert DNA, 1:1, 1:3 and 3:1 molar ratios of vector:insert were tried when cloning a fragment into a plasmid vector. Ligation reaction was carried out at 16°C overnight as described above.

2.11.3. Preparation of DH5 competent cells

2.11.3.1. SOB plate

SOB agar medium (20 g tryptone, 5 g yeast extract, 5.8 g NaCl, 1.9 g KCl, pH 7.2 - 7.3, 15 g bacto-agar/litre) stock was melted in a microwave oven. When the medium had cooled to 60°C, $MgSO_4$ and Ampicilin were added at final concentrations of 10 mM and 50 µg/ml respectively, mixed and poured into the plates. The plates were covered,

swirled and left on bench for 1 - 2 hours. Then the plates were transferred to 37°C and inverted without lids to dry for 30 minutes (Sambrook *et al*, 1989).

2.11.3.2. Competent cells

DH5 strain (supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1) was streaked on a SOB plate without Ampicilin overnight. A colony from the plate was inoculated into 25 ml of SOB medium and incubated at 37°C overnight with vigorous shaking. 5 ml of cells from the overnight culture was inoculated into 500 ml of SOB medium with shaking at 150 - 200 rpm at 37°C until OD_{600} reached 0.45 - 0.55. Further manipulation was as described above for preparation of competent JM101 cells (Sambrook *et al*, 1989).

2.11.4. Transformation of DH5 competent cells with the ligated pEMBL vector

An aliquot of DH5 competent cells was thawed on ice. 100 µl of the cells was combined with the ligated DNA, gently mixed with a pipette and incubated on ice for 30 minutes. The mixture was heat-shocked for 90 seconds at 42°C. The tubes were then cooled on ice for 1 minute. 1 ml of SOB medium was added and the tubes were shaken gently at 37°C for 1 hour to allow the cells to recover. Cells were plated on the SOB plates containing 50 µg/ml Ampicilin and incubated at 37°C overnight.

2.11.5. Minipreparation of plasmid DNA from the transformed cells

Usually ten well isolated colonies from the Ampicilin selective plate were individually inoculated into 5 ml SOB medium containing 50 µg/ml Ampicilin and incubated at 37°C overnight. 1.5 ml of each culture was placed into an eppendorf tube and centrifuged at 14,000 rpm for 1 minute. The medium was removed by aspiration and the pellet was resuspended by vortexing in 100 µl of ice-cold miniprep lysis buffer (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM glucose) and incubated for 5 minutes at room temperature. 200 µl of a freshly prepared solution containing 0.2 M NaOH and

1% SDS was added, mixed by inversion and incubated on ice for 5 minutes. 150 µl of potassium acetate solution (3 M potassium acetate, pH 4.8) was added, mixed and incubated on ice for 5 minutes. The supernatant was transferred into a new tube after centrifugation at 14,000 rpm for 5 minutes, and digested with RNase A at final concentration of 20 µg/ml, incubating at 37°C for 20 minutes. The mixture was extracted with an equal volume of phenol:chloroform and DNA was precipitated, washed and resuspended in H_2O. A portion of plasmid DNA was digested with appropriate restriction enzymes and run on an agarose gel to check if there was an insert of the correct size.

2.11.6. Storage of *E. coli* cells transformed with the target gene

In order to store the bacterial culture, 1 ml was transferred into 1.5 ml sterile tube. 200 µl of sterile glycerol was added to the culture. The mixture was then vortexed to ensure that the glycerol was evenly dispersed in the medium. The storage tube was equipped with a screw cap and labelled. The tube was frozen in liquid nitrogen and then transferred to -70°C for long term storage.

2.11.7. Large-scale preparation of plasmid DNA

A single colony of plasmid-harbouring bacteria was inoculated into 5 ml of SOB medium containing 50 µg/ml Ampicilin, and shaken at 37°C overnight. 400 ml of SOB was inoculated with 1 ml of the overnight culture, and shaken vigorously at 37°C overnight. The cells were harvested by centrifugation at 4,000 rpm for 15 minutes at 4°C in a Sorvall GS3 rotor. The cell pellet was washed and well resuspended in 10 ml of Solution I (50 mM glucose, 25 mM Tris-HCl (pH8.0), 10 mM EDTA, pH8.0) after the supernatant was removed. 20 ml of freshly prepared Solution II (0.2 M NaOH, 1% SDS) was added and mixed thoroughly by gently inverting. 15 ml of ice-cold Solution III (3 M potassium acetate, pH 4.8) was then added and the mixture was mixed and stored on ice for 10 minutes. The lysate was centrifuged at 4,000 rpm for 15 minutes at

4°C in a Sorvall GS3 rotor. The supernatant was then transferred into a new bottle and mixed well with 0.6 volumes of isopropanol and stored for 10 minutes at room temperature. The nucleic acids were recovered by centrifugation at 5,000 rpm for 15 minutes at room temperature in a Sorvall GS3 rotor. The supernatant was decanted carefully and the pellet was rinsed with 70% ethanol at room temperature. The pellet was dissolved in 12 ml of TE (pH 8.0) (Sambrook *et al*, 1989).

2.11.8. Purification of supercoiled DNA by equilibrium centrifugation in CsCl-ethidium bromide

12 ml solution was transferred to a small centrifuge tube suitable for a Sorvall SS34 rotor and spun at 10,000 rpm for 10 minutes, and the supernatant was transferred to a new tube. 12.7 g CsCl, 960 µl of 10 mg/ml ethidium bromide and 120 µl of 10% Triton were added. To remove debris and precipitation, the solution was spun at 3000 rpm for 10 minutes. The clear, red solution was then transferred to a Beckman Quick-Seal centrifuge tube, sealed and spun at 50,000 rpm at 20°C for 24 hours in NVT65 rotor of a Beckman L7 ultracentrifuge. To collect the band of closed circular plasmid DNA, a 21-gauge hypodermic needle was inserted into the top of the tube to allow air to enter. A second needle (18-gauge hypodermic needle) was inserted into the tube so that the open, bevelled side of the needle was positioned just below the plasmid DNA band. The plasmid DNA was sucked with a 3 ml syringe in a dark room under UV light. The plasmid DNA was transferred to a 20 ml tube. The solution was then aliquoted into eppendorf tubes and the ethidium bromide was removed by repeated extraction with water-saturated 1-butanol. To remove CsCl from the DNA solution, the solution was diluted with 3 volumes of water and the DNA was then precipitated with 2 volumes of ethanol for 15 minutes at 4°C followed by centrifugation at 15,000 rpm for 15 minutes at 4°C. The pellet was dissolved in 500 µl of TE. The concentration of DNA was determined by measuring the OD_{260}. The purified plasmid DNA was stored at -20°C.

2.11.9. Isolation of DNA probes

60 µg of plasmid DNA was incubated with 60 units an appropriate restriction enzyme(s) at 37°C for 8 hours. The digested plasmid DNA was loaded onto a 0.75% ultrapure agarose gel and run along with DNA size maker in 1 x TAE buffer overnight. The gel was then stained with ethidium bromide at final concentration of 0.5 µg/ml for 30 minutes. The slice of agarose containing the probe band was cut out with a razor blade. The DNA probe was obtained by the freeze squeeze method as described above.

2.12. Computer analysis of DNA sequences

For analysis of restriction enzyme recognition sites, determination of the presence or absence of other specific sequence and to compare sequences, the program DNA Strider was used on an Apple Macintosh. Comparisons of sequences with the Genbank or EMBL database were made using BLAST (Altschul *et al* 1990) or FASTA (Pearson & Lipman, 1988), available within the Genetics Computing Group (GCG) package by network connection to MRC Human Genome Mapping Project Resource Centre (Hinxton).

2.13. Statistical analysis

The binomial probability distribution was used to analyse the distribution of breakpoints in the vicinity of ABL exon 2 to determine whether the breakage is random or not.

CHAPTER THREE ESTABLISHMENT OF BUBBLE PCR PROCEDURE

3.1. Introduction

To understand better the mechanism responsible for the reciprocal translocation between chromosomes 9 and 22 that occurs in CML and to detect minimal residual disease at the genomic level, a rapid method to characterise the BCR-ABL and ABL-BCR breakpoints at the DNA level is required. Classically, this would have been done by constructing genomic DNA libraries for individual CML patient, and then screening and isolating the breakpoints from the libraries. Although technically feasible, this approach would be very labour intensive, time consuming and expensive. I decided therefore to explore the possibility of PCR-based methods for cloning of BCR-ABL genomic breakpoints. Since the position of genomic breakpoint in the ABL gene for individual patient is spread over more than 200 kb in length (Bernards *et al* 1987), a new method must not be completely sequence-dependent. When I started this project there were several published PCR procedures which had been developed to clone small regions of unknown sequence next to a region of known sequence. These methods were developed initially for different purposes, for example, vectorette mediated PCR was established to facilitate rapid cloning of cosmid of YAC clone ends for use as probes in chromosome walks (Riley *et al* 1990). These techniques can however be adapted to the cloning of chromosomal translocation breakpoints. Each method is described briefly below.

3.1.1. Inverse PCR

Inverse PCR was described independently by three laboratories (Ochman *et al* 1988; Triglia *et al* 1988; Silver & Keerikatte, 1989) before I started this study. The approach to inverse PCR is to cut double-stranded target DNA with an appropriate restriction

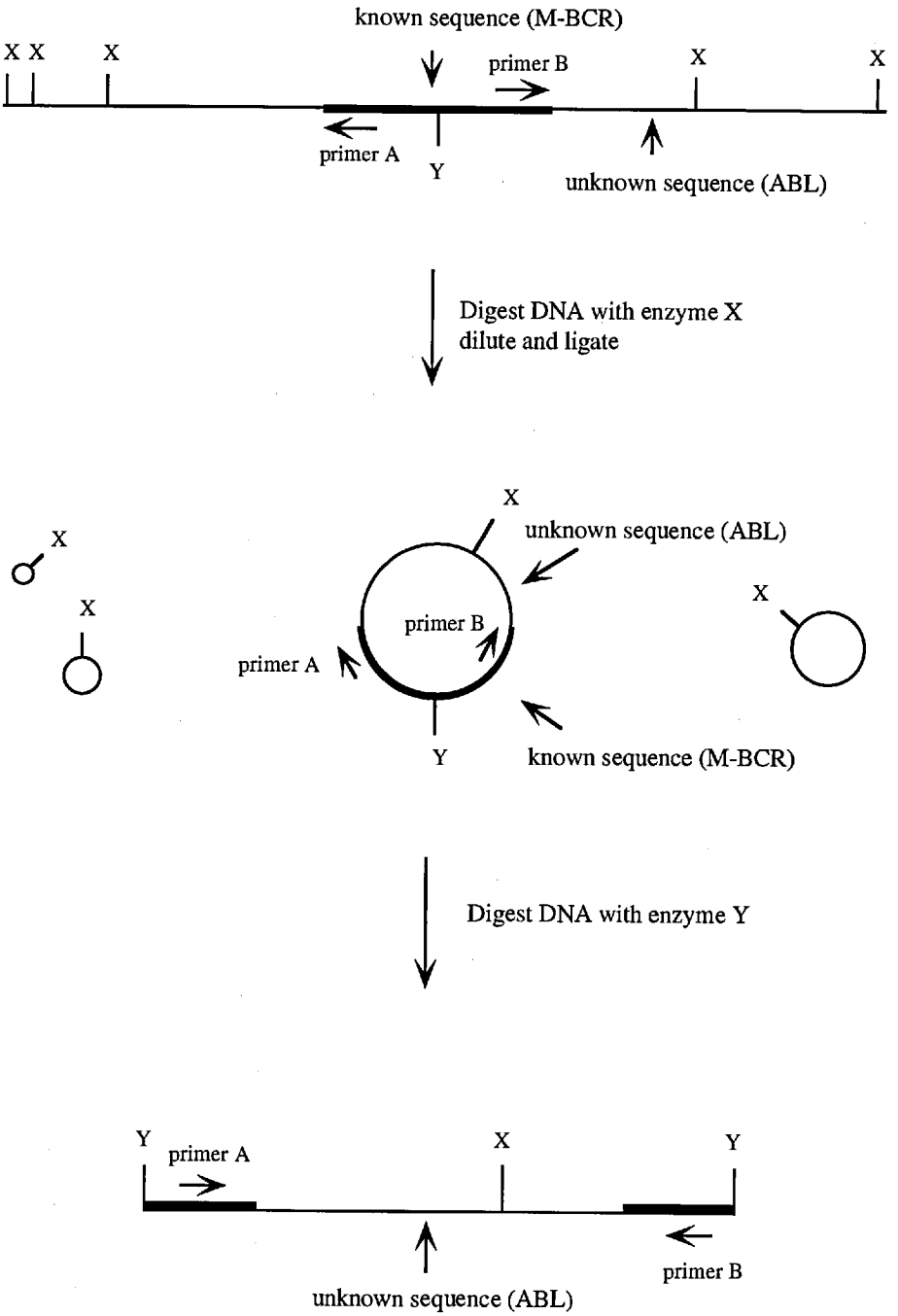


Figure 3-1. Inverse PCR by circular permutation.

A DNA strand with a segment of known sequence (heavy line) is cut with a restriction enzyme X, diluted and ligated to form circles. Circles containing the region of known sequence plus flanking DNA are linearised by cutting with a restriction enzyme Y. Primers A and B are then oriented to amplify the unknown sequence.

enzyme, for example, enzyme X (Figure 3-1), followed by dilution and self-ligation to form circles. One of these circles should contain the unknown sequence next to the region of known sequence. An additional enzyme Y is used to linearise the covalently closed circular double stranded DNA within the region of known sequence. The unknown sequence is now flanked by regions of known sequence and so two primers (primer A and primer B) can be designed complementary to the known sequences to amplify the unknown sequence.

3.1.2. Fors linker ligation PCR

Fors linker ligation PCR (Fors *et al* 1990) was developed to clone the shark protein zero (Po) promoter in a study of vertebrate nervous system. It is an alternative way to clone unknown region by PCR (Figure 3-2). In this procedure, genomic DNA was cleaved by a restriction enzyme that makes a staggered cut. Modified T7 Polymerase (Sequenase™) was used to specifically generate a blunt ended DNA fragment of the target region with a primer to the known sequence after denaturation. A blunt ended double stranded linker was then ligated to the DNA fragments with the modified ends. The linker can only ligate to the end of the target DNA fragment since only this fragment will have a blunt end. PCR was then performed with one primer annealing to the linker and the other annealing to the area of the known sequence.

3.1.3. Single primer mediated PCR

Single primer-mediated PCR (Parks *et al* 1991) employs only one primer for the reaction (Figure 3-3). A PCR primer is synthesised to anneal to the known sequence within a target region. Under low stringency conditions (a low annealing temperature) the primer may also anneal to a region of unknown sequence downstream of the known sequence in the opposite direction. Thereby, the target sequence can be amplified after subsequent cycles of PCR. In addition, non-specific amplification products are also produced at the same time since the primer can anneal non-specifically to many

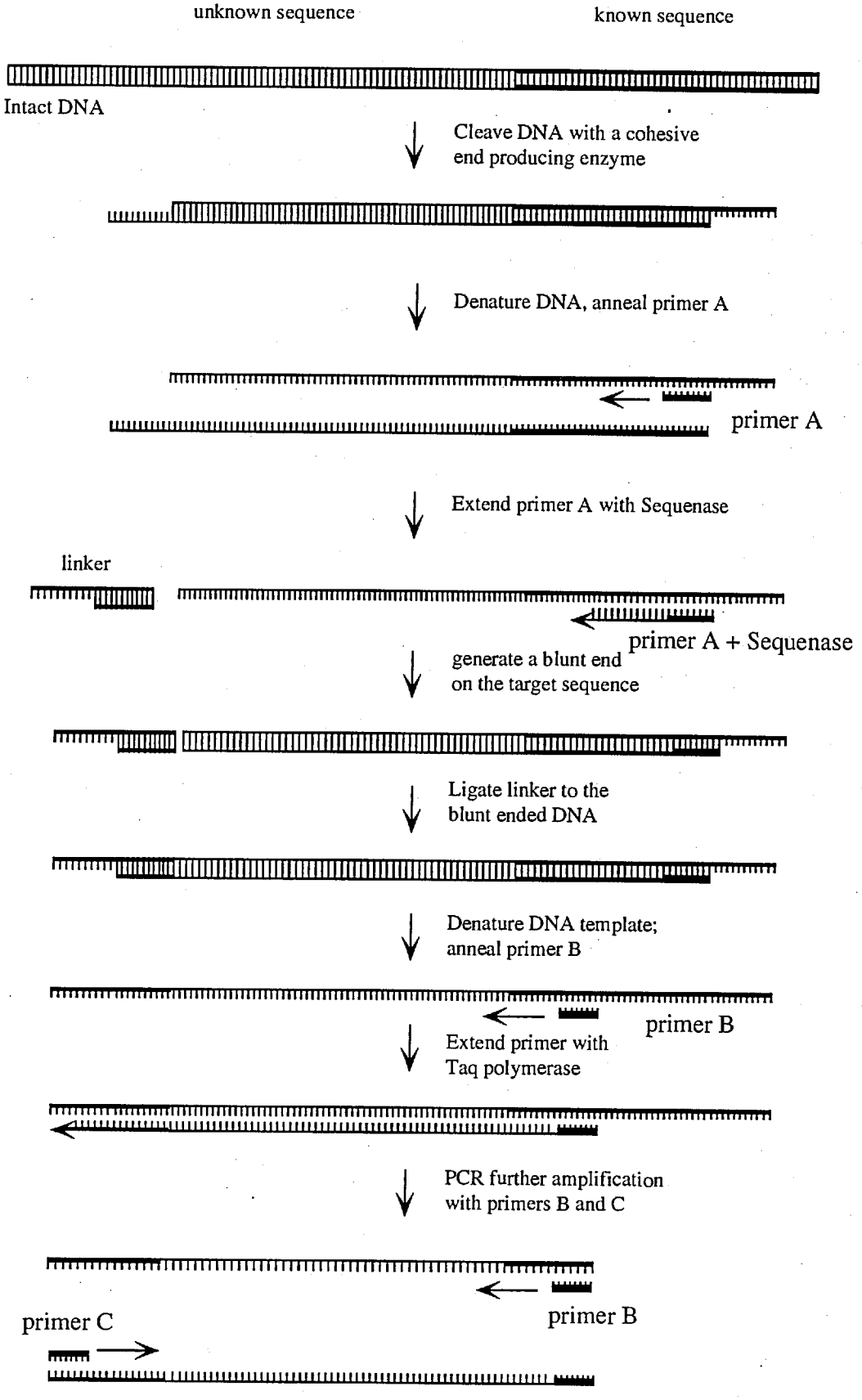


Figure 3-2. Fors linker ligation PCR

Genomic DNA is digested with a cohesive end producing enzyme. The DNA is denatured and primer A is annealed and extended with Sequenase. Then a blunt ended linker is ligated to the blunt ended DNA segments modified by Sequenase. Subsequently the DNA is amplified with primers B and C.

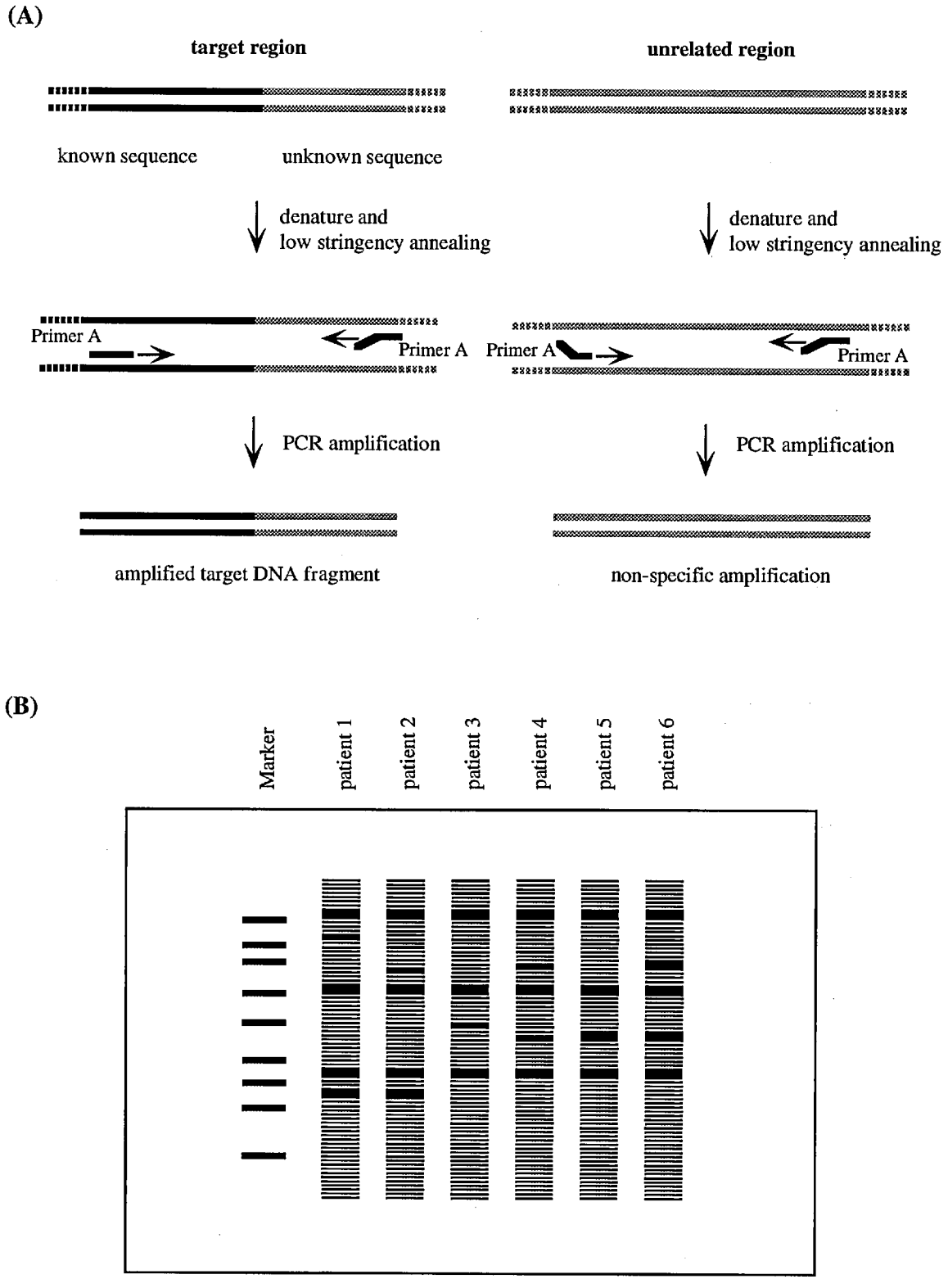


Figure 3-3. Diagrams for single primer mediated PCR

(A) By use of a low annealing temperature primer A anneals specifically to its complementary sequence, and also non-specifically to other sites. (B) PCR amplification products appear as a smear on an agarose gel.

different sites. Compared to other PCR methods to amplify an adjacent sequence of known DNA fragment, this method requires no enzymatic manipulation or linker ligation. This technique has been demonstrated as suitable for limited chromosome walking, and therefore it has also the potential for cloning a genomic breakpoint.

3.1.4. Panhandle PCR

Panhandle PCR (Jones & Winistorfer, 1992) is a novel method that requires the generation of a template shaped like a pan with a handle. In this method, a known DNA segment is synthesised downstream of the unknown sequence by copying the known sequence after the target DNA is 'intra-strandedly' circularised into a pan-like structure (Figure 3-4A). Generation of this template permits specific amplification of the unknown sequence, since known sequence now flanks the unknown region. The procedure is briefly described as follows: genomic DNA is subjected to digestion with a restriction endonuclease X. Then DNA fragments are dephosphorylated by alkaline phosphatase and then this enzyme is removed by the gene clean (Bio 101) procedure. The DNA is ligated with T4 DNA ligase to a single stranded oligonucleotide that is complementary to part of the known sequence. The ligated DNA is denatured and allowed to self-anneal to form a pan-like structure DNA (Figure 3-4A). The annealed end is then extended by DNA polymerase and PCR amplification performed with primers annealing the panhandle portions. Finally nested PCR with internal primers is used to specifically amplify the target region (Figure 3-4B).

3.1.5. Bubble/Vectorette mediated PCR

The Bubble/vectorette mediated PCR was described previously for cloning an unknown DNA region next to a known DNA sequence from yeast artificial chromosome (YAC) library (Riley *et al* 1990). The technique is essentially the same as Bubble PCR (Smith, 1992; Green, 1993) which was established with λ DNA. The principle of the technique is as follows.

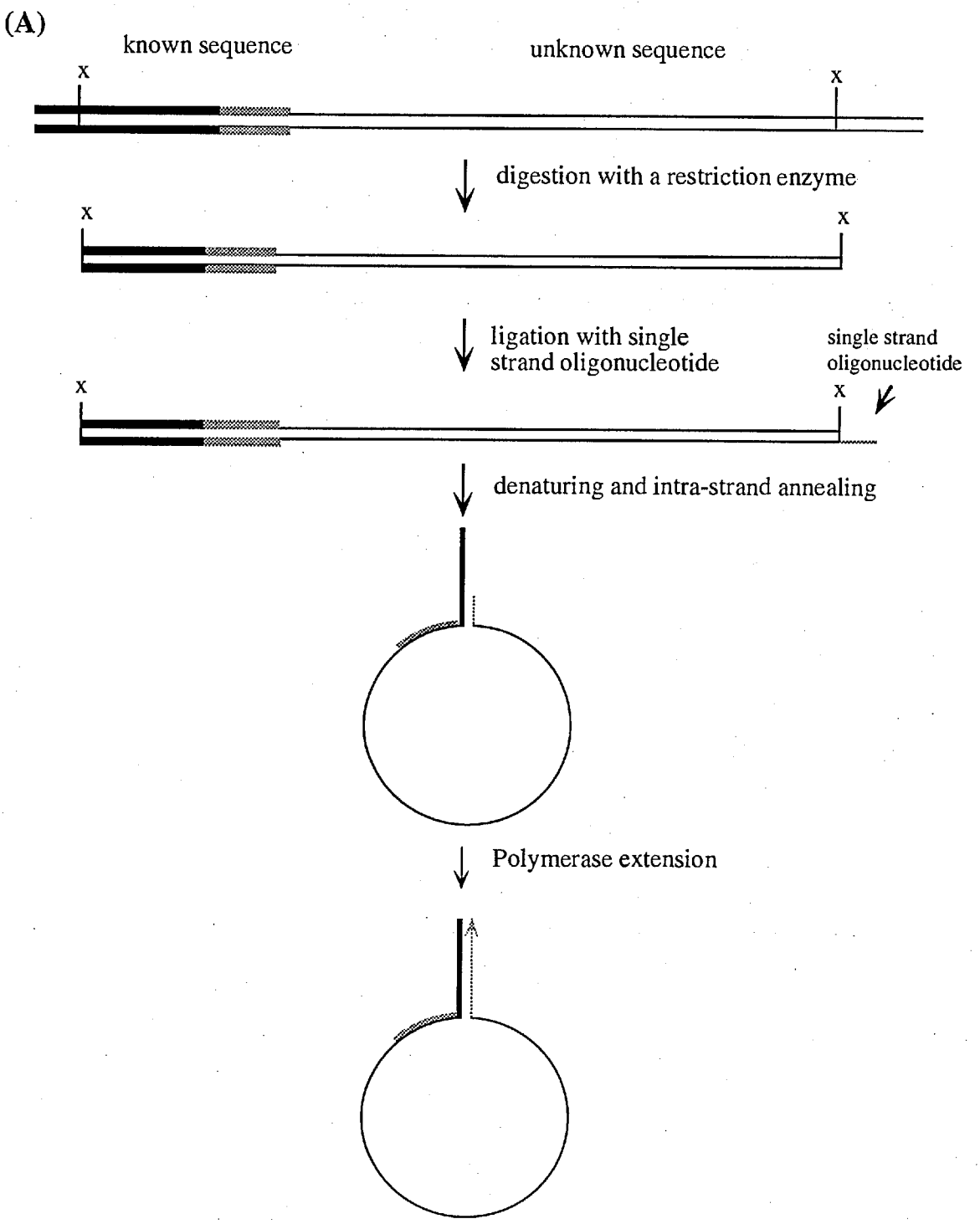


Figure 3-4A. Panhandle PCR. Double stranded known DNA (▬ complementary with the single stranded oligonucleotide and Panhandle region; ▩ non-complementary region) flanking an unknown DNA (– unknown DNA region) is cut with a restriction enzyme x. The DNA is ligated to a single oligonucleotide (— the single oligonucleotide and complementary to the region in the handle). Then the DNA template is denatured and intra-strandedly annealed to form the Panhandle structure.The ligated single stranded oligonucleotide is extended by DNA Polymerase.

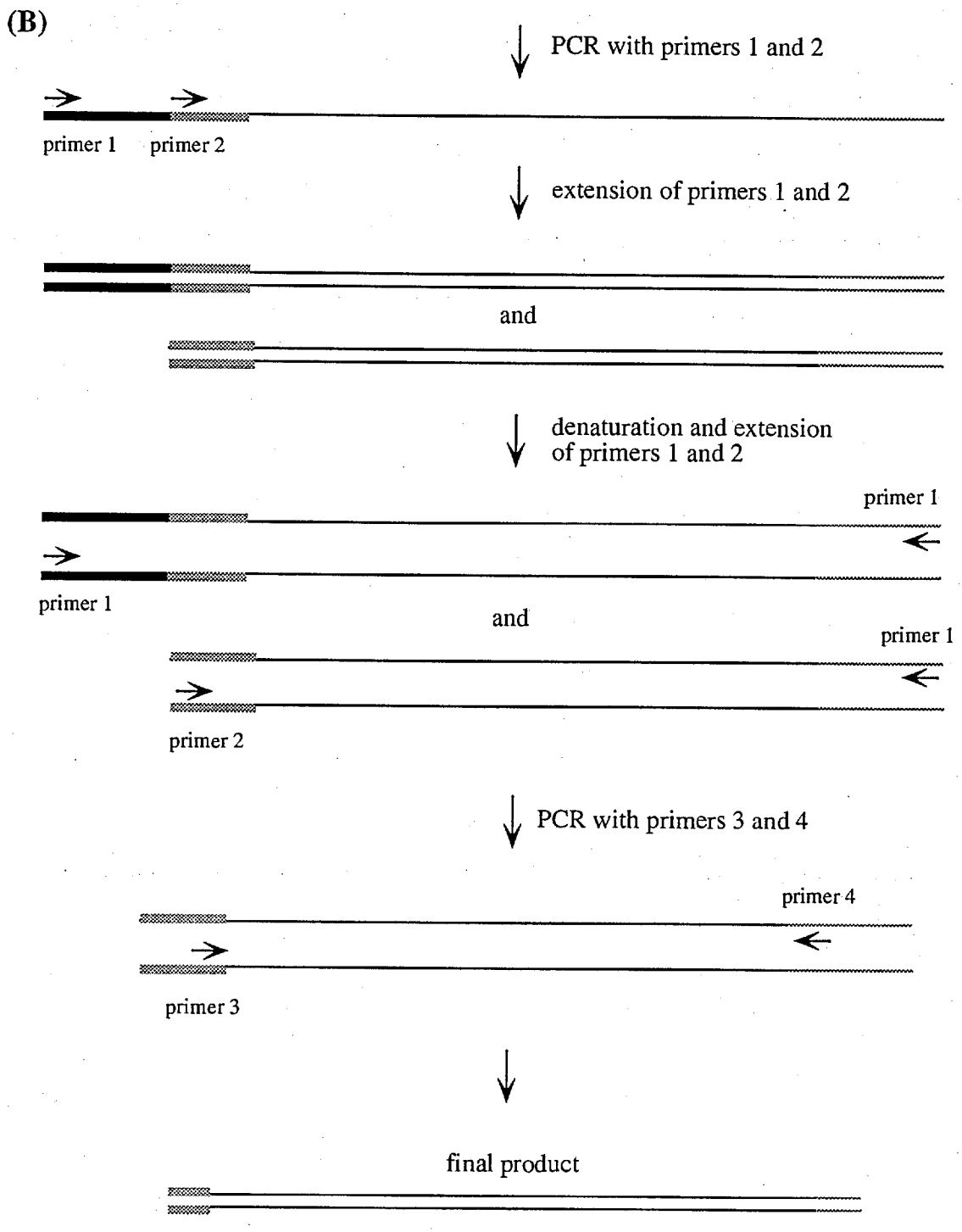


Figure 3-4B. Panhandle PCR. PCR amplification with primers 1 and 2 is performed after denaturation of the DNA strands. Two products are generated: one is amplified with primer 1 alone and the other is amplified with primers 1 and 2. The following PCR with the nested primers 3 and 4 amplify a specific product from the target region.

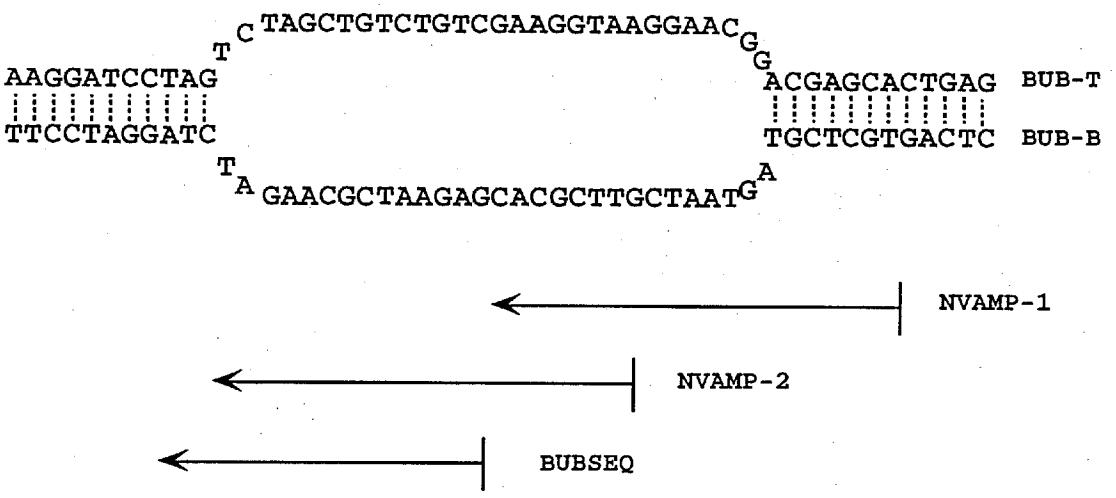
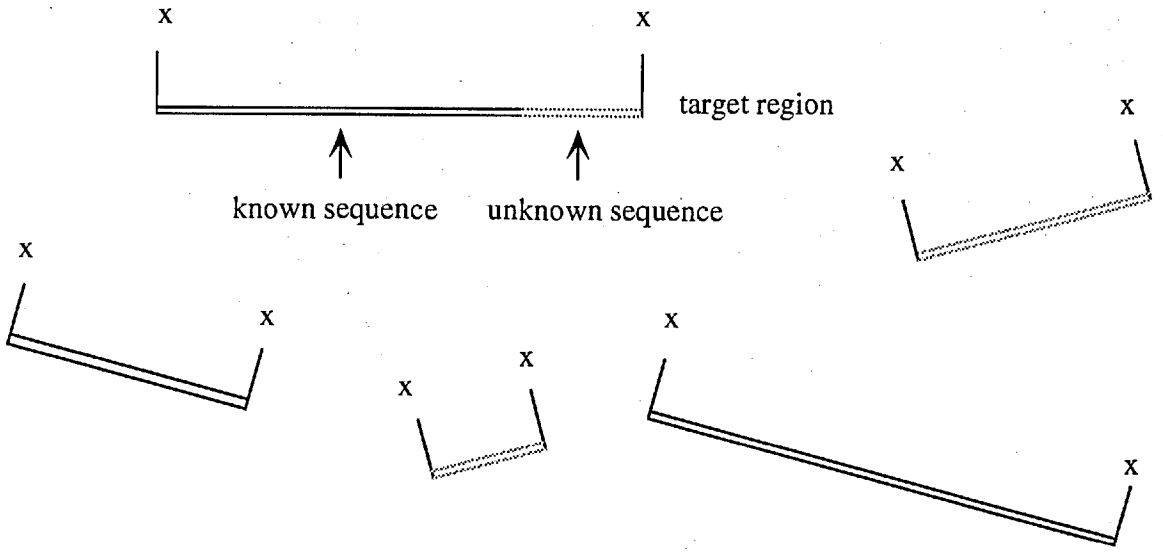


Figure 3-5. Heteroduplex structure of Bubble Oligonucleotide

Bubble PCR primers, NVAMP-1 and NVAMP-2, and sequencing primer, BUBSEQ, are indicated by arrows.

The 'Bubble' oligonucleotide was a duplex that contained a complementary region at each end and a non-complementary sequence in the middle. To make the Bubble oligonucleotide, the two oligonucleotides (BUB-T and BUB-B; Figure 3-5) are annealed. Genomic DNA is digested with a restriction enzyme and ligated to the double stranded Bubble oligonucleotide (Figure 3-6, A and B). The DNA is denatured at 100°C and a specific primer (primer A) anneal to the target DNA. The primer is extended by Taq polymerase to the end of the Bubble oligonucleotide (Figure 3-6C). Amplification of the target strand was achieved by PCR using the same primer and a second primer (primer B) which was complementary to the newly synthesised unique sequence of the Bubble oligonucleotide (Figure 3-6D). This provided the specificity of the reaction since the unique Bubble sequence will only be copied in the target DNA fragments, and not for any other fragments.

A. Digestion with restriction enzyme x.



B. Ligate Bubble oligo

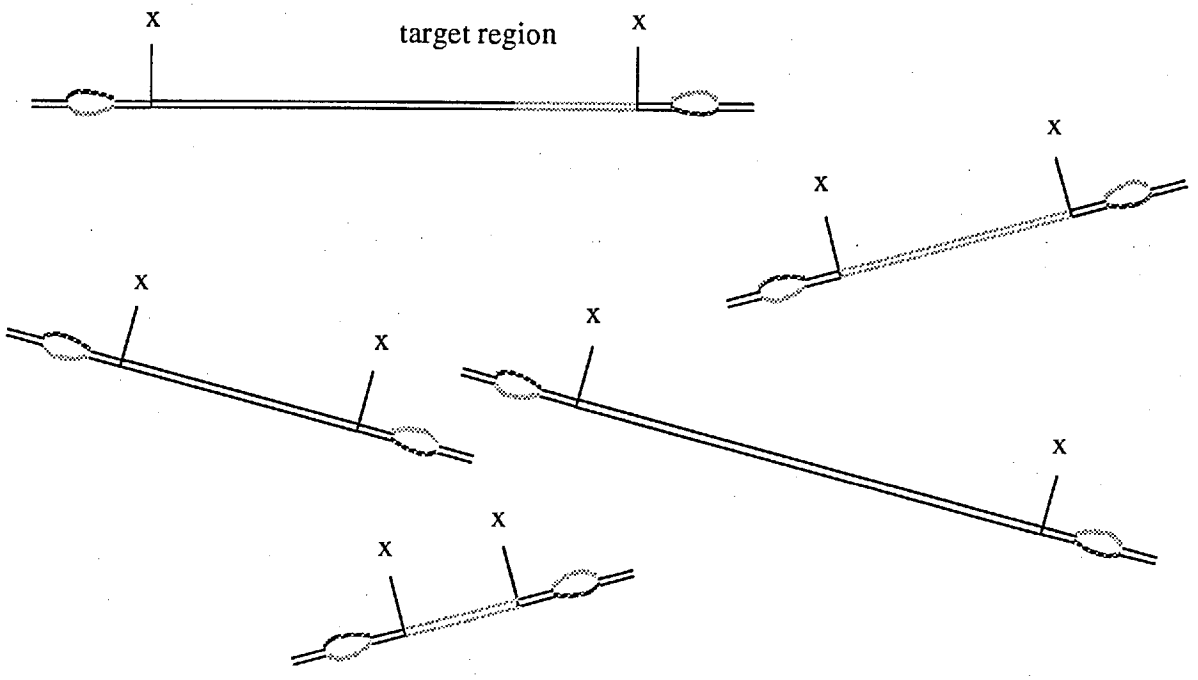
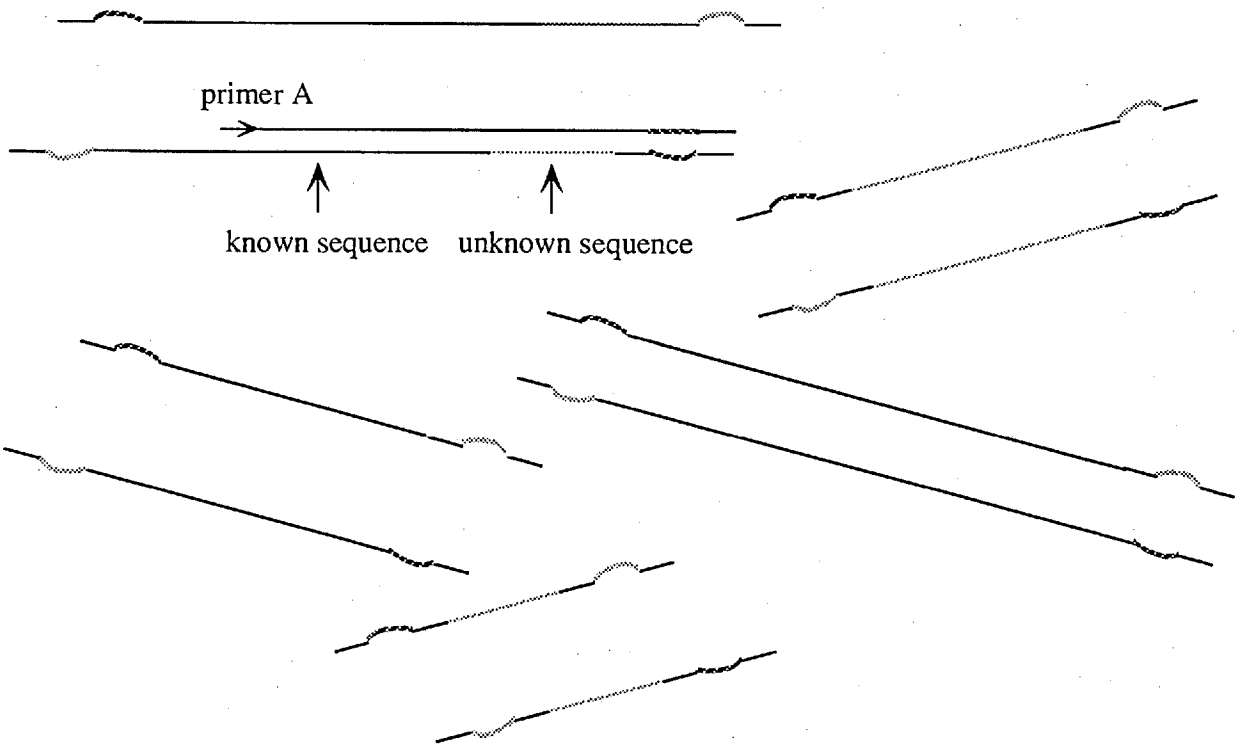


Figure 3-6 (a). Bubble PCR Scheme

(A) Genomic DNA is digested with a restriction enzyme x. (B) The DNA fragments are ligated to Bubble oligos. At this stage all fragments are ligated with Bubble oligos at the both ends.

C. Denature DNA. Anneal and extend primer A



D. PCR using oligos A and B to amplify the target region

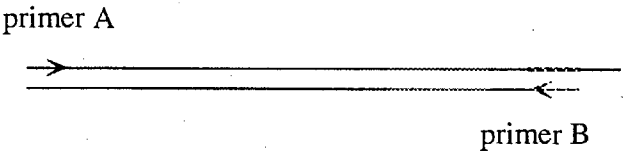


Figure 3-6 (b). Bubble PCR Scheme

(C) A specific primer A is specifically annealed to the known sequence next to the unknown region and then extended by DNA Polymerase. At the ends of the target fragments an unique sequence complementary to the Bubble is synthesised. (D) Primer B is annealed at this unique sequence and the target fragment with an unknown region is amplified.

3.2. Results

3.2.1. General strategy for developing a site specific cloning method

One-sided PCR techniques consist of two primary phases: 1) a selection phase during which the desired DNA fragment is specifically targeted and 2) an amplification phase during which the copy number of the desired DNA fragment increases exponentially. For low complexity templates, such as plasmids, cosmids, or previously amplified products, the screening stage is vastly simplified and are thus likely to amplify with a broader set of conditions than genomic DNA. For this reason, I initially utilized a plasmid construct containing the M-BCR (p4.8Bgl-1) to develop a system suitable for the amplification of BCR-ABL breakpoints..

After the expected size band was obtained from p4.8Bgl-1, I tested the procedure on genomic DNA samples from the HL60 cell line and from CML patients. HL60 is a human promyelocytic leukaemia cell line; it does not have the BCR-ABL translocation but does have a normal BCR gene. This cell line has routinely been used as a BCR-ABL translocation negative control (Melo *et al* 1993a; Melo *et al* 1996). The procedure was shown to be working when the expected bands were obtained on an agarose gel after the one-sided PCR procedure was performed on HL60 genomic DNA cleaved by different restriction enzymes.

The reproducibility of the system was checked using various enzymes and various BCR primers. Amplified products from HL60 and patients with CML were gel purified, cloned and sequenced. The sequence of the fragment obtained from sequencing reaction was compared with the known sequence of the M-BCR (David Leibowitz, personal communication; Sowerby *et al*, 1993; Chissoe *et al*, 1995).

3.2.2. Construction of pBgl 4.8-1 plasmid

To construct a plasmid clone containing the M-BCR, DNA from cosmid CA22 (kindly provided by Dr. G. Grosveld) (Groffen *et al* 1984) was digested by Bgl II. A 4.8 kb M-BCR DNA fragment [subsequently found to be 5.0 kb from sequence data (Sowerby *et al,* 1993; Chissoe *et al,* 1995)] was isolated and subcloned into the BamH I polycloning site of a pEMBL 8(+) vector. The new construct was named as p4.8Bgl. Thus the DNA template was simplified for establishment of Bubble PCR procedure.

To confirm that the insert was cloned into the vector, 5 individual p4.8Bgl transformants were selected and DNA isolated by the miniprep method (Sambrook *et al,* 1989). 8 µl of each DNA suspension from p4.8Bgl was digested with restriction enzymes Hind III only and Hind III combined with EcoR I. There are no EcoR I sites in the M-BCR insert but there is a single site for EcoR I in pEMBL vector. pEMBL vector has a single site for Hind III but the M-BCR has two sites for Hind III (Figure 3-7A). Miniprep DNA was digested and run on a gel to check the M-BCR insert. DNA from 3 colonies showed the expected size bands (Figure 3-7B). Colony 1 was selected and designated p4.8Bgl-1. This plasmid was grown up in bulk culture and the insert further confirmed by restriction enzyme digestion with Hind III and BamH I in terms of the positions of the sites defined previously by Southern blotting (Groffen *et al,* 1984; Jaubert *et al,* 1990; Mills *et al,* 1991b) and the partial known sequence of the M-BCR.

3.2.3. Initial attempts to establish a PCR-based technique

3.2.3.1. Initial attempt 1: Adaptation of Fors linker ligation PCR

Initially I considered the Fors linker ligation PCR (Fors *et al* 1990) to be a potentially useful technique to characterise BCR-ABL breakpoints for CML. The technique has a number of advantages. First, any enzyme making cohesive ends of DNA fragments can be used for cleavage of the BCR or ABL gene. The range of possible enzymes is

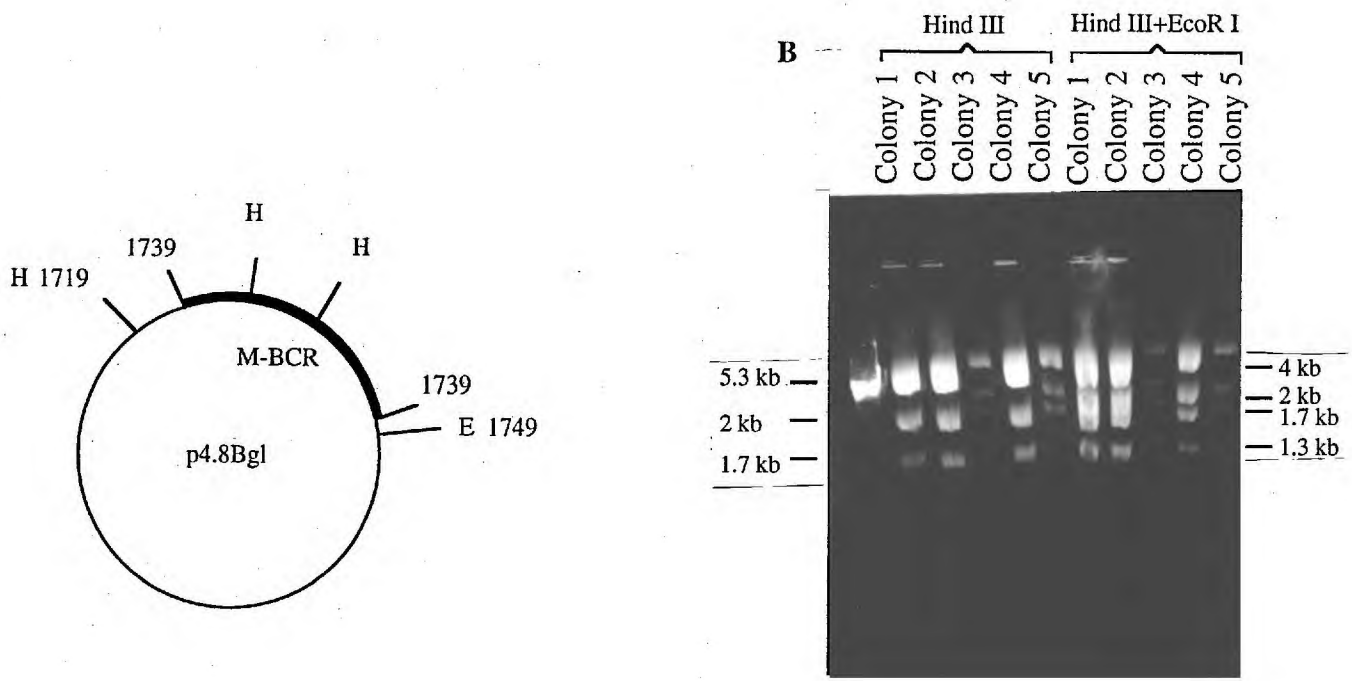


Figure 3-7. Construction of p4.8Bgl

A) 5.0kb M-BCR fragment was cloned into the BamH I site at position 1739 of the EMBL plasmid vector. H and E indicate Hind III and EcoR I sites respectively. B) DNA from 5 colonies were digested with Hind III only, Hind III and EcoR I respectively. Plasmids in colonies 1, 2 and 4 were with a correct insert.

therefore large. For cloning of the BCR-ABL breakpoint from individual CML patients, a BCR primer is selected as close to the breakpoint as possible for PCR amplification. Therefore, flexibility of possible restriction enzymes would increase a chance to amplify the breakpoint in each patient. The second advantage is that PCR amplification is allele specific. This specificity is provided by the ligation with the modified blunt ended DNA fragments that are synthesised by Sequenase from specific primer. The third advantage is that fragments of the normal allele (BCR) and the abnormal allele (BCR-ABL) should be distinguishable in size when the PCR products are run on a gel, since BCR and BCR-ABL will have different restriction patterns. Hence it should be possible to identify the BCR-ABL fragment in an agarose gel for gel purification and DNA cloning. For these reasons, this is the first method that I tried.

Initially I started the system with the frequent cutter Taq I and two M-BCR primers (B2A and B3A). 20 ng DNA from plasmid p4.8Bgl-1 was digested with 1 unit of Taq I (GIBCO) at 65°C for 6 hours. 5 ng DNA was aliquoted into 4 tubes. One tube was tested with primer B2A, the second tube with primer B3A, the third without primer and the fourth without Sequenase. At the time I knew that there were four sites for Taq I in the 2,541 bp known M-BCR sequence (size range 119 bp - 968 bp) and five sites for Taq I in the vector pEMBL (size range 357 bp - 1,440 bp). All the known fragments were within the amplification range of PCR. Amplification of the fragment from primer B2B to the next Taq I site was expected to produce a band of 520 bp (Figure 3-8). A fragment of 310 bp was expected to be amplified from primer B3B. Primers B2A and B3A are located immediately upstream of the primers B2B and B3B respectively. The primers B2A and B3A for making a blunt end of the target fragment were mixed separately in a reaction tube with 5 ng of Taq I-digested p4.8Bgl-1 DNA in Sequenase buffer (100 mM $MgCl_2$, 200 mM Tris-HCl, pH 7.5, 250 mM NaCl) in a volume of 14.5 µl. The DNA was denatured in microtubes at 95°C for 5 minutes and the primer was annealed at 45°C for 30 minutes on a Programmable Thermal Cycler (M.J. Research, Inc.). When the annealing temperature was reached in the tubes, 3.5 µl of 5 mM

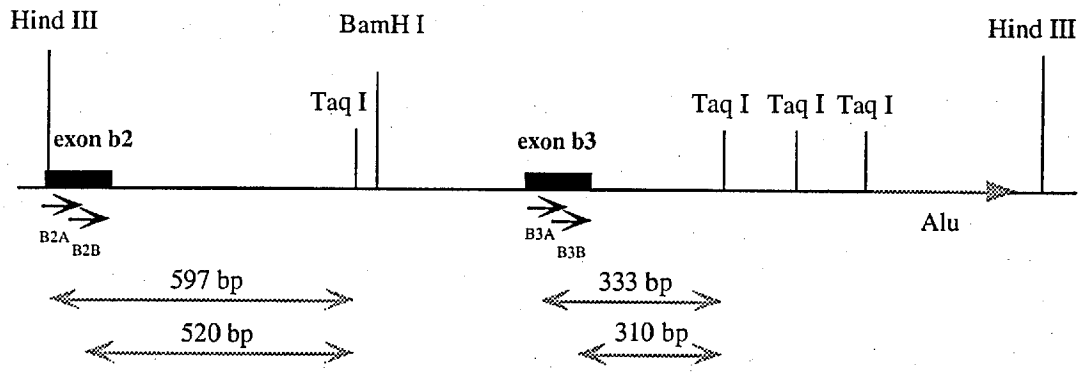


Figure 3-8. Strategy for amplification of M-BCR with enzyme Taq I in p4.8Bgl-1

Taq I recognition sites in the M-BCR. Primer sites are indicated by solid arrows. The distance between primers B2A, B2B, B3A and B3B and the next Taq I sites is indicated.

dithiothreitol (DTT), 100 nM of each deoxynucleotide triphosphate (dNTP) and 2 µl (6.5 units) of freshly diluted Sequenase (version 2.0, USB) [1:3 in enzyme dilution buffer (10 mM Tris-HCl, pH 7.5, 5 mM DTT, 0.5 mg/ml BSA, USB)] were added to the tubes. The mixture was incubated at 45°C for 5 minutes. The modified DNA fragment was then ligated with linker BVEC (Figure 3-9). The sequence of the linker (BVEC) I used was different from the published one (Figure 3-2). However, the structure (blunt ended double stranded oligonucleotide) was similar. To facilitate subsequent cloning, there is a BamH I site at the 5' end. 14 µl (3.5 ng) of the modified DNA fragments were mixed with 4 µl of 10 x ligation buffer (300 mM Tris-HCl, pH7.8, 100 mM MgCl2, 100 mM DTT and 5 mM ATP), 1 µl (5.9 fmoles) of linker BVEC and 1 µl (1 unit) of T4 DNA ligase (GIBCO BRL). The mixture was incubated at 16°C overnight. PCR amplification of the ligated p4.8Bgl-1 DNA with primers B2B, B3B in combination with primer Vecamp-1, designed to anneal to the linker, carried out along with a negative control, the one tube without modified DNA. The PCR product was run on a gel with 0.5% ethidium bromide and visualised under UV light. However, no specific amplification was obtained on multiple attempts (not shown).

To check whether the Sequenase really worked in the reaction to generate a blunt ended target, the activity of the enzyme was monitored with α-^{32}P-dCTP. The four reactions were repeated as above with both p4.8Bgl-1 DNA and HL60 genomic DNA respectively. The reaction was performed at 37°C and 45°C respectively for both p4.8Bgl-1 and HL60. The modified DNA was then directly run on a 6%

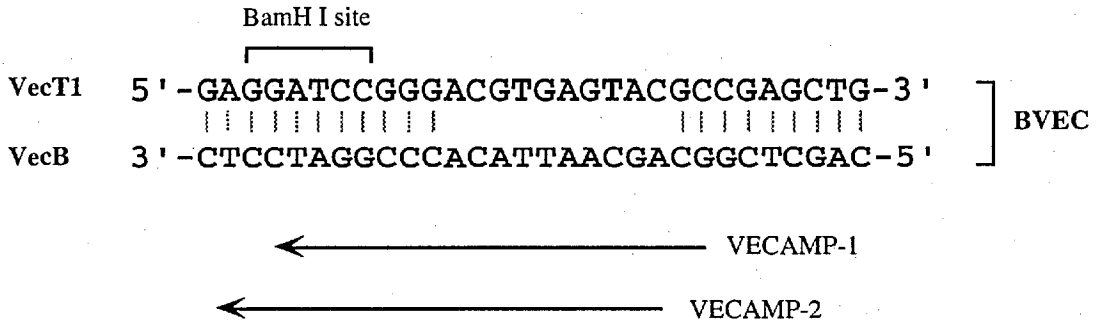


Figure 3-9. BVEC oligonucleotide.

BVEC oligonucleotide consists of top strand (VECT1) and bottom strand (VECB). BamH I recognition site is indicated. There is an uncomplementary region in the central and two complementary regions at the both ends. VECAMP-1 and VECAMP-2 are PCR primers.

polyacrylamide gel to see any ^{32}P had been incorporated. After running, the gel was fixed to 3MM paper, dried and exposed to a film. The modified DNA from p4.8Bgl-1 and HL60 all were strongly radioactive at both temperatures. The reason why the procedure failed was unclear but could have been due, for example, to a problem in ligating the modified DNA with the linker.

3.2.3.2. Initial attempt 2: Single stranded oligonucleotide-mediated PCR

Whilst considering the problems mentioned above, an idea for a novel single stranded linker method occurred to me. Genomic DNA is digested with a restriction enzyme and then a specific PCR primer (primer A) is annealed to the M-BCR after denaturation. The primer is extended to produce single stranded DNA fragments. A single stranded oligonucleotide (oligonucleotide C) can be ligated to the newly synthesised single stranded DNA fragments. Then a new complementary strand is generated with primer B

by copying the strand ligated with oligonucleotide C. Then the target DNA is amplified further with primers A and B (Figure 3-10). If the first PCR primer is specifically annealed to the region of interest, then the amplification achieved with those two primers should be faithful to the original template.

This approach should be feasible. According to previous publications, T4 RNA ligase can be used to ligate single stranded DNAs under certain conditions (Snopek *et al* 1976; Tessier *et al* 1986). The stringency for the first PCR primer specifically annealing can be easily controlled by variation of the annealing temperature.

There were various theoretical advantages of this method. First, there is no constraint on the number or type of restriction enzymes which can be used. Any enzyme which can perform complete digestion of human genomic DNA can be used. Second, any potential problem specific to double stranded oligonucleotides will be eliminated, such as, instability of double stranded linker structure due to nucleotide pairs mismatching, leading to low efficiency ligation. Third, manipulation of single stranded oligonucleotide might be easier than double stranded oligonucleotide since there is no need to maintain a duplex structure. Therefore, a new protocol for this single stranded oligonucleotide-mediated PCR was designed.

Oligonucleotide VecB (Figure 3-9) was used as this linker. The sequence in VecB strand of BVEC oligonucleotide was randomly designed, and was searched to the Genbank database. No significant homology with the human genome were found at that time. Therefore, a primer designed for VecB strand would hopefully not result in significant nonspecific annealing.

The oligonucleotide used in the ligation reaction must first be modified to phosphorylate the 5' hydroxyl group of the deoxyribose at the 5' end of VecB

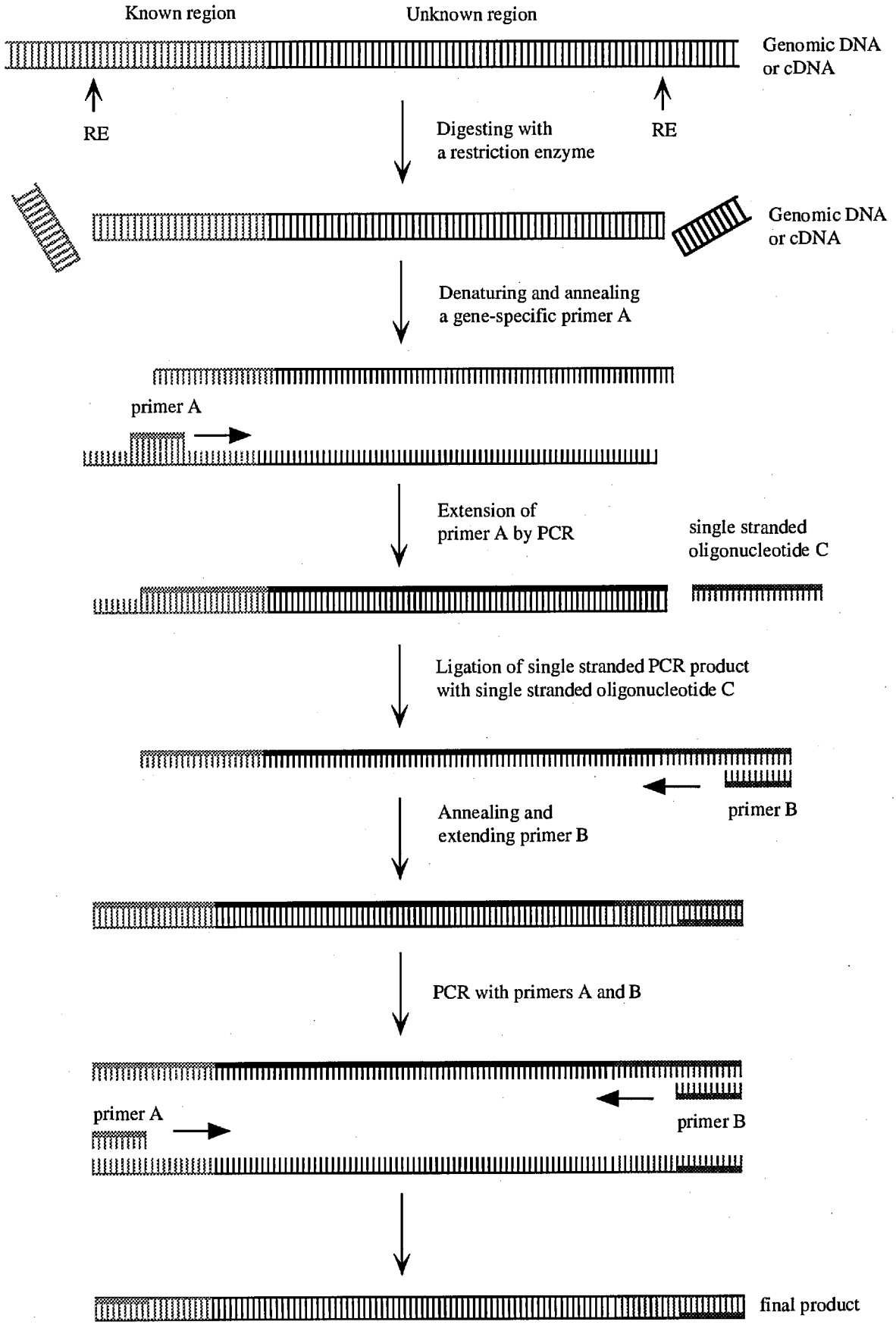


Figure 3-10. Strategy for single-stranded DNA ligation and PCR amplification

Primer A is specifically annealed to the known region of the gene and extended by PCR to generate single stranded DNA. An arbitrary oligonucleotide C is ligated to the single stranded DNA. The oligonucleotide C can be recognised by primer B. Therefore, amplification of the target region of DNA can be performed by PCR with primers A and B. RE: Any restriction enzyme.

oligonucleotide by T4 polynucleotide kinase. Thereby VecB became the donor oligonucleotide to covalently link to the 3' end of the previously digested DNA fragments. The second point to be considered is that the 3'-hydroxyl group of the deoxyribose at the 3' end of the oligonucleotide needs to be blocked by a dideoxyribonucleotide with terminal deoxynucleotidyl transferase (TdT) to avoid formation of oligonucleotide polymers.

The procedure I used was as follows: 5 μg of VecB was phosphorylated in 50 μl reaction with 2 μl of 10 mM ATP and 2 units T4 polynucleotide kinase in buffer (70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT) at 37°C for 20 minutes and inactivated at 65°C for 10 minutes. To remove the residual ATP molecules, the phosphorylated VecB was precipitated, washed and resuspended in 20 μl water. 10 μl of the phosphorylated oligonucleotide VecB was incubated with 1 μl of 100 μM ddATP and 15 units TdT in buffer (100 mM cacodylate buffer, pH 6.8, 1 mM $CoCl_2$, 0.1 mM DTT) in 20 μl final volume at 37°C for 60 minutes and then at 65°C for 10 minutes. 21 ng of the denatured and digested p4.8Bgl-1 DNA was ligated with 1 μl of the modified VecB oligonucleotide and 3 units T4 RNA ligase (New England Biolabs) in buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 10 μg/ml BSA, 41.6 μM PEG6000, 1.0 mM HCC, 200 μM ATP) at 22°C overnight. Subsequently PCR amplification was performed with the primers, B2A or B3A with Vecamp-3 respectively.

Initially, I tried the technique with the 5' end of VecB oligonucleotide phosphorylated but I found that the procedure failed, possibly because the ligation efficiency was still rather low. Subsequently the 3' blocking step was introduced. This time, amplification of the target fragments from p4.8Bgl-1 was successful with primers B2A or B3A combined with Vecamp-3 (not shown). However, no clear bands could be obtained from HL60 genomic DNA modified with VecB oligonucleotide in spite of repeated attempts with various changes of single strand oligonucleotide linkers, restriction enzymes and ligation conditions.

3.2.3.3. Initial attempt 3: Single primer mediated PCR

Single primer mediated PCR (Figure 3-3) has the advantage that amplification of the unknown region can be performed without ligation of any linkers, thereby having the potential to circumvent some of the problems encountered above. I tried the B2A and B3A primers on both p4.8Bgl-1 and HL60 DNA but obtained either multiple bands or smears on the gel. I concluded that this technique was not suitable for characterisation of the BCR-ABL breakpoints.

3.2.4. Establishment of Bubble PCR

After abandoning the strategy for the single stranded oligonucleotide-mediated PCR and single primer mediated PCR, I started to try to exploit Bubble/Vectorette mediated PCR.

3.2.4.1. Bubble PCR with BVEC oligonucleotide

Initially I used a Bubble oligonucleotide called BVEC (Figure 3-9), produced by hybridisation of the two oligonucleotides VecT1 and VecB. The 11 bases at the 5' end of BVEC are complementary between VecT1 and VecB. The following 10 bases are not complementary. The 9 bases at the 3' side of BVEC are complementary again. Thereby, the ends of BVEC are blunt after the two individual strands were annealed. A BamH I site was designed at the 5' end of complementary region of the heteroduplex oligonucleotide for subsequent cloning. Two PCR primers, Vecamp-1 and Vecamp-2, were made for annealing at the bottom strand VecB of the BVEC to generate specificity by nested amplification.

The BVEC duplex was made by annealing at equal volumes of 10 μM VecT1 and 10 μM VecB at 15°C overnight in distilled water. Therefore, concentration of BVEC was

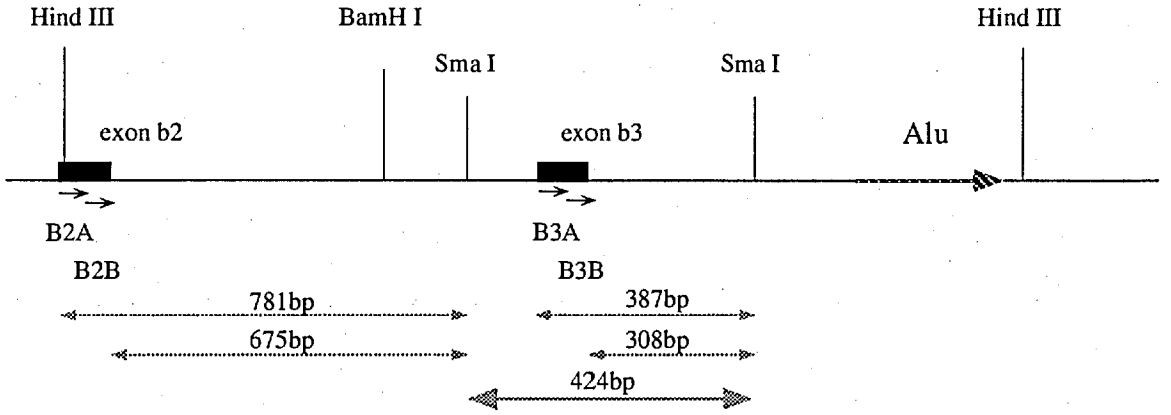
6.023 x 10^{12} molecules/µl, which was enough for ligation of 1 µg (4.8 x 10^{11}ends) of genomic DNA digested with a 6 bp-cutter.

Initially Sma I was selected because this enzyme is a blunt ended cutter with a single site (at position 1,764) in pEMBL8(+). In the M-BCR, there were four sites within the known 2,541bp of sequence that was known at the start of this study (Dr. David Leibowitz, personal communication). Therefore, at least 5 fragments can be generated from p4.8Bgl-1 by Sma I. One Sma I site is located 675 bp downstream of exon b2. The expected fragment would be 781bp in size from exon b2 to the Sma I site if primer B2A is used for amplification (not including the amplified size of BVEC). In zone 3 there is a single site for Sma I as well. It is situated at 308 bp downstream of exon b3. The length of the fragment will be 387 bp from exon b3 to the only Sma I in zone 3 if primer B3A is used for PCR amplification (not including the amplified size of BVEC). All these sizes of the fragments are within the range of conventional PCR amplification (Figure 3-11A). For DNA extracted from a CML patient, the Bubble PCR procedure after digestion with Sma I is outlined in Figure 3-11.

135 ng of p4.8Bgl-1 was digested with Sma I in 20 µl at 25°C overnight and then the enzyme was inactivated at 65°C. Ligation of the restriction digested p4.8Bgl-1 DNA with BVEC was carried out in 50 µl with 1 unit T4 DNA ligase (GIBCO BRL) at 16°C overnight in ligation buffer. The ratio of BVEC and the number of the fragments of BVEC generated by Sma I is about two to one (506 fmoles : 256 fmoles). Similarly, Sma I digested HL60 DNA was ligated to BEVC. PCR amplification was performed with either B2A or B3A and Vecamp-1.

p4.8Bgl-1 bands were seen at the correct sizes (781 bp and 387 bp respectively) but specific amplification was rather weak, particular for B2A, and smearing was also seen on the gel (Figure 3-12A). Furthermore, the results were not consistent. For HL60 only smears appeared (Figure 3-12B).

A. Partial M-BCR region



B. Digest with enzyme Sma I

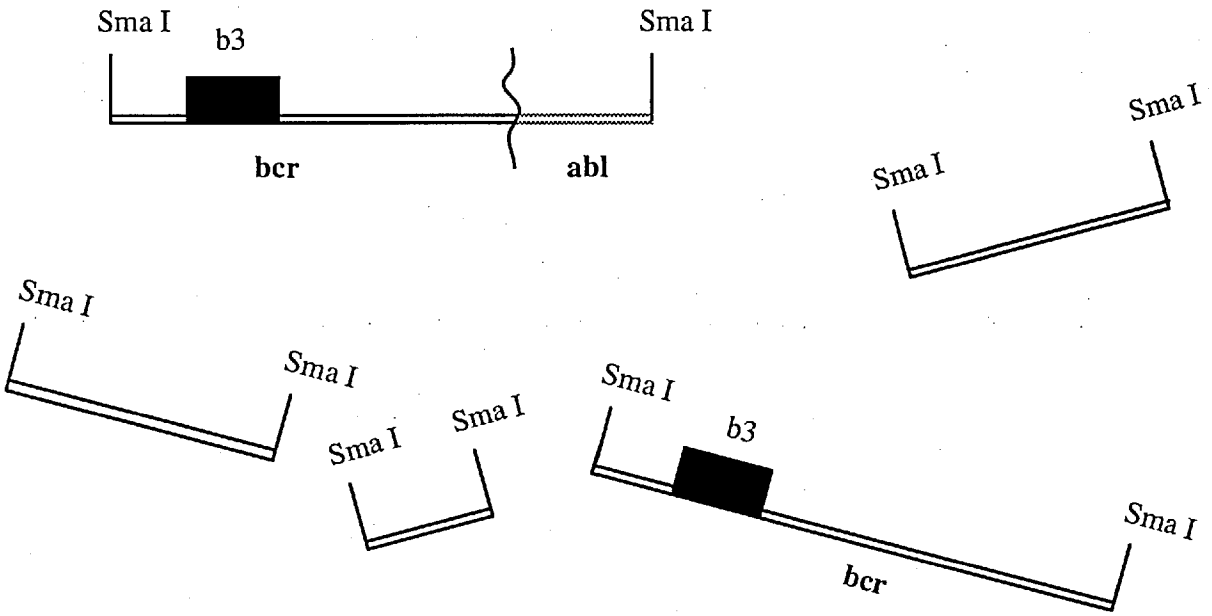
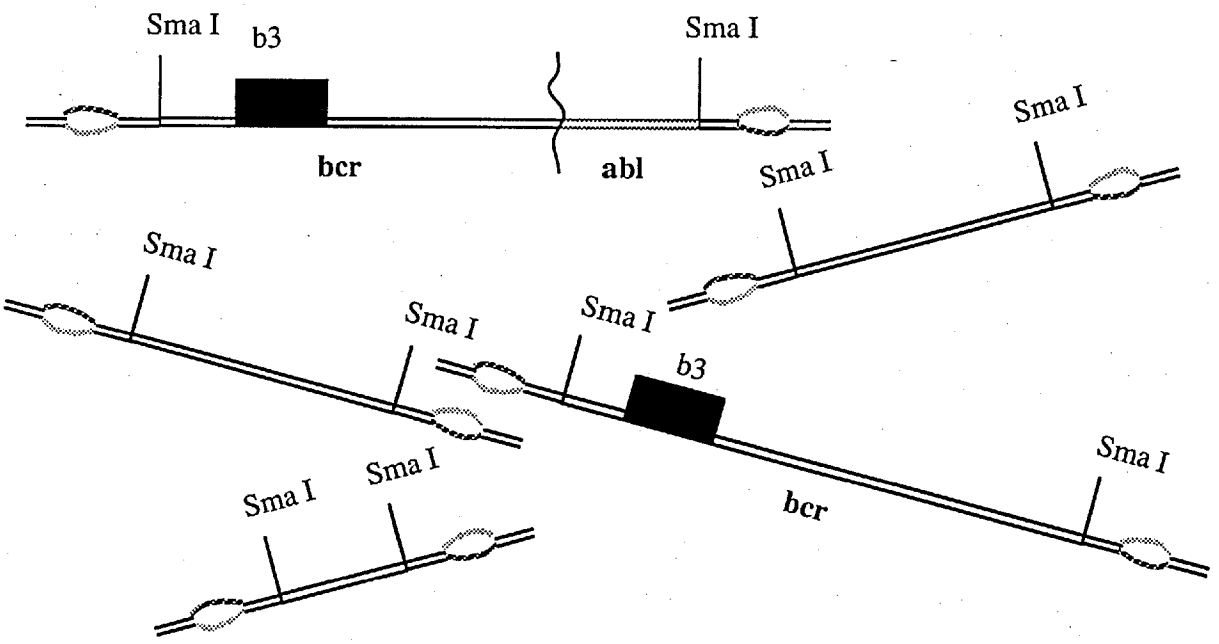


Figure 3-11 (a). Exploration of Bubble PCR with BVEC oligonucleotide

A) A map of M-BCR with restriction endonuclease Sma I. BCR primers are used to anneal to exons b2 and b3 respectively. The normal BCR allele amplified with primers B2A or B3A in combination with primer Vecamp-1 is expected to result in products of 781bp or 387bp respectively. B) DNA is digested with Sma I. Exon b3 and a partial sequence from the zone 3 should be included in a 424 bp Sma I fragment in the normal allele. If DNA from a CML patient is subject to Bubble PCR, then the BCR-ABL breakpoint should also be included in a fragment from the abnormal allele.

C. Ligate with Bubble oligonucleotide



D. Denature DNA, anneal and extend BCR primer B3A

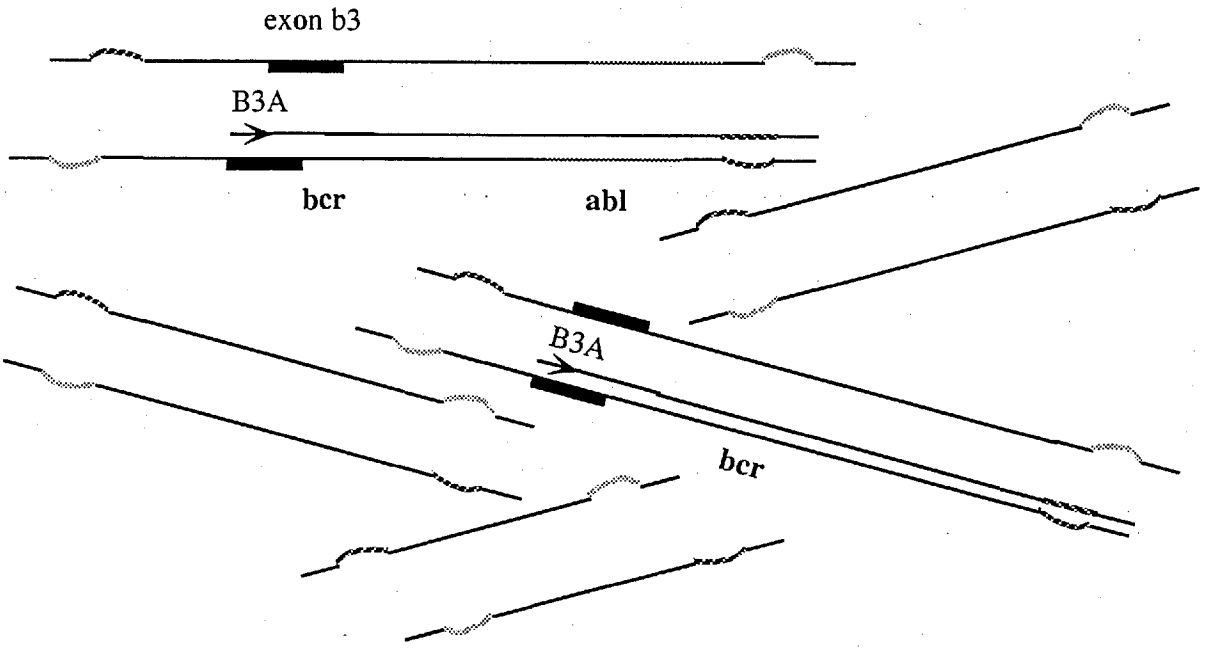
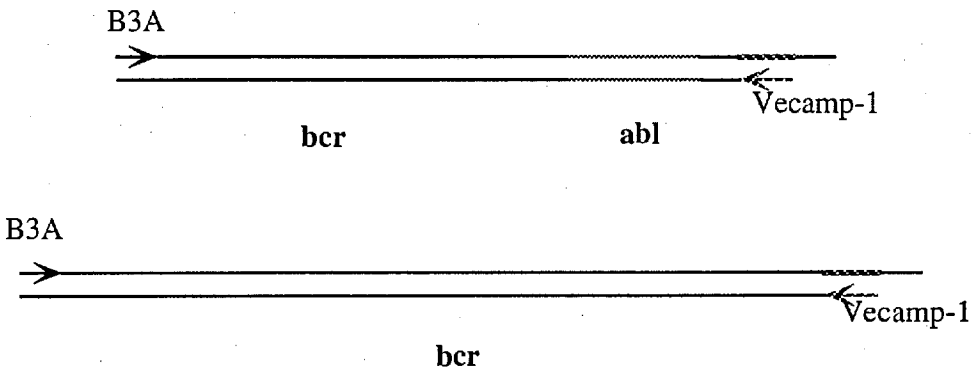


Figure 3-11 (b). Exploration of Bubble PCR with BVEC oligonucleotide

C) Digested DNA is ligated to Bubble oligonucleotide. D) DNA is denatured. A BCR primer, B3A, is then annealed to the exon b3 on the both BCR and BCR-ABL fragments and extended by Taq polymerase. A unique sequence is generated at the 3' end on the new strands for primers Vecamp-1 and 2 annealing.

E. PCR using primers B3A and Vecamp-1



F. Nested PCR using primers B3B and Vecamp-2

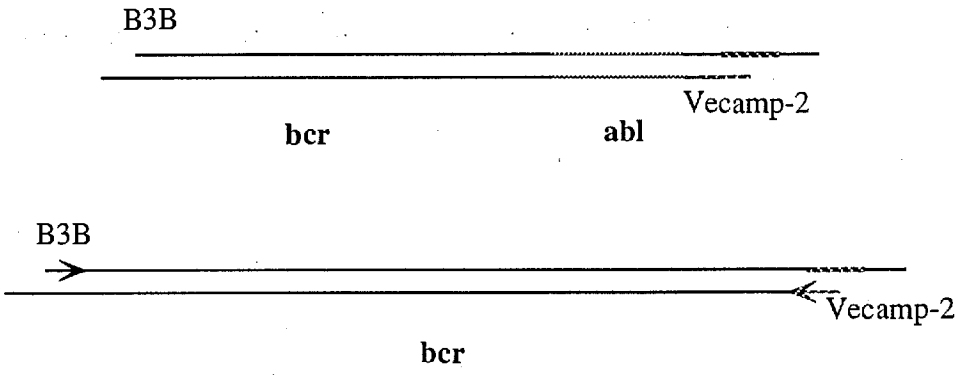


Figure 3-11 (c). Exploration of Bubble PCR with BVEC oligonucleotide

E) PCR amplification of the target DNA with primers B3A and Vecamp-1 at the first step. F) The fragment is reamplified with primers B3B and Vecamp-2 at the second step. If a Sma I site is not too far away from the breakpoint in the abnormal allele, then two PCR products are expected to be amplified at the end. One is the normal BCR; the other is BCR-ABL.

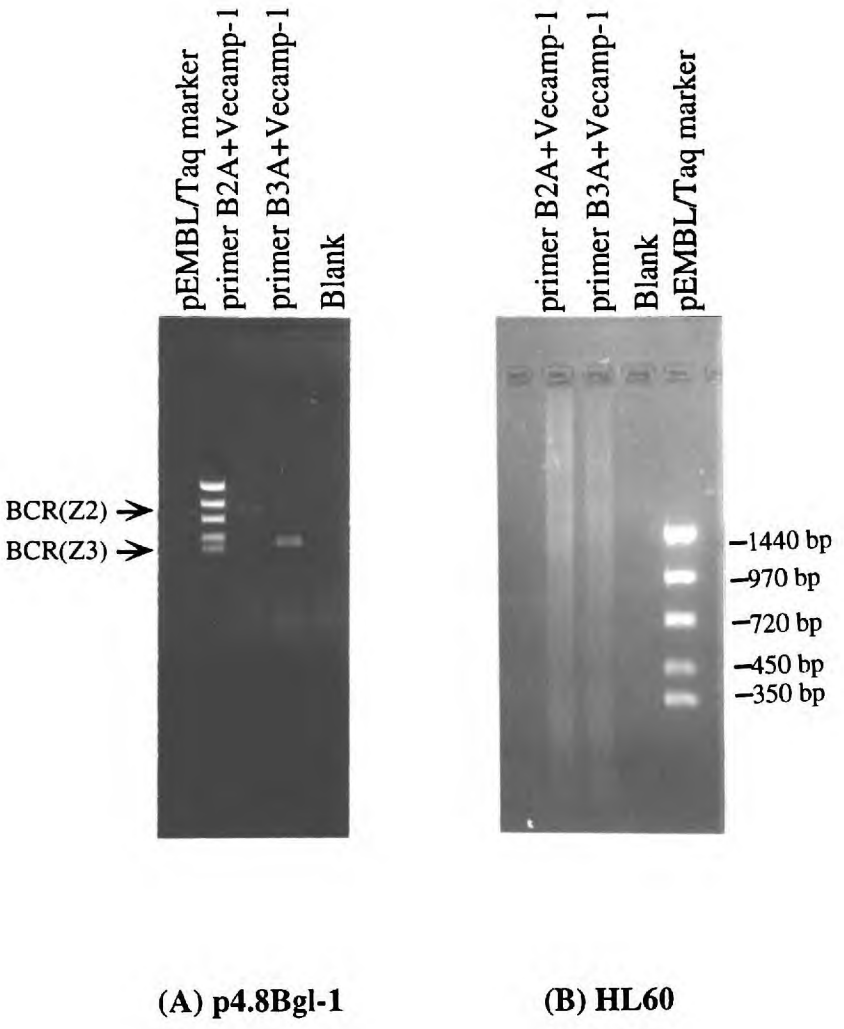


Figure 3-12. Bubble PCR with BVEC oligonucleotide

A) Specific amplification from p4.8Bgl-1 can be seen at the correct size but the bands were weak and smearing was also seen on the gel. BCR(Z2) and BCR(Z3) were the BCR products amplified from Zone 2 and Zone 3 respectively. B) Bubble-PCR products from HL60 DNA appeared only as a smear with primers B2A or B3A and vecamp-1 respectively.

It was possible that the smearing was caused, at least in part, by residual unligated BVEC oligonucleotides acting as PCR primers in the amplification reaction. To test this possibility, I used a Geneclean kit (Bio101) or micro-concentrator (Amicon) to remove unligated BVEC from the DNA templates before the PCR was set up. At the same time several aliquots of the ligation products were kept without removing BVEC as control. Single step PCR amplifications with primers B2A and B3A in combination with

Vecamp-1 respectively were carried out (Figure 3-13). The first lane on the gel was the molecular marker, λ DNA digested with Hind III. The second lane was an amplification from a sample without p4.8Bgl-1 DNA. In the third to fifth lanes were single primer amplifications with B2A, B3A or Vecamp-1 on p4.8Bgl-1 DNA after micro-concentrator cleaning. The sixth and seventh lanes were the amplifications with B2A or B3A combined with Vecamp-1 for micro-concentrator cleaned p4.8Bgl-1. Similarly the

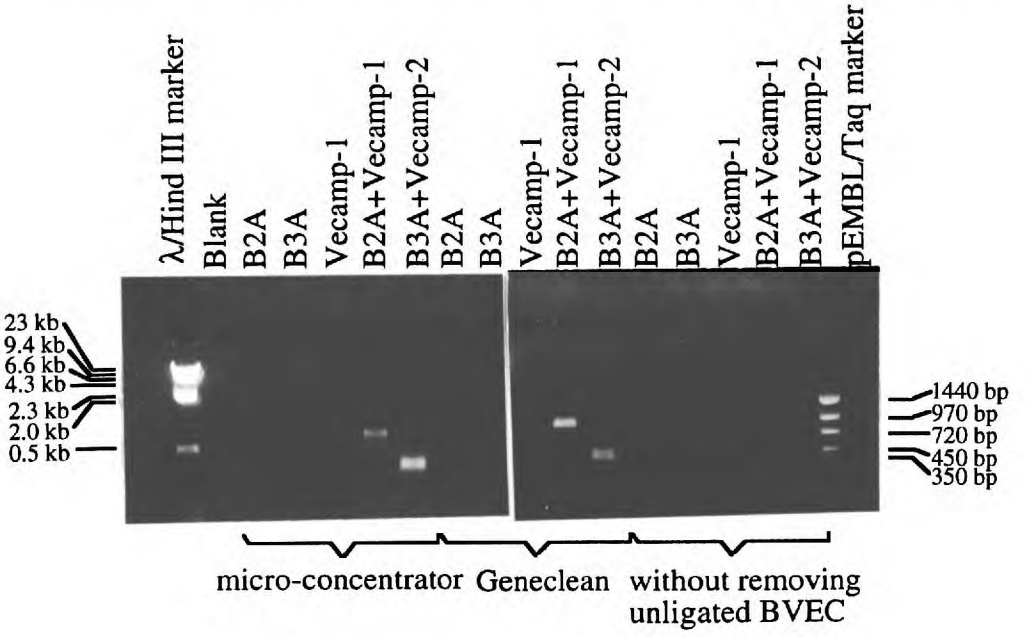


Figure 3-13. Bubble PCR trial with BVEC oligonucleotide for p4.8Bgl-1 DNA

PCR amplifications of p4.8Bgl-1 DNA after removing unligated BVEC with micro-concentrator (lanes 3 to 7); removing unligated BEVC by Geneclean (lanes 8 to 12) and without removing unligated BVEC (lanes 13 to 17). In each group, PCR amplifications are performed with B2A, B3A, Vecamp-1, B2A + Vecamp-1 or B3A + Vecamp-2 primer combinations.

lanes eight to twelve were the amplification from Genecleaned p4.8Bgl-1 with the same primer combinations as the lane three to seven. The lane thirteen to seventeen were amplified from the DNA without removing unligated BVEC. The last lane was a molecular marker, pEMBL DNA digested with Taq I. These results demonstrated a much better yield of specific PCR products when either Geneclean or micro-concentrator was performed prior to PCR. Therefore, Geneclean or micro-concentrator should be included in the protocol for this PCR procedure. From that point Bubble PCR started to work consistently with plasmid DNA.

In order to test the reproducibility of the procedure, the DNA digestion, ligation and removal of unligated BVEC were repeated. PCR was carried out with different BCR primers, JGZ1, JGZ2, JGZ3 and JGZ4 (Figure 4-6) (see Chapter 4). Bands of the expected size were generated in each lane and the results were reproducible. The annealing temperature was optimised for the primers. The sensitivity of PCR for amplification of p4.8Bgl-1 DNA fragments was tested from 2×10^5, 2×10^4, 2×10^3, 2×10^2, 2×10^1, 2×10^0 molecules per reaction. I found that the correct size band could be amplified reproducibly with both sets of primers when 2×10^3 target molecules were included in the reaction. Also the efficiency for removing the unligated BVEC oligonucleotide was compared between Geneclean and micro-concentrator. I found that the volume and concentration of the cleaned DNA were easily adjusted by Geneclean method. The micro-concentrator worked well but the final volume of DNA solution was usually large (200 µl - 500 µl) and somewhat variable after centrifugation. For this reason I chose Geneclean as the simplest method for removing unligated oligonucleotides.

To evaluate the reliability of the PCR, the sequence of the fragment generated by the PCR needed to be confirmed. For cloning purpose, all the BCR nested primers (B2B, B3B, JGZ2 and JGZ4) were designed with an EcoR I site at the 5'-end. The BVEC Bubble oligonucleotide was designed with a BamH I site at the 5'-end. The strands extended from primers Vecamp-1 and Vecamp-2 which anneal at BVEC will generate BamH I site. Therefore all fragments amplified will have an EcoR I at one end and a BamH I site at the other end. M13 vectors (mp18 and mp19) were digested with BamH I and EcoR I so that the digested fragments with both BamH I and EcoR I cohesive ends could be ligated into the cloning site with a defined orientation. The phosphate groups at both 5' termini were removed from the linearized M13 vectors to prevent religation of any molecules that had been digested with only one enzyme. The fragments amplified by Bubble PCR with p4.8Bgl-1 DNA ware digested with both

BamH I and EcoR I, and cloned into M13 mp18 and mp19 respectively. The sequences cloned were confirmed to be the M-BCR portion immediately after the PCR primers from BCR in the M13mp19 clones and from the BVEC oligonucleotide in M13mp18 clones by sequencing (Figure 3-14).

3.2.4.2. Bubble PCR for HL60 genomic DNA

The procedure, including Geneclean, that worked with p4.8Bgl-1 was repeated with genomic DNA from HL60. Genomic DNA was digested with Sma I, ligated with BVEC oligonucleotide, genecleaned and amplified. The results were greatly improved: bands of the correct size could be seen clearly on the gel (Figure 3-15). However, on several occasions non-specific bands were also amplified.

I considered it highly desirable to eliminate the non-specific bands because with CML patients, the size of the BCR-ABL product was unknown. Non-specific amplification would be confused with specific amplification from the true abnormal allele. Although the specific amplification product of BCR-ABL could be localised by Southern blotting with an internal BCR probe, this would be time-consuming and might increase the chance of producing artifacts at the end. Furthermore, a background smear amplification would also give rise to problems with the cloning and sequencing.

Non-specific multiple amplifications were constantly seen in HL60 DNA with different PCR primers for BCR. However, when those primers were used in combination with other reverse BCR primers in a normal amplification reaction on HL60 DNA, clear specific bands without non-specific amplification were seen (not shown), indicating that the BCR primers were working well. Neither the VecB nor VecT1 oligonucleotide matched any sequence in the Genbank database (released in August 1996) but it was possible, of course, that these oligonucleotides could hybridise to unknown sequences.

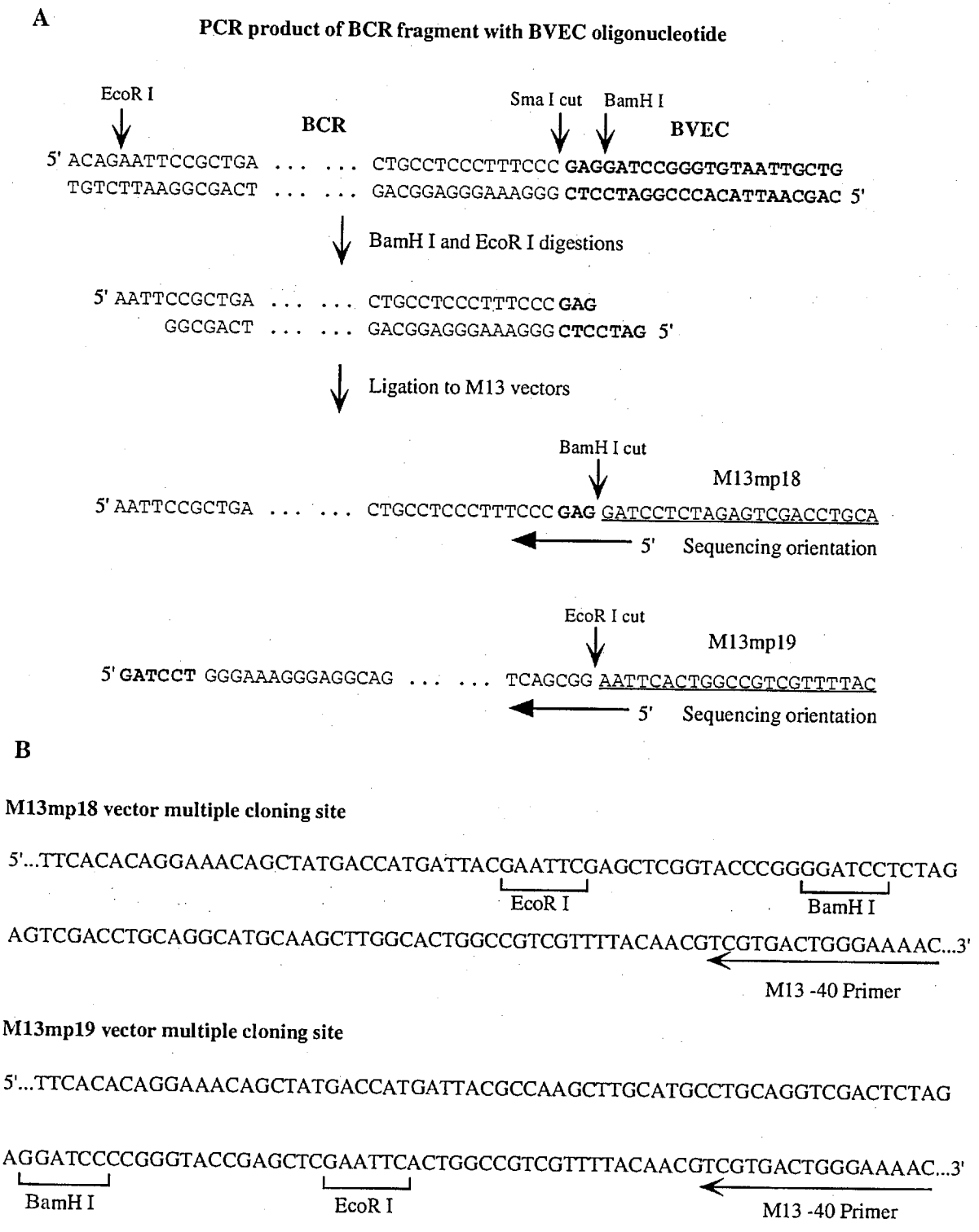


Figure 3-14. Sequencing for PCR products amplified from p4.8Bgl-1 DNA

A) Sequences obtained from PCR amplification for p4.8Bgl-1 DNA with M13 -40 primer. B) Multiple cloning sites of M13mp18 and M13mp19 vectors. DNA fragments amplified were cloned into the site between BamH I and EcoR I recognition sites before sequencing. M13 -40 primer in the mp18 vector primes from 3' end of the cloned fragment; M13 -40 primer in the mp19 vector primes from 5' end of the fragment.

Despite the initial success of the technique, I found that the reactions frequently failed (Figure 3-16). The PCR primers were checked and all amplified well with the other primer combinations. Although difficult to prove, I suspected that the ligation efficiency was still not good enough. One explanation for the low efficiency could be

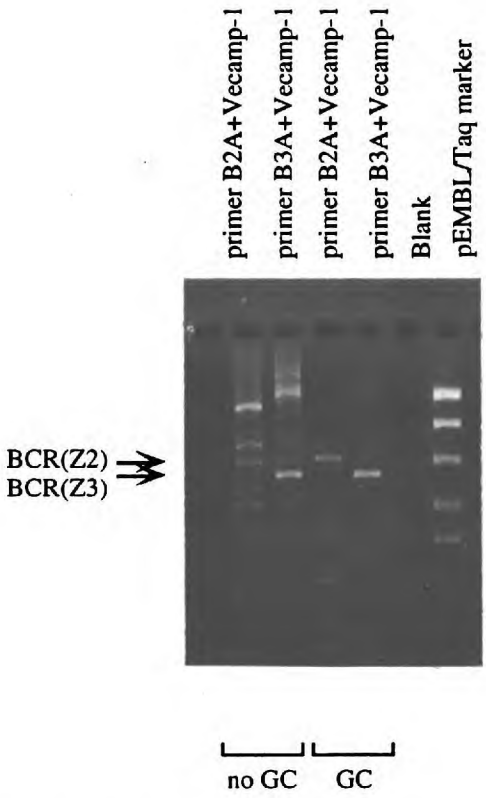


Figure 3-15. Bubble PCR trial for HL60 DNA

Specific amplification from zone 2 and 3 of the M-BCR i s indicated by an arrow. Other bands on the gel are non-specific. Geneclean prior to PCR was not performed in lane 1 and 2. Geneclean was done before PCR in lane 3 and 4. Negative control in lane 5. DNA molecular weight marker in lane 6. PCR primers for zone 2 and 3 of BCR in each reaction are indicated. GC: Geneclean. BCR(Z2) and BCR(Z3) indicated are the same as Figure 3-12.

that only a small proportion of VecT1 and VecB was annealed to form the BVEC duplex, and therefore only a small proportion of target DNA fragments could be ligated. All fragments are blunt ended and therefore compete with the target fragments for the BVEC oligonucleotides. Therefore, amplification may be negative in some reactions due to insufficient number of target fragments ligated to BVEC.

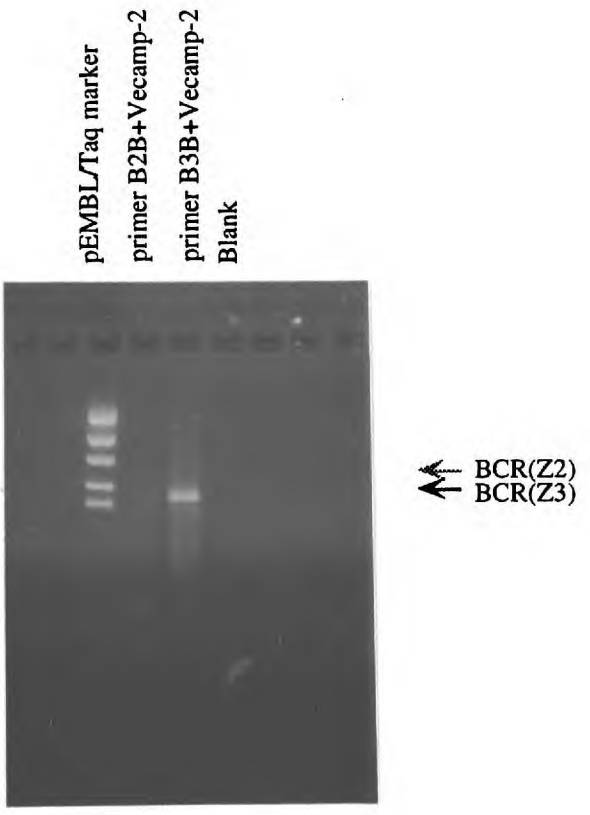


Figure 3-16. Frequent failure of the amplification with Bubble oligonucleotide BVEC

Sometimes no amplification product was obtained from Bubble PCR with oligonucleotide BVEC for HL60 DNA. DNA molecular weight marker was run in lane 1, amplification product from zone 2 in lane 2, product from zone 3 in lane 3 and negative control in lane 4. The failure amplification is indicated by a dotted arrow. The others symbols indicated are the same as Figure 3-12.

3.2.4.3. Bubble PCR with a new Bubble oligo for p4.8Bgl-1 DNA

A new Bubble oligonucleotide was designed in an attempt to increase the reliability of the technique. In particular I considered the following points to increase the stability of the Bubble oligonucleotide. 1) A salt containing buffer rather than water was used for oligonucleotide annealing because the ionic strength would help the two individual oligonucleotides to anneal to each other, 2) Annealing was performed with a high concentration of each oligonucleotide, 3) The length of the oligonucleotides was increased, and 4) The temperature for annealing and ligation to DNA fragments was decreased to 4^{o}C.

The new oligonucleotide was composed of two strands, BUB-T and BUB-B. The length of the oligonucleotide for both strands was increased to 52 nucleotides. There were 11 bases of complementary region at the 5' end, and 12 bases complementary region at the 3' end with a 29 base single stranded loop in the centre (Figure 3-5). A BamH I site was placed at the 5' end in the complementary region, which was to facilitate later cloning. The sequence of the bottom strand was randomly designed and the Genbank database was searched with the sequence. No significant homologous sequences were found in the human genome. Primers NVECAMP-1 and NVECAMP-2 were designed complementary to the unique sequence copied from single stranded region for PCR amplification. Primer BUBSEQ was made for direct sequencing (Figure 3-5).

The new Bubble oligonucleotide was formed by annealing unphosphorylated oligonucleotides BUB-T and BUB-B at a concentration of 136 μM at 4°C in 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl for 16 hours. After ligation of p4.8Bgl-1 with the new oligonucleotide, any unligated Bubble oligonucleotide was removed by the Geneclean protocol. PCR was then performed with primers B2A, B3A and Z4A in combinations with NVECAMP-1. A clear band of the expected size was amplified in each reaction by one step PCR (not shown).

3.2.4.4. Bubble PCR with a new Bubble oligonucleotide for genomic DNA from CML patients

Sma I was not a good choice for cleavage of genomic DNA, partly because this enzyme is methylation sensitive (Nelson & McClelland, 1991) and partly because its recognition site (CCCGGG) is relatively infrequent. In order for the technique to work well, a restriction site must occur on the ABL side of the translocation fairly close to the breakpoint (e.g. < 1 kb). In order to increase this chance, a 4 bp cutter was chosen. Inspection of the sequence of the M-BCR revealed that there was under-representation

of Rsa I sites (4 bp blunt ended cutter). This enzyme was therefore selected to develop the technique on genomic DNA.

5 μg of genomic DNA from HL60 cell line and three CML patients were digested with restriction enzyme Rsa I in 50 μl. 14 μl (1.03×10^{13} ends) of the digested DNA from each sample was ligated with 1.5 μl (1.2×10^{14} molecules) of 136 μM Bubble oligonucleotide in a final volume of 20 μl at 4°C for 36 - 48 hours. The ligated DNA was heated at 65°C for 5 min. The volume of DNA solution was then topped up to 50 μl with distilled water. 150 μl of NaI (Bio 101 kit) was added to each digested DNA, and 5 μl of glassmilk (Bio 101 kit) was mixed, and the mixture was placed on ice for 5 minutes. The Geneclean procedure was completed by washing and elution steps as

Table 3-1. Bubble PCR primer combinations

PCR steps / M-BCR	First step		Second step	
Zone 2	JGZ1	Nvamp-1	JGZ2	Nvamp-2
Zone 3	JGZ3	Nvamp-1	JGZ4	Nvamp-2
Zone 4	Z4A	Nvamp-1	Z4B	Nvamp-2

Note: Nvamp-1 and Nvamp-2 are the primers for Bubble oligonucleotide

recommended by the manufacturer. The Geneclean procedure should remove excess Bubble oligonucleotide and also the top strand of the ligated oligonucleotide since the glass fragments only bind DNA of approximately > 200 bp. Finally, the DNA was eluted in 15 μl of water. 200 ng (6.3×10^4 genomes) of DNA was put into each PCR mixture. A series of nested PCR amplifications were performed for each PCR cycle so that most of the M-BCR could be spanned. Combinations of primers used were listed in Table 3-1 (the sequences of all primers are shown in Table 2-1; see Chapter 2). An annealing temperature at 62°C for the primers was used initially was found to result in multiple bands. After changing the annealing temperature to 66°C, specific amplifications for the normal allele were achieved in all samples and there was a different size band in some of the patient samples (Figure 3-17). The number of patient samples were enlarged to 5. All the bands derived from the normal alleles were

reproducible; the novel bands in the CML samples were also reproducible. Amplified bands were gel purified and sequenced. As expected the bands common to all individuals were derived from the M-BCR. The other amplified bands contained M-BCR sequence followed by novel sequence. Subsequently I was able to confirm that the novel sequence was indeed derived from the ABL gene (see Chapter 6).

3.3. Discussion

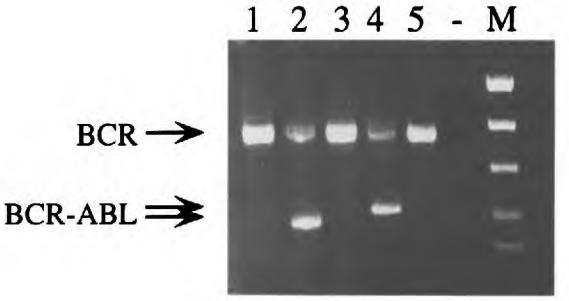
PCR is a good method to rapidly obtain BCR-ABL or ABL-BCR breakpoints from CML patients, since it can rapidly and specifically amplify a particular target region of genomic DNA. PCR results in products that are easily visualised on an agarose gel and the amount of DNA produced is sufficient for cloning and sequencing.

3.3.1. Bubble PCR is the best strategy for characterisation of the BCR-ABL or ABL-BCR breakpoints in CML

I explored several strategies associated with PCR techniques for site specific cloning. Several techniques have been published which are of potential use for characterisation of BCR-ABL or ABL-BCR breakpoint in CML. 1) inverse PCR (Ochman *et al* 1988; Triglia *et al* 1988; Silver & Keerikatte, 1989); 2) Fors linker ligation PCR (Fors *et al* 1990); 3) Bubble/Vectorette mediated PCR (Riley *et al* 1990; Smith, 1992; Green, 1993); 4) Single-primer PCR (Parks *et al* 1991); 5) Panhandle PCR (Jones & Winistorfer, 1992) and 6) Single-stranded DNA ligation PCR (Zhang & Chiang, 1996). At the time I started this study, inverse PCR, Fors linker ligation PCR, single-primer PCR, and Bubble/Vectorette mediated PCR were available.

Inverse PCR had been described (Figure 3-1) when I started this project. From the available protocol, this technique requires the presence of three conveniently placed sites for two restriction enzymes, one site in the unknown sequence (ABL) and two in

A



B

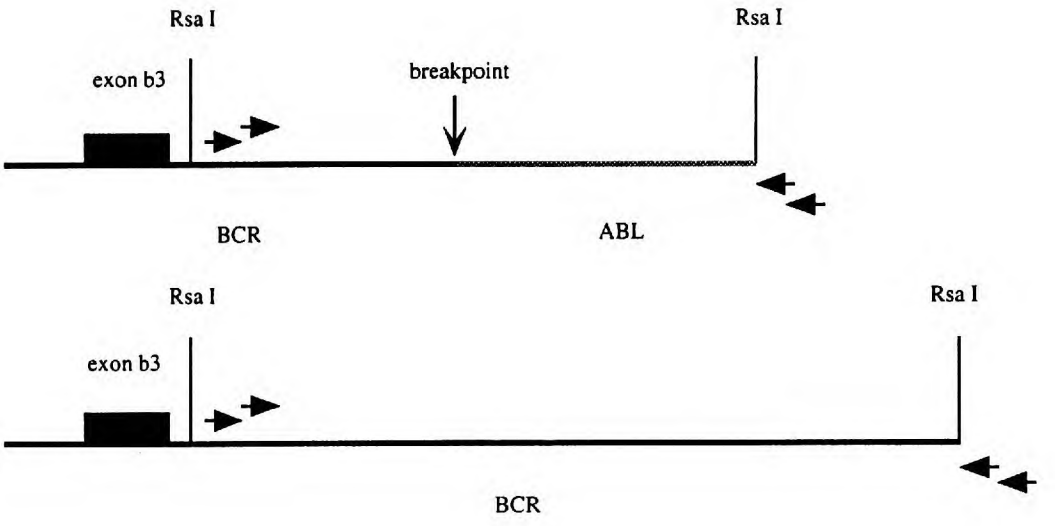


Figure 3-17. Amplification of BCR-ABL breakpoint fragments by Bubble PCR

A) PCR amplification of BCR-ABL breakpoint fragments in two out of the five patients in zone 3 of the M-BCR. The upper band in each patient is BCR normal allele. The lower band is the abnormal allele amplified between the BCR primer site immediately after the RsaI site in BCR gene and the closest RsaI site to the breakpoint in ABL. The breakpoints in the remaining three patients did not amplify, either due to lack of a convenient Rsa I site within the ABL sequence, or because the breakpoints did not fall within this region.

B) Diagram for this amplification with the BCR primers in the zone 3 of the M-BCR region by nested bubble PCR. ◄ indicates PCR primer position and synthesis orientation. The primers at the ABL side annealed to the bubble oligonucleotide.

the known sequence (BCR) (Figure 3-1). This considerably limits the applicability of the technique. Several considerations affect the choice of the first restriction enzyme. First, it should generate a fragment of appropriate size for circulation and PCR amplification. Fragments shorter than 200 - 300 bp are difficult or impossible to circularise (Shore *et al* 1981), and fragments larger than about 3 kb are difficult to amplify from complex mixtures (genomic DNA), although recently several 'Long PCR' protocols had been developed that enabled amplification of larger fragments (Schwarz *et al* 1990; Ponce & Micol, 1992). Second, the enzyme should produce a non-degenerate cohesive end to facilitate subsequent self-circularisation. Third, there should be no site for the enzyme X used to form the circle in the M-BCR between the unique restriction site Y and the translocation breakpoint (Figure 3-1). In practice, these limitations were too great to allow a general BCR-ABL translocation cloning protocol to be developed. In addition, it was unclear whether self-circularisation of each digested DNA fragment was really practical for high complexity DNA.

The advantage of the Fors linker ligation PCR (Fors *et al* 1990) (Figure 3-2) is that in theory any restriction enzyme that produces a cohesive end could be used to digest the genomic DNA. This means that a range of enzymes can be used, thereby giving a greater chance of success. Another advantage is that this PCR would result in two products at the end: one is the normal BCR band and the other would be abnormal with BCR-ABL breakpoint on a agarose gel. This will provide convenience for later gel purification and cloning. In the initial steps of this procedure, Sequenase is used to generate a blunt ended DNA initiated from a primer to known sequence after denaturation. The specificity of this method is theoretically generated by linker ligation at the unique blunt end of DNA fragment. The linker consists of two strands that are different in length with the top strand longer than the bottom strand (Figure 3-2). After the first PCR primer extension, the sequence complementary to the top strand of the linker is synthesized and is the only sequence recognised by the second PCR primer. Conceptually, this is quite similar to Bubble/Vectorette PCR.

However, in practice the unligated linkers in the PCR reaction may cause a lot of non-specific amplification. The optimal temperature for Sequenase is 37°C. At this temperature the initial primer will anneal non-specifically at many different locations on DNA templates. All specifically and non-specifically annealed primers will be extended by Sequenase and subsequently amplified. This explains why the amplification products were always seen as a smear on the gel, including the one in the published paper (Fors *et al* 1990).

Single-primer mediated PCR (Parks *et al* 1991) does not require digestion of the template DNA with restriction enzymes. The only information required is a short stretch of sequence used to design a gene-specific primer. Therefore I have designed JGZ1, JGZ2, JGZ3, JGZ4, Z4A, and Z4B primers at the M-BCR side. However, the product of single-primer PCR on an agarose gel appeared as a smear, and it was not possible to judge which band was actually derived from the normal allele or the abnormal allele (Figure 3-3). Although this simple method could be useful for substrates of low complexity, such as plasmid or cosmid DNA, I concluded that it would not be easily adaptable to cloning of the BCR-ABL breakpoints.

To enlarge the range of restriction enzymes and increase the chance of encompassing the breakpoint, I explored a single stranded oligonucleotide mediated PCR at the beginning of 1992. I found that method worked with plasmid DNA but I failed to establish the technique for human genomic DNA. Eventually after much effort I abandoned this method. Subsequently, a very similar method was published (Zhang & Chiang, 1996). This technique was used to isolate the PAL gene from genomic DNA of loblolly pine, based on the sequence of a partial PAL cDNA clone. The protocol is briefly described as follows: a specific primer designed to the 5'-end of the coding region in the antisense orientation was annealed and extended by PCR using genomic DNA as a template. Single-stranded DNA extension products were ligated to an

arbitrary oligonucleotide with T4 RNA ligase to create templates with 5'- and 3'-ends of known sequence. These templates were then reamplified to make double-stranded DNA for further cloning and sequencing. This approach is generally same as the one I attempted. The only difference is that DNA digestion is required at the beginning (Figure 3-10).

Panhandle PCR (Jones & Winistorfer, 1992; Jones, 1995) is also a novel method for PCR amplification of unknown DNA (Figure 3-4). I did not try this method principally because I was concerned about the low efficiency with which a single stranded oligonucleotide can be ligated to the double stranded DNA fragments. Also, the method requires several enzymatic manipulations. Very recently, however, this method has been adapted to clone breakpoints in the MLL gene at 11q23 (Kim *et al* 1996).

There were also many other PCR based techniques published at the time, for instance, linker-adaptor PCR, anchor PCR. Linker-adaptor PCR (Saunders *et al* 1989; Vooijs *et al* 1993) was created to make probes for chromosome painting and FISH study. No specificity is applied to a particular region in a chromosome with this technique. Anchor PCR (Loh *et al* 1989; Quinn Laquer *et al* 1992) has been used to characterise cDNA populations. This method made use the poly $(A)_n$ tail of mRNA to convert RNA to DNA and then amplified the sequence of interest. However, neither of these techniques could easily be adapted to the cloning of translocation breakpoints.

Finally I decided to try Bubble/Vectorette mediated PCR (Riley *et al* 1990; Smith, 1992; and Green, 1993). This method turned out to be the most successful strategy although it did take time and effort to establish.

3.3.2. Considerations of Bubble oligonucleotides

After many experiments with several different oligonucleotides, I concluded that the essential requirements for the Bubble PCR were: a) the oligonucleotides should be efficiently ligated to DNA fragments, b) the oligonucleotides should be of a specific sequence which can be copied and then this copied sequence can be recognised by one of PCR primers. This is the reason why a loop structure is required in the Bubble oligonucleotide. c) The oligonucleotide sequence and PCR primers should not be homologous to any repetitive sequences.

Eventually I designed the Bubble oligonucleotides. The Bubble oligonucleotides consist of two strands which have non-complementary regions in the central and matched regions at the both ends (Figure 3-14). The length of the oligonucleotides, the ratio between the complementary and uncomplimentary regions, and actual sequences of the oligonucleotides are critical. Also a restriction enzyme site needs to be placed into 5' end of the oligonucleotide, so that PCR product can easily be cloned into a suitable vector.

3.3.3. Technical factors affecting efficient ligation

Efficient ligation of the Bubble oligonucleotide to digested DNA is a critical step in the technique. Good efficiency ligation requires that the two strands of the Bubble oligonucleotides are completely and correctly annealed.

Salt is required to stabilise the Bubble oligonucleotide duplexes. The buffer used for annealing the two strands should not interfere with the subsequent ligation and PCR reactions. I found that the solution 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl worked well as an annealing buffer. The ionic strength in the buffer helps the DNA strands anneal to each other, for instance, high concentrations of Mg^{2+} stabilise

double stranded DNA and prevent complete denaturation. However, excess Mg^{2+} could also stabilise spurious annealing of PCR primers to incorrect template sites. In my study, 10 mM MgCl2 and 50 mM NaCl were used in the annealing buffer. The buffer gave rise to good ligation frequency which was confirmed by very good amplification results from both normal and abnormal alleles.

The initial Bubble oligonucleotide I used was annealed in distilled water at a relatively low concentration (10 μM). It is likely that this step was very inefficient, leading to a poor efficiency of ligation. For the p4.8Bgl-1 DNA amplification, a low efficiency of ligation would not be discovered because the PCR technique is very sensitive, and the target DNA is of low complexity. For genomic DNA, however, the problem became apparent.

Temperature is another major factor for the stability of the Bubble oligonucleotide. Even at room temperature, the Bubble oligonucleotides may denature. Initially I set up the ligation reaction at room temperature and performed the reaction at 16°C. Subsequently, I always kept the annealed Bubble oligonucleotides at 4°C or on ice. The ligation reaction was also carried out at 4°C. I found that these steps dramatically increased the efficiency of the procedure.

The efficiency of ligation could have been increased by designing a sticky ended Bubble oligonucleotide, such as that described in the original Vectorette PCR. A cohesive ended fragment ligation is more efficient than a blunt ended one. However, a cohesive ended Bubble oligonucleotide would restrict the number of enzymes that could be used for amplification of BCR-ABL breakpoints.

The ligation reaction volume and concentration of heteroduplex oligonucleotides to be used for ligation reaction are also factors that affect the efficiency of the reaction. To

increase the ligation efficiency, I tried to minimise the reaction volume and use the Bubble oligonucleotide at a high concentration.

Most of factors mentioned above were not relevant when the technique was performed with p4.8Bgl-1. The reason is that the digested plasmid DNA has very little DNA background. The target DNA fragments were typically present at a frequency of one in every four or five. However, in human genomic DNA the target fragments would be estimated to be present at a frequency of only one in 10^7 if a 4 bp cutter is used to digest the DNA.

3.3.4. Critical factors contributing to non-specific amplification of the PCR

The unligated oligonucleotides are one of the important factors which contribute to subsequent non-specific PCR amplification, either because they anneal randomly to genomic DNA or because they anneal to the ligated Bubble oligonucleotide. These oligonucleotides can be removed by a Centricon device or by a Geneclean kit. I found that Geneclean was the best method.

A "Hot start" for PCR is also an important element in the protocol. The modified DNA is first denatured, and then a M-BCR oligonucleotide is specifically annealed to the DNA and extended. Without "Hot start" the M-BCR primer may prime non-specifically leading to multiple products. This may result from the initial primer annealing non-specifically to DNA at low stringency. If this happens, every DNA template with the sequence copied from BUB-B at the ends will subsequently be amplified since all DNA fragments have been ligated with the Bubble oligonucleotide. The result is amplification of multiple products leading to a smear on the gel. Therefore, the first primer annealing site is crucial for the specificity of Bubble PCR amplification. The temperature in the first cycle of the PCR is the most important for controlling this specificity. A conventional PCR program is not suitable for restricting the specificity

because when the reaction is heating up at the very first stage, a new non-specific strand can be generated even before the denaturing temperature is reached. Specificity can, however, be achieved by hot-start PCR, i.e. by only adding the Taq polymerase once the reaction tube has reached the denaturing temperature. Subsequently, the specificity of primer annealing can be controlled by cooling the mixture to a defined temperature.

Even with the Hot start and Geneclean I found that it was still necessary to perform a nested PCR in order to generate specific products. The nested primers provide an extra round of specificity and eliminate all or most of the non-specific products from the first step PCR. The specific bands may not be clearly seen on a gel by one step PCR because specific amplified fragments are not dominant over the genomic DNA background. When 1 µl of the first step PCR product is transferred to the second reaction mixture, non-specific amplification from DNA background is diluted. Thus DNA templates for the amplification with the nested primer pair easily are simplified and the fragments are easily amplified in the reaction.

I found that the number of cycles of the PCR reaction should be limited to 30 cycles in the first step PCR. If cycle number is greater than 35 or 40, extra bands appear as products from the second PCR. These extra bands presumably result from non-specific amplification. The greater the number of cycles performed the greater chance that primers will anneal non-specifically to the other DNA regions.

3.4. Potential applications of Bubble PCR

Bubble PCR can be used amplify unknown DNA that flanks a known segment directly from human genomic DNA. This method is suitable for gene cloning without going through laborious DNA library construction and screening as long as a short sequence of the target gene is known. The Bubble oligonucleotides can be used for universal amplification of different genes, thus reducing the need to synthesise a large number of primers.

Amplification of DNA segments from bulk human genomic DNA has the potential to supplant current cloning-base strategies for sequencing the human genome, and will permit genome walking into currently unclonable regions of DNA (Wyman *et al* 1985). Amplification of fragments adjacent to cDNA regulatory regions and intron-exon junctions, and viral and transposon integration sites can be determined.

3.4.1. Bubble PCR in CML

The mechanism by which the reciprocal translocation between chromosomes 9 and 22 occurs in CML is unclear. Knowledge of a large number of flanking sequences and locations of the BCR-ABL and ABL-BCR breakpoints may contribute to our understanding of the mechanism. Bubble PCR can be used to characterise these sequences and provide a base for analysis of the breakpoint distribution. Similarly, the technique could be used to clone other translocation breakpoints.

Knowledge of sequences flanking the translocation breakpoints can be used to design patient-specific primers for DNA-PCR in CML. Patient specific PCR primers, therefore, can serve as a tool to monitor a leukaemic clone during or after new treatment regimens. When a new treatment for CML is developed, such as interferon-α or donor lymphocyte transfusion following relapse post BMT, one needs to ascertain whether the treatment can reduce or eradicate leukaemic progenitor cells in the patient. DNA-PCR is potentially a useful tool to detect and quantify CML cells. In theory the number of tumour cells can be quantified more precisely by DNA-PCR than by RT-PCR because there is only one abnormal allele at the DNA level in each leukaemic cell, whereas the number of BCR-ABL mRNA molecules is uncertain. If some leukaemic progenitor cells do not actively express BCR-ABL mRNA, DNA-PCR would be able to recognise them, while RT-PCR would not. Therefore, DNA-PCR may be of particular value to evaluate the results of new treatments.

DNA-PCR with patient specific primers can be used to study the growth kinetics of leukaemic clones during haemopoiesis. DNA-PCR can be used as an efficient tool to track a given clone of leukaemic cells when it proliferates, differentiates and dies by specific amplification of the breakpoint as a genetic marker. This PCR could be applied to haemopoietic colonies and other small samples. In contrast, RT-PCR may not always give reliable PCR results from single colonies in semi-solid cell culture due to very limited copies of abnormal mRNA molecules available for conversion to cDNA (Diamond *et al* 1995).

3.4.2. Potential therapeutic strategies based on DNA sequence

Bubble PCR has also a potential application to DNA based therapeutic strategies which rely on knowing the breakpoint sequence for each patient, e.g. triplex oligonucleotides (Cooney *et al* 1988; Rajagopal, 1989) or rationally-designed sequence specific DNA binding proteins (Choo *et al* 1994).

Triplex-forming oligonucleotides (TFOs) have been shown to bind in a sequence-specific manner to polypurine/polypyrimidine sequences in several human gene promoters, including the MYC P1 promoter (Kim & Miller, 1995). TFOs have been shown to inhibit transcription in vitro and the expression of target genes in cell culture. Some evidence suggested that triplex forming ^{125}I labelled oligonucleotides could induce sequence-specific DNA double-strand breaks (Panyutin & Neumann, 1994). If a TFO is used to target the breakpoint of a chimaeric oncogene in tumour cells, Bubble PCR can help to localise, amplify, sequence the breakpoint and to design the sequence of TFO for specific targeting.

A DNA-binding peptide comprising three zinc-fingers was engineered to bind specifically to a unique 9 bp region of a BCR-ABL cDNA and, in a model system, this

protein repressed BCR-ABL mRNA expression (Choo *et al* 1994). This work indicates that there is a possibility that a similar protein could be engineered to target the genomic BCR-ABL oncogene. For CML, the location of genomic breakpoints is variable, the sequence flanking the breakpoint should be known in each individual patient. Therefore, Bubble PCR could be used to amplify the breakpoint and provide sequence information for engineering the chimaeric protein to specifically bind to the BCR-ABL fusion gene.

3.4.3. The position of the breakpoint and FISH analysis

The breakpoint location in ABL gene in CML characterised by Bubble PCR can also provide a base for evaluating factors that influence the false positive and negative rates of BCR-ABL fluorescence in-situ hybridisation (FISH) (Chase *et al* 1997). Variability in false positive rate (FPR) and false negative rate (FNR) in FISH studies often give rise to difficulties for the interpretation of results. In CML, the breakpoint on the chromosome 22 is found almost always in a 5.8 kb region, i.e., the M-BCR of the BCR gene. In contrast the breakpoints on chromosome 9 may be spread over approximately 200 kb region between exon Ib and exon a2 (Figure 1-2). Thus, the extent to which BCR and ABL signals are separated in a Ph chromosome positive cell may be influenced by the precise position in the ABL gene but not in the BCR gene. For example, if the breakpoint is located in a region around exon Ib, the BCR and ABL signals would be separated by about 200 kb, whereas if the breakpoint is situated close to exon a2, the two signals would be separated by only about 10 - 15 kb. To investigate whether the breakpoint position affects the assessment of FNRs, CFU-GM colonies from three patients with 5' breakpoints (close to the 5' end of the ABL2) and two with 3' breakpoints (close to the exon a2), characterised by Bubble PCR in my study, were analysed. The FNRs were indeed higher for the patients with 5' ABL breakpoints. On the basis of these results, the criteria for a correct estimation of Ph positivity or Ph negativity were proposed in this FISH study (Chase *et al* 1997).

In summary Bubble PCR is a powerful tool that has potential applications in both basic research and clinically therapeutics.

CHAPTER FOUR CHARACTERISATION OF BREAKPOINTS WITHIN THE M-BCR

4.1. Introduction

The previous chapter described the establishment of the Bubble PCR procedure for human genomic DNA. I showed that this technique is potentially useful for characterisation of the translocation breakpoints between chromosomes 9 and 22. In order for the breakpoint between BCR and ABL genes to be identified, the sequence of the M-BCR needs to be analysed in detail with respect to the positions of potentially useful restriction enzyme sites. For individual patients, the approximate position of the breakpoint can be determined by a combination of Southern blotting and RT-PCR. In this chapter I am going to present the use of the established Bubble PCR technique to amplify and sequence M-BCR breakpoints in 17 CML cases. Bubble PCR is further confirmed to be a useful tool to clone BCR-ABL breakpoints from CML patients.

4.2. Results

4.2.1. Strategy considerations

In the initial stage of this study, I used Southern blotting and RT-PCR to localise approximately the positions of the breakpoints within the M-BCR prior to performing Bubble PCR. Southern blotting was also used in some cases to determine which restriction enzyme would be suitable for digestion prior to Bubble PCR, i.e. one that could generate a fragment that spans the breakpoint but is sufficiently small for PCR amplification. Once it was clear that the technique was indeed working, Bubble PCR

was performed on randomly selected CML patients without having first localised the approximate position of the breakpoint.

4.2.2. Localisation of the BCR-ABL breakpoint

Southern blot, RT-PCR and Bubble PCR were performed independently. Patient selection was essentially random in the initial stages of this study when I simply requested peripheral blood samples from CML patients when they visited the clinic. Subsequently patients were selected on the basis that they had undergone allogeneic BMT and that there were stored cells or DNA available from the same patient prior to BMT. Of 64 patients (Table 4-1) in this study, 38 patients were analysed by Southern blotting, 48 patients were screened by RT-PCR and 42 underwent Bubble PCR.

4.2.2.1. Localisation of the breakpoint by RT-PCR

RT-PCR was performed by single step amplification with BCR and ABL primers (Figure 4-1). Oligonucleotide primers used in the RT-PCR for BCR-ABL chimaeric gene were usually B2A and CA3- (Table 2-2), designed to hybridise to BCR exon b2 and ABL exon 3 respectively. A cDNA control PCR for the ABL gene with primers A2 and CA3- was always included for each sample to confirm adequate cDNA quality (Cross *et al* 1993b). In some cases, samples were analysed using a multiplex PCR procedure in which BCR-ABL is co-amplified with normal BCR, the latter serving as a control for adequate cDNA (Cross *et al* 1994b). A PCR product showing the b2a2 chimaeric type on a gel indicate that the genomic break probably falls in zone 2, 3, 4 or 5. The product b3a2 in a patient suggests that the genomic breakpoint falls in zone 3, 4 or 5 (Figure 4-3). Of the 48 patients in chronic phase screened by RT-PCR (Table 4-1), 27 (56%) patients expressed b3a2 BCR-ABL. 18 (38%) patients expressed b2a2 BCR-ABL. Two out of the 48 (4%) patients co-expressed both b2a2 and b3a2 types of BCR-

Table 4-1. Localisation of BCR-ABL breakpoints within the M-BCR

No	patient	RT-PCR	Southern blot	Bubble PCR	No	patient	RT-PCR	Southern blot	Bubble PCR
1.	CS	b3a3	BCR-	R-	33.	BO	b2a2	zone 1/2	zone 2
2	MS	b2a2	zone 1/2	nd	34	TL	b2a2	zone 4 Rs-	nd
3.	MT	b3a2	zone 4 Rs+	zone 4	35.	BG	b3a2	zone 4 Rs-	R-
4.	WS	b3a2	zone 3 Rs-	R-	36.	BL	b2a2	nd	nd
5.	EE	b3a2	zone 5	nd	37.	BB	b3a2	nd	nd
6.	AE	b3a2	zone 4 Rs+	zone 4	38.	LA	b2a2	nd	R-
7.	AJ	b3a2	zone 3 Rs+	zone 3	39.	GV	b2a2	nd	nd
8.	SL	b2a2	zone 1/2	zone 2	40.	DL	b3a2	zone 4 Rs+	nd
9.	AD	b2a2	zone 3 Rs-	R-	41.	CM	b2a2	zone 1/2	nd
10.	HS	b3a2	zone 3 Rs+	nd	42.	SR	b2a2	nd	nd
11.	MP	b3a2	zone 4 Rs+	nd	43.	IM	b3a2	nd	nd
12.	JG	b3a2	zone 4 Rs+	nd	44.	MC	b3a2	nd	nd
13.	FS	b3a2	zone 4 Rs-	nd	45.	HJ	b2a2	nd	nd
14.	IA	b3a2	zone 4 Rs+	R-	46.	HR	b2a2	nd	nd
15.	NR	b3a2	zone 4 Rs-	nd	47.	ER	b3a2	nd	nd
16.	CC	b3a2	zone 3 Rs+	zone 3	48.	EK	both	nd	R-
17.	CP	b2a2	BCR-	R-	49.	PS	nd	zone 3 Rs+	zone 3
18.	SS	both	zone 3 Rs-	nd	50.	BY	nd	nd	zone 4
19.	PB	b2a2	zone 1/2	R-	51.	AH	nd	nd	zone 4
20.	GR	b2a2	BCR-	R-	52.	ML	nd	nd	zone 2
21.	PJ	b3a2	zone 4 Rs-	R-	53.	GM	nd	nd	zone 3
22.	MM	b2a2	zone 3 Rs-	R-	54.	GL	nd	nd	zone 3
23.	RP	b3a2	zone 3 Rs+	nd	55.	SM	nd	nd	zone 3
24.	GI	b3a2	zone 5	nd	56.	RK	nd	nd	zone 4
25.	CU	b2a2	zone 3 Rs-	R-	57.	BD	nd	nd	R-
26.	VD	b3a2	zone 5	R-	58.	SA	nd	nd	R-
27.	DD	b3a2	zone 4 Rs+	R-	59.	TN	nd	nd	R-
28.	BA	b3a2	BCR-	zone 4	60.	BT	nd	nd	R-
29.	BM	b2a2	zone 1/2	zone 2	61.	AA	nd	nd	R-
30.	SI	b3a2	zone 4 Rs+	R-	62.	HT	nd	nd	R-
31.	DM	b3a2	zone 3 Rs+	zone 3	63.	NI	nd	nd	R-
32.	JB	b3a2	zone 4 Rs+	R-	64.	LS	nd	nd	R-

Note: both: a patient with both b2a2 and b3a2 mRNA expressions. nd: not done; R-: a re-arranged band failed to amplify; Rs+/-: rearrangement positive or negative as determined by Southern blot after digestion with Rsa I. BCR-: no rearrangement detected in the M-BCR by Southern blotting after digestion with BamH I, Bgl II and Hind III. All the patients in the table above were either Ph chromosome positive or BCR-ABL positive.

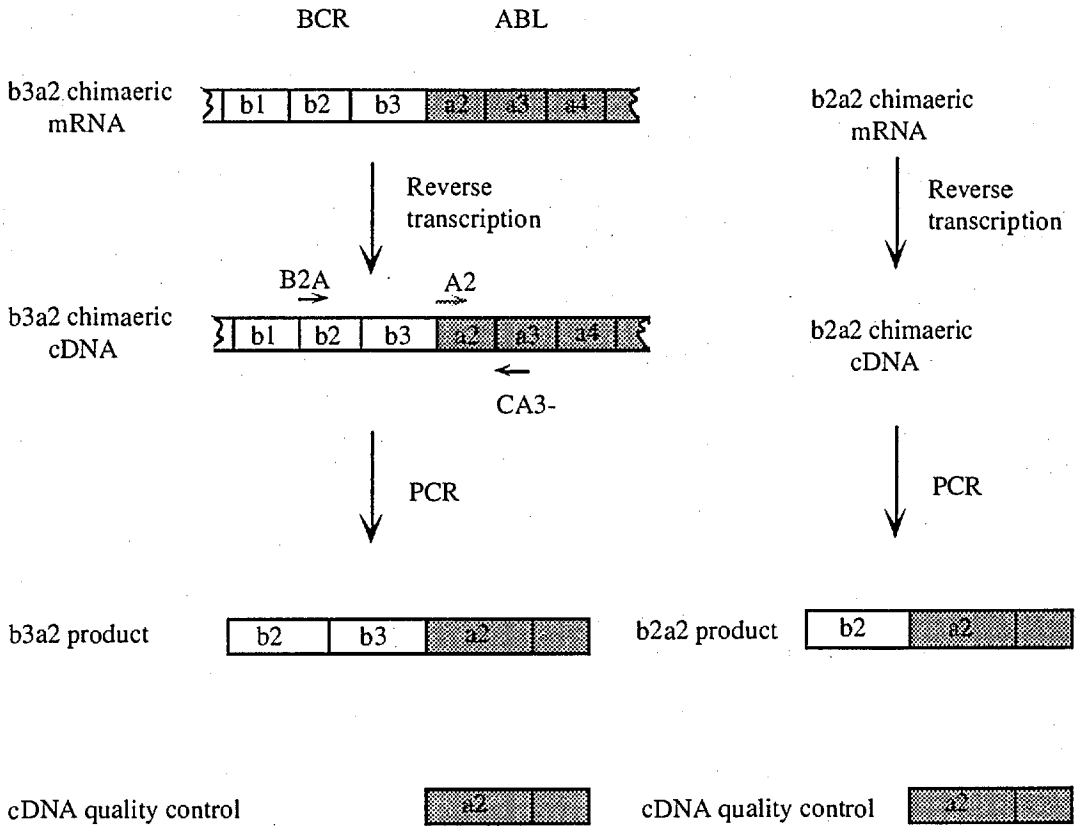


Figure 4-1. RT-PCR for CML patients

The chimaeric BCR-ABL mRNA is reverse-transcribed into cDNA. PCR is used to amplify b2a2 or b3a2 BCR-ABL fusion products. The horizontal arrows indicate position and orietation of the PCR primers.

ABL. The remaining one (2%) patient showed neither the b2a2 and b3a2 types. Its size was smaller than b2a2 and b3a2 (Figure 4-2).

4.2.2.2. Localisation of the breakpoint by Southern blot

The BCR-ABL translocation breakpoints could be virtually anywhere in the 5.8 kb M-BCR (Groffen *et al* 1984; Sowerby *et al* 1993) and in more than 200 kb of the first intronic region of ABL (Bernards *et al* 1987). There is no evidence to suggest that there is any subclustering of the breakpoints within the M-BCR region. Southern blotting was used to localise the BCR-ABL breakpoint within the M-BCR. On the basis of

approximate position determined by Southern blot and RT-PCR, a BCR primer was designed as close as possible to the breakpoint to maximise the chance that a Rsa I fragment harbouring the breakpoint will be within the size range for PCR amplification.

The M-BCR is divided into five zones according to the recognition sites of the restriction enzymes, BamH I, Hind III and Bgl II (Groffen *et al* 1984; Jaubert *et al* 1990; Mills *et al* 1991b; Figure 4-3). Genomic DNA from a patient was digested with

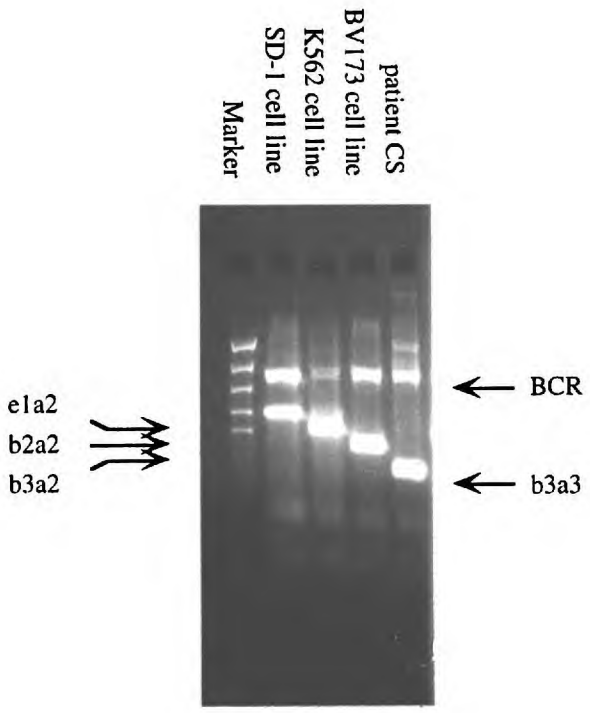


Figure 4-2. An irregular BCR-ABL detected by multiplex RT-PCR in CML patient CS

these three enzymes. The blot was hybridised with the 5' and 3' probes respectively. The position of the breakpoint was determined as previously described (Jaubert *et al* 1990; Inokuchi *et al* 1991). The 5' probe hybridised to the sequence between the 5' Bgl II and the 5' Hind III sites; the 3' probe hybridised to the sequence between the Hind III and the Bgl II sites at the boundaries of zone 4 (Figure 4-3). After BamH I digestion, the 5' probe detects a rearrangement in zone 1 or 2 (Table 4-2) because the 5' BamH I

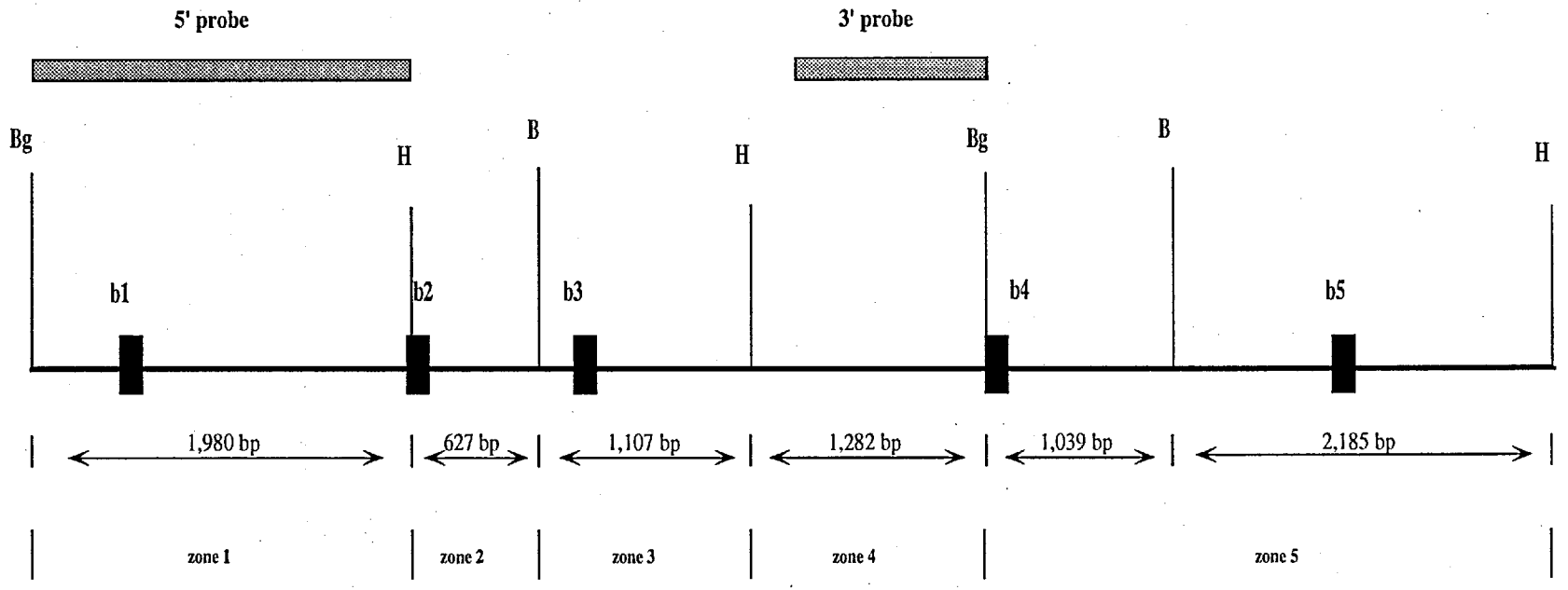


Figure 4-3. Restriction map of M-BCR

Positions of exons b1-b5 are shown as filled boxes. Zones are divided by the sites of restriction enzymes, defined by Groffen et al (1984) and positions of the probes used in this study are indicated by a stippled box. H=Hind III, B=BamH I, Bg=Bgl II.

site is at the 3' end of zone 2. The normal size of this BamH I fragment is expected to be 12 kb (the 5' BamH I site is located outside of the M-BCR and not shown in Figure 4-3). Fragments with a different size are considered to indicate a rearrangement in zone 1 or 2. In Hind III digestion the 5' probe can detect a rearrangement only in zone 1. The 5' Hind III site is outside the M-BCR as well and not shown in Figure 4-3. Its normal size is expected to be 10.5 kb. Thereby, if a rearranged band is found after BamH I digestion, whether the break falls in zone 1 or 2 can be distinguished by Hind III

Table 4-2. Localisation of the BCR-ABL breakpoints by Southern Blot

5' probe		3' probe	
Restriction enzymes	Rearranged Zones	Restriction enzymes	Rearranged Zones
BamH I	1, 2	BamH I	3, 4, 5
Hind III	1,	Hind III	4, 5
Bgl II	1, 2, 3, 4	Bgl II	1, 2, 3, 4

digestion. After Bgl II digestion, both the 5' and 3' probes can detect rearrangements in zones 1, 2, 3, and 4 because both probes hybridise to the same one (Figure 4-3). The normal size is 5 kb (Groffen *et al* 1984; Sowerby *et al* 1993; Chissoe *et al* 1995). For the 3' probe after BamH I digestion, a rearrangement within zone 3, 4 and part of zone 5 can be found. The normal size is 3.4 kb with BamH I. If a rearrangement falls into anywhere in zone 4 or 5, it can be found with the 3' probe after Hind III digestion. The germline size of the 3' Hind III band is 4.5 kb. Of 38 patients (Table 4-1) screened by Southern blot, the breakpoint was successfully localised in 34 (89%). Six (18%) patients had a breakpoint in the zone 1 or 2, 11 (32%) patients had a breakpoint in the zone 3, fourteen (41%) patients in the zone 4, and the three (9%) had breaks in the zone 5. The reason that the position of the breakpoint could not be determined for four patients may have been due to extensive deletions of BCR sequence or breakpoints located outside the M-BCR (Popenoe *et al* 1986; Saglio *et al* 1988).

4.2.2.3. Comparison of results obtained by both RT-PCR and Southern blot

Of the patients described above, 37 patients were analysed by both Southern blot and RT-PCR (Table 4-3). All the six patients with genomic breakpoints in zone 1 or 2 expressed the b2a2 type of mRNA. However, of the 24 breakpoints localised to zones 3 and 4, 4 patients expressed b2a2 BCR-ABL (three for zone 3; one for zone 4) and 19 expressed b3a2 (seven for zone 3; twelve for zone 4). The remaining one patient who

Table 4-3. Results of localisation of BCR-ABL breakpoint

		zone					All zones
		1+2	3	4	5	BCR-	
DNA breakpoint*		6	11	13	3	4	37
RNA	b2a2	6	3	1		2	11
	b3a2		7	12	3	1	22
	b2a2+b3a2		1				1
	other					1	1

* DNA breakpoints were localised by Southern blot.

expressed both b2a2 and b3a2 BCR-ABL had a genomic breakpoint in zone 3 [the other one in the only two patients who co-expressed both b2a2 and b3a2 mRNAs was not localised by Southern blot (Table 4-1)]. The patients whose breakpoints were found in zone 5 all expressed b3a2 BCR-ABL. In four patients for whom a rearrangement was not detectable by Southern blot, b3a2 mRNA was expressed in one patient, and b2a2 was expressed in two patients and an irregular mRNA in the remaining one patient (Table 4-1).

4.2.3. Sequence analysis of an unusual size RT-PCR product

BCR-ABL amplified by RT-PCR from patient CS for localisation of the breakpoint (Table 4-1) was purified and the DNA was sequenced by an automatic sequencer (373A DNA sequencer, Applied Biosystems) in both orientations with primer B2B and CA3-

with an ABI PRISM™ dye terminator cycle sequencing kit. The BCR-ABL was found to be that BCR exon b3 was juxtaposed with ABL exon a3. The sequence is illustrated in Figure 4-4. This figure is in frame and has been previously reported as a rare variant BCR-ABL (Iwata *et al* 1994).

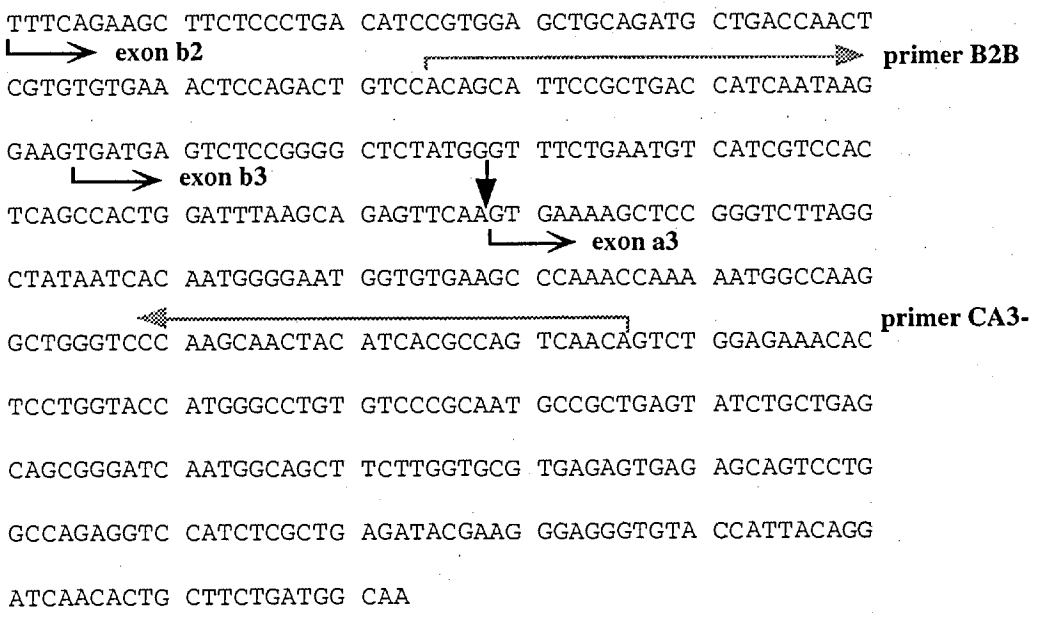
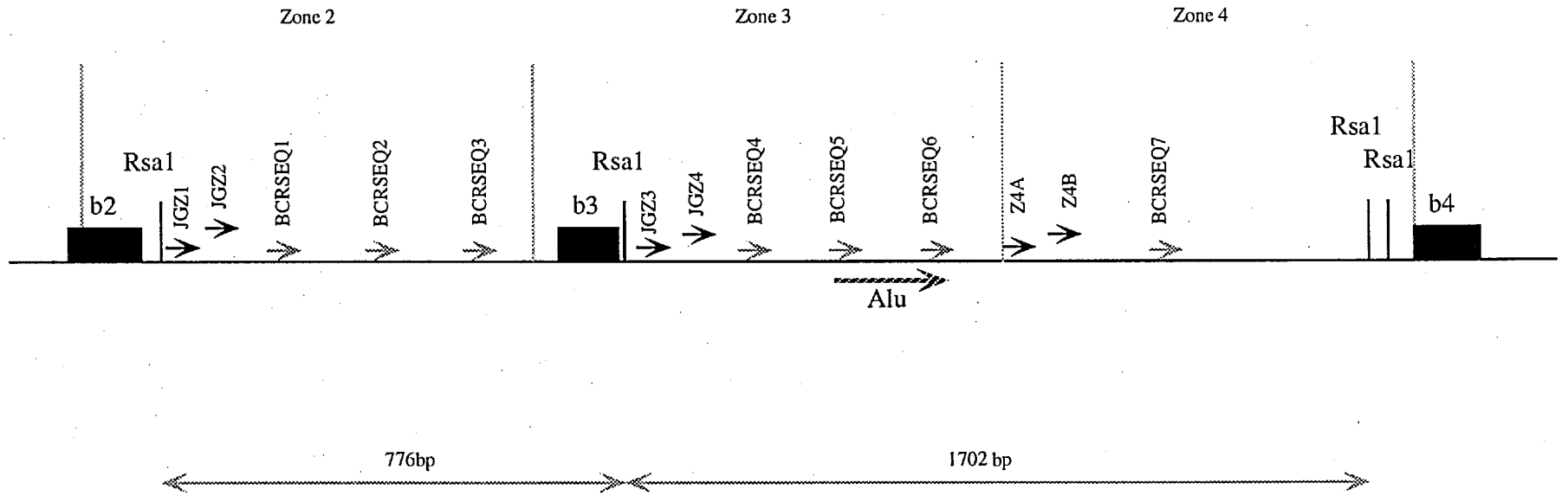


Figure 4-4. b3a3 BCR-ABL amplified from patient CS by RT-PCR

The vertical arrow in the sequence points the b3a3 junction site. The stippled arrows indicate positions of sequencing primers. The solid arrow show the positions of exons. Exons b2 and b3 are from the M-BCR in the BCR gene and exon a3 from ABL gene.

4.2.4. Rsa I enzyme is selected for characterisation of BCR-ABL breakpoint

Rsa I was chosen as the cutter for Bubble PCR. Rsa I is a 4 base pair cutter, recognising the sequence GTAC. This site theoretically occurs once in every 256 bp of genomic DNA. However, in the sequenced region of the M-BCR, Rsa I is underrepresented. There is one site situated 20 bp downstream of the exon b2 (Figure 4-5). The next site is 776 bp downstream and 5 bp away from the exon b3. In zone 4 there are two Rsa I sites, 100 bp apart. However, the region between the site in zone 3 and the first one in zone 4 is 1.7 kb. The spacing of Rsa I sites means that it is relatively easy to design a set of nested PCR primers for Bubble PCR amplification that cover most of the M-



Fiure 4-5. Positions of Primers for Bubble PCR Amplification

A short solid arrow indicates Bubble PCR primers; a short stippled arrow indicates sequencing primer. The striped arrow shows the position and orientation of Alu element. Black boxes indicate M-BCR exons. Rsa I enzyme sites are underrepresented within this region. The size between the first two Rsa I sites is 776bp and between the second and third Rsa I is 1,702bp.

BCR. Moreover, Rsa I generates blunt ended fragments of DNA so that the ends of DNA fragments are compatible with the Bubble oligonucleotide. Rsa I is only partially methylation sensitive. $GT^{m6}A^{m5}C$ can not be cleaved by Rsa I, but this modification is very rare in human genomic DNA. Rsa I is unaffected by both ^{m4}C and ^{m5}C modifications. Therefore, broadly speaking, Rsa I is not sensitive to site-specific methylation and particularly useful for achieving complete digestion of methylated DNA (Nelson & McClelland, 1991).

The size of the fragments created by Rsa I digestion are theoretically within PCR amplification range if Rsa I cut DNA in every 256 bp. Therefore, chance for catching the breakpoint may be increased if they frequently occur in the ABL side. Southern blotting gel was performed with selected CML patient genomic DNA digested with Rsa I. Many rearranged bands, when detectable, were indeed smaller than the genomic 1.7 kb Rsa I band (Figure 4-6).

4.2.5. Characterisation of BCR-ABL breakpoints on chromosome 22q- by Bubble PCR

Three sets of PCR primers were designed to span most of the M-BCR immediately after each Rsa I site. Two primers were made for nested PCR in each zone (Figure 4-5). Nested PCR primers JGZ1 and JGZ2 were for zone 2, JGZ3 and JGZ4 for zone 3, Z4A and Z4B for zone 4 (Table 4-4). The primers NVamp-1 and NVamp-2 were designed to the unique sequence, copied from the bottom strand of the Bubble oligonucleotide, formed after strand synthesis initiated at the first of the BCR primers used (Figure 3-5; Figure 3-11(c)). Primers JGZ1, JGZ3, Z4A and NVamp-1 were used for the first round PCR and JGZ2, JGZ4, Z4B and NVamp-2 for the second round PCR (Table 4-4). After Rsa I digestion, the normal BCR gene amplified with the zone 2 primers, JGZ2 and NVamp-2, should be 776 bp in size (plus 33 bp from the Bubble oligonucleotide). The size of the normal BCR gene amplified with zone 3 primers, JGZ4 and NVamp-2,

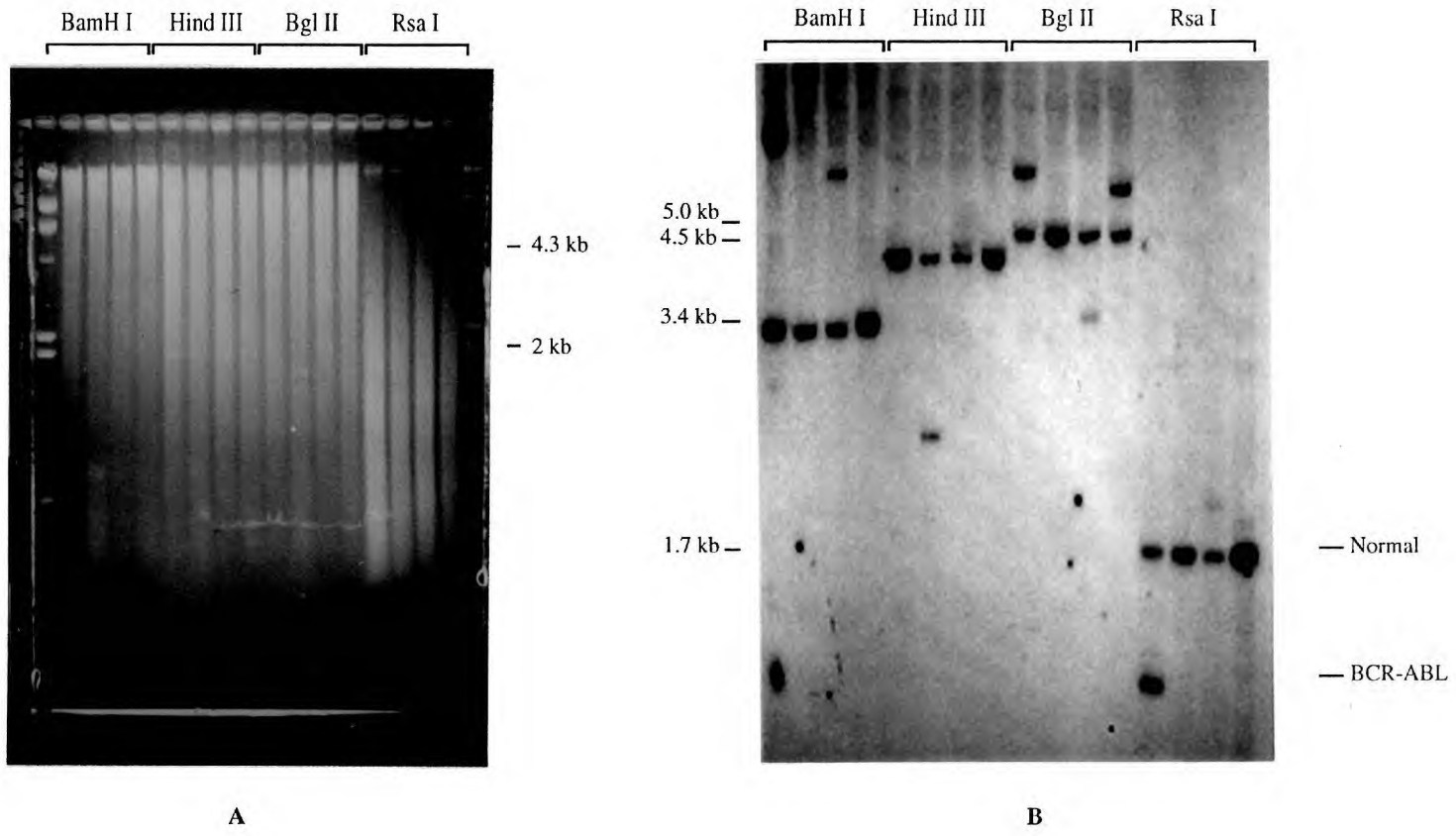


Figure 4-6. Genomic DNA after Rsa I digestion

A) Rearranged DNA fragments after Rsa I digestion are smaller than 1.7 kb in size. B) A rearrangement in a CML patient AH was detected with Rsa I enzyme. The size of the fragment is within the range of PCR amplification.

should be 1,699 bp. For zone 4 the size should be 879 bp. The DNA fragment amplified with the zone 3 primers actually includes the fragment amplified with the zone 4 primers since there is no Rsa I site located between zones 3 and 4. However, I found it preferable to perform PCR separately for zone 4 since the fragment produced by the zone 3 primers is relatively large.

Forty two Ph-positive CML patients in chronic phase (40 patients) or blast crisis (2 patients) tested by Bubble PCR were selected essentially at random as described above. Products spanning the BCR-ABL breakpoints were successfully amplified from 17 patients (40%) (Table 4-1). The breakpoints from patients BO, ML, BM and SL were

Table 4-4. Bubble PCR primer combinations

PCR steps / M-BCR	First step		Second step	
Zone 2	JGZ1	Nvamp-1	JGZ2	Nvamp-2
Zone 3	JGZ3	Nvamp-1	JGZ4	Nvamp-2
Zone 4	Z4A	Nvamp-1	Z4B	Nvamp-2

amplified in zone 2 with PCR primers JGZ1 and NVamp-1 for the first round PCR and with primers JGZ2 and NVamp-2 for the second round. Similarly the primers JGZ3, JGZ4, NVamp-1 and NVamp-2 were used for two step amplification of the breakpoints in patients GM, AJ, GL, SM, DM, MT, PS in zone 3. The breakpoints in patients BA, AE, RK, AH, BY and CC were amplified with primers Z4A, Z4B, NVamp-1 and NVamp-2 in zone 4 (Table 4-5). The breakpoint in patient PS was identified with zone 3 PCR primers but it was actually located at the beginning of the zone 4. It could not in fact be amplified by zone 4 PCR primers because the break site in this patient occurred at the same position as primer Z4B. Several examples of successful Bubble PCR amplification are shown in Figure 4-7. The remaining 25 samples were tested with all three nested primer combinations but a rearranged band failed to amplify, presumably due to lack of a convenient RsaI site on the ABL side of the translocation (Figure 3-17B), or because the breakpoints fell downstream of the first zone 4 Rsa I site or upstream of the Rsa I site in zone 2.

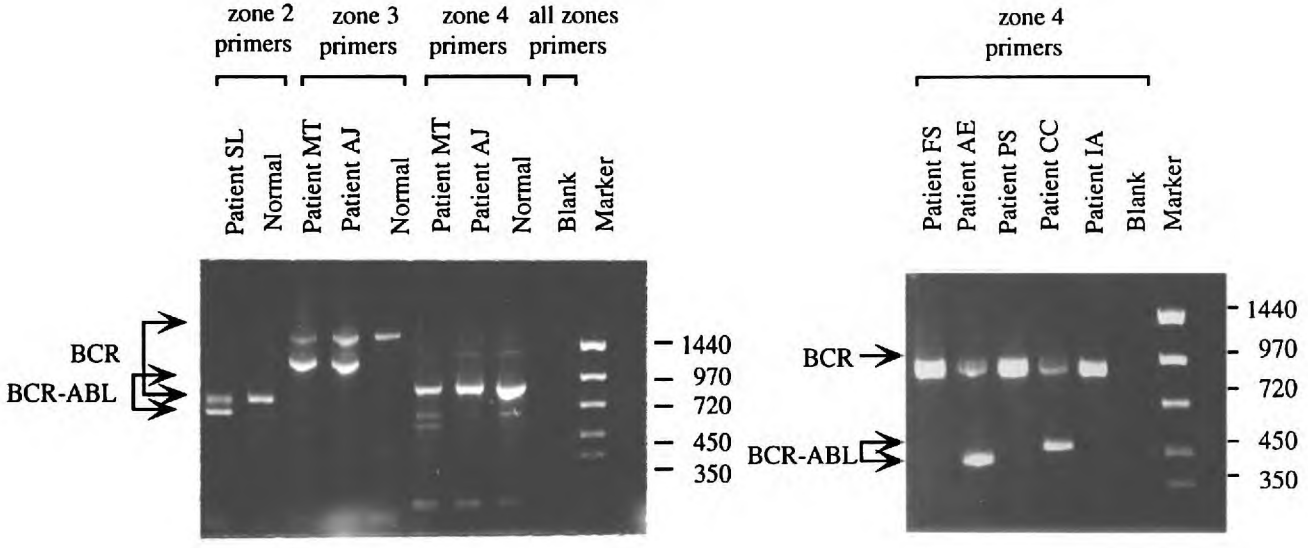


Figure 4-7. BCR-ABL breakpoints amplified from CML patients by Bubble-PCR

BCR-ABL breakpoints amplified by Bubble PCR for CML patients with zone 2, zone 3, and zone 4 primers in M-BCR region.

Table 4-5. Results of BCR-ABL breakpoints amplified by Bubble PCR

Patients	Region	BCR primers	Primers for Bubble oligo
1. BO	zone 2	JGZ1 + JGZ2	NVamp-1 + NVamp-2
2. ML	zone 2	JGZ1 + JGZ2	NVamp-1 + NVamp-2
3. BM	zone 2	JGZ1 + JGZ2	NVamp-1 + NVamp-2
4. SL	zone 2	JGZ1 + JGZ2	NVamp-1 + NVamp-2
5. GM	zone 3	JGZ3 + JGZ4	NVamp-1 + NVamp-2
6. AJ	zone 3	JGZ3 + JGZ4	NVamp-1 + NVamp-2
7. GL	zone 3	JGZ3 + JGZ4	NVamp-1 + NVamp-2
8. SM	zone 3	JGZ3 + JGZ4	NVamp-1 + NVamp-2
9. DM	zone 3	JGZ3 + JGZ4	NVamp-1 + NVamp-2
10. MT	zone 3	JGZ3 + JGZ4	NVamp-1 + NVamp-2
11. PS	zone 4	JGZ3 + JGZ4*	NVamp-1 + NVamp-2
12. BA	zone 4	Z4A + Z4B	NVamp-1 + NVamp-2
13. AE	zone 4	Z4A + Z4B	NVamp-1 + NVamp-2
14. RK	zone 4	Z4A + Z4B	NVamp-1 + NVamp-2
15. AH	zone 4	Z4A + Z4B	NVamp-1 + NVamp-2
16. BY	zone 4	Z4A + Z4B	NVamp-1 + NVamp-2
17. CC	zone 2	Z4A + Z4B	NVamp-1 + NVamp-2

Note: (JGZ3 + JGZ4*) are zone 3 primers. The breakpoint in patient PS was amplified with zone 3 PCR primers because it was exactly located within the zone 4 primer Z4B.

Sequencing of amplified fragments was performed by one of two different protocols: i) Fragments were gel purified and cloned into M13mp18 and 19 or ii) Fragments were gel purified and sequenced directly with a Δ-Taq cycle sequencing kit (USB). The compete sequence of each fragment was not usually determined. I aimed to identify unambiguously the position of the breakpoints within the M-BCR and also obtain sufficient ABL sequence to enable the design of patient-specific PCR primers (see Chapter 7). Initially all the inserts were sequenced in both orientations with the 5' and 3' sequencing primers. Because the fragments amplified by Bubble PCR were usually too

large for the breakpoint to be identified in a sequencing reaction primed with either the M13 -40 primer or the second step BCR primer, a series of new sequencing primers were designed. BCRSEQ1, BCRSEQ2 and BCRSEQ3 were made for the zone 2 of the M-BCR. The breakpoints for patients BM and SL were obtained by sequencing walks primed by these three primers. BCRSEQ4, BCRSEQ5, BCRSEQ6 were synthesised for the zone 3. For patients GL, SM, DM, MT and PS, the breakpoints were identified by those sequencing primers. The breakpoints for patients BY and CC were characterised by sequencing primer BCRSEQ7. The locations of the sequencing primers in the M-BCR are illustrated on Figure 4-5 and the sequences of the primers are listed in Table 4-6.

Table 4-6. Sequencing primers for the M-BCR region

Name	Sequence
BCRSEQ1	5'-CACAAGGCTGCAGCAGA-3'
BCRSEQ2	5'-TCCCACATCACCCCGAC-3'
BCRSEQ3	5'-AGATCCCCAAGACAGAA-3'
BCRSEQ4	5'-GGGAGCTGGTGAGCTG-3'
BCRSEQ5	5'-AGCTACTGGAGCTGTC-3'
BCRSEQ6	5'-TTGGAGACCAGCCTGAC-3'
BCRSEQ7	5'-TGTCTCTGGCTGCCTC-3'
BUBSEQ 8	5'-GAGAATCGCAAGATCTA-3'

The sequences of all Bubble PCR products were compared with that of the M-BCR, and the positions at which the sequences diverged, i.e. the site of the M-BCR breakpoint for each patient are shown on Figure 4-8. Table 4-7 shows the sequences 20 bp either side of the break for each case. The breakpoints in patients DM, MT and SM is located within the Alu in M-BCR (Table 4-8).

At the time I performed this analysis, the sequence of the ABL introns were completely unknown. However, during the course of this study, Chissoe *et al* (1995) published the sequence of most of the ABL gene and then I was able to confirm directly for many cases that the amplified sequences distal to the breakpoints were indeed from the ABL

gene (see Chapter 6). Confirmation that the sequence were genuinely BCR-ABL junction fragments was also made in some cases by designing patient-specific sets of primers (Table 4-9). As expected, each primer pair only generated a specific product from the patient for whom the primers were designed (see Chapter 7).

Table 4-7. Flanking Sequences of the Breakpoints characterised by Bubble PCR

1. AE	ATGGATGAATTACATGACAT/TTGCAGAATCAGAAAAAGTA
2. CC	CCCGGGCTTGTCTCTCCTTG/GGTGCAGTGGCTCACACCTG
3. GL	AATTGCAGGGGTTTGGCAAG/CACCTTTTCATATGTTTACT
4. BA	CTAAAATTCTTTAAACCCTA/AAGCGGATTTACTCTAAGGC
5. DM	GATTAGCCAGGCTAGGCAGT/TTTAAATCCTGGCTTTCCCC
6. MT	GCATTCCAGCCTGGGCGACA/ATGCAAGAAAGAATAGTGAG
7. ML	TTGATGGGACTAGTGGACTT/ATGACAAAATAACAATATGG
8. GM	CTCATCGGGCAGGGTGTGGG/TCTTCACTTTCGTAGCTTCT
9. AJ	TGGGAGCTGGTGAGCTGCCC/ATAATGACATGATTTGGGAT
10. PS	GGGATTGTTGGGGAATGGGG/CTGCAGTGAGCCGAGATCGT
11. AH	CCTCATAAACGCTGGTGTTT/GGCTTGTCATAGAAGGGCAT
12. BO	GCACTTTTGATGGGACTAGT/CCTTATCCTTAGATTAGATC
13. SM	TCATGCCTGTAATCCCAGCA/AGGATCCTAGATCTTGCGAT
14. AL	ACACAGTGTCCACCGGATGG/ATTTTATAAGTTGATCCAAC
15. RK	TCTCTGGCTGCCTCATAAAC/AATGGTCCCCCCACCCTCAC
16. BY	CTCGTGGGCCTCCCTGCATC/TCCTCATTTTGGAGATATTT
17. BM	AGACAGAAATCATGATGAGT/TACAGAAAGGGGCACAAATT

Note: The sequences underlined are the BCR portion. / indicates the BCR-ABL breakpoint.

An average of 116 bp of ABL sequence (range 30 - 244 bp) was determined at each BCR-ABL junction (Table 4-10) from various size of PCR product (Table 4-11). There sequences were used to search the Genbank database (release 81; Feb.1994). For patients CC and PS, the breakpoints fell within an ABL Alu element. For patient GM, an Alu element was found 158 bp downstream of the breakpoint. For patient GL, the breakpoint fells in a L1-like sequence (Figure 4-9). No other significant sequence features were found, except for patient AE where the break fell in an A-T rich region.

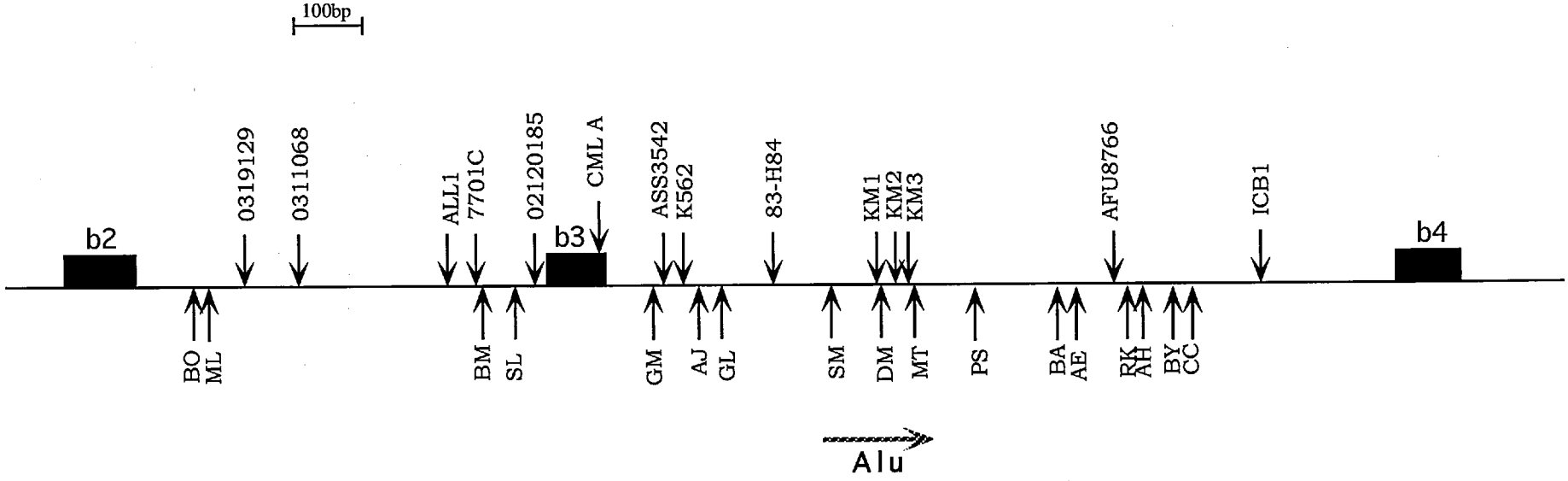


Figure 4-8. BCR-ABL breakpoints in the M-BCR

The arrows pointing downward show the positions of the BCR-ABL breakpoints identified by others (see text). The arrows upward indicate the positions of the breakpoints characterised in my study. The Alu element in the M-BCR is presented as the longer arrow along the horizontal line.

Table 4-8. Sequence feature analysis for the breakpoint flanking sequence cloned from chromosome 22q-

Patient	Alu in BCR	Alu in ABL	L1 in ABL	AT-rich
AE				+
AH				
AJ				
AL				
BA				
BO				
BM				
BY				
CC		+		
DM	+			
GL			+	
GM		close to Alu		
ML				
MT	+			
PS		+		
RK				
SM	+			

Figure 4-9. Comparison of ABL sequence in patient GL from the BCR-ABL breakpoint with L1 element

>GB:H72652 H72652 yu05d12.r1 Homo sapiens cDNA clone 232919 5' similar to contains L1 repetitive element.

Length = 484

Plus Strand HSPs:

Score = 127 (35.1 bits), Expect = 0.011, P = 0.011
Identities = 39/56 (69%), Positives = 39/56 (69%), Strand = Plus / Plus

```
Query:     1 CACCTTTTCATATGTTTACTGGCCATTTGGAATATATCTCTCAGGATCCTAGAGTC 56
             ||||||| || ||| | | ||||||||||||     |||| ||  ||   | ||||
Sbjct:   179 CACCTTTCCAAATGATAATTGGCCATTTGGACATCATCTTTCTTGAAGATCAAGTC 234
```

Table 4-9. Confirmation of ABL sequences by PCR reamplification

Patient	Bubble PCR				Reamplification PCR			
	BCR primer	Bubble primer	Positivity	Sample	BCR primer	ABL primer	Positivity	Sample
AE	Z4A, Z4B	NVAMP-1&2	+	CP	Z4A, Z4B	1489ABL	+	CP
AH	Z4A, Z4B	NVAMP-1&2	+	CP	Z4A, Z4B	1620BA	+	PBMT
AJ	JGZ3&4	NVAMP-1&2	+	CP	B3A, B3B	1535ABL	+	PBMT
SL	JGZ1&2	NVAMP-1&2	+	CP	B2A, B2B	1572BA	+	PBMT
BA	Z4A, Z4B	NVAMP-1&2	+	CP	Z4A, Z4B	1546ABL	+	CP
BM	JGZ1&2	NVAMP-1&2	+	CP	B2A, B2B	1797BA	+	PBMT
BY	Z4A, Z4B	NVAMP-1&2	+	CP	Z4A, Z4B	1818BA	+	PBMT
CC	JGZ3&4	NVAMP-1&2	+	CP	B3A, B3B	1638ABL	-	CP
DM	JGZ3&4	NVAMP-1&2	+	CP	B3A, B3B	1854ABL1&2	+	PBMT
GM	JGZ3&4	NVAMP-1&2	+	BC	B3A, B3B	GLMNABL	+	BC
ML	JGZ1&2	NVAMP-1&2	+	CP	B2A, B2B	MaisABL	+	PBMT
MT	JGZ3&4	NVAMP-1&2	+	CP	B3A, B3B	1472ABL1&2	+	PBMT
RK	Z4A, Z4B	NVAMP-1&2	+	CP	Z4A, Z4B	1732BA1&2	+	PBMT

Postivity (+/-): specific amplification is positive or negative. CP: a sample from the patient in chronic phase prior to BMT. PBMT: a sample from the patient post BMT.

Table 4-10. ABL sequences obtained from chromosome 22q- by Bubble PCR

patient	sequence
AE	TTGCAGAATCAGAAAAAGTAAAAATAAAAAAAAAGGTTTTTAACCTTTT
AH	GGCTTGTCATAGAAGGGCATTTAAGTGACTTTGCCAAGAGAAACAGT
AJ	ATAATGACATGATTTGGGATTACTCTTTCAGAGAAACAGTTTCCTAAAGGTT TACTTCAGTTAGTGATTTTTATTTTTATT
SL	*ATGG*ATTTTATAAGTTGATCCAACCATGGTATGACTTCTGTGGGTAAGGATC CTGAGATCCCCAAGACAGAAATCATGATGAGTATGTTTTTGGCCCATGACAC TGGCTTACCTTGTGCCAGGCAGATGGACGCCACACAGTGTCACGATGATTTT ATAA
BA	AAGCGGATTTACTCTAAGGCAGTTCAGATTTGGTCCCCAGCTGAGAATTATA GCCTGGAAATACCAACAGGAAAATCAGTGTCATTTGAAGGACAGTCATCTGT GCAGCCTGTGCATGAAATCATGGGTCTGAATTAGGCCCCCCCATTCAAGATG CGGGGGTGTGGGGTTTCTCTTTTGCTGAA
BO	CCTTATCCTTAGATTAGATCTATATCTCCTCCACCCCCCCGTCAACTTT
BM	TACAGAAAGGGGCAGAAATTGTAAATGTAAGGATCCCCGGGTACCGAGCTCG AATTCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCA CAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGC CTAAT
BY	**TCCTC**ATTTTGGAGATATTTGGTGGACATTGGGCATTATACATACCCAGTAA GATATTAGCACCATGAAAACTCCACAAAAGGTCCGAGCGCCTAGAACAGT
CC	GGTGCAGTGGCTCACACCTGTAATCCCAGCACTTTGGGAACCCAAGGTGGGA CGGTCCTCTGAGACCAGGAGTTTGAGAGAGTGGCCTGGACAACATAGCAACC ACCCTATTAGT
DM	TTTAAATCCTGGCTTTCCCCTTAACTTGGGTGGGATCACACCATTAACCGTG TTACCCCTCTGAAGCTTTGGCTCCCACACCTGTGAGATGTGAATGTTGATAA CACACACCCACCTCATAGGATTAATGAGAACATGCCAAGATATAATGCGCTG AAGGTCTTAGCATGCCTGCGCAGAGCCCCAGCAT
GL	CACCTTTTCATATGTTTACTGGCCATTTGGAATATATCTCTCAGGATCCTAG AGTCGACC
GM	TCTTCACTTTCGTAGCTTCTAGGTGGGTATGAAATTGATCTGATTTGAGACT GGTGCTCCCAGACCATTGCCGTCAGCAGAAAGGCTATCTGTGCTTCAGAGAT GGAGTGGCACTTGGACTTCTCTGTTGTTTTCAACTAAGAATATAAGAGTAAA AATGTGGCCGGGCGCGGTGGCTCAAGCCTGTAATCCCAACTCTTTGGGAGGC CGAGACGGTGGATTATTTGAGGTCAGGATTTCGAGA
ML	ATGACAAAATAACAATATGGTTTTAAGTGGAGTGAGAGGCACTTTTGAAGAT CCAGATCGA
MT	ATGCAAGAAAGAATAGTGAGGGAAAAGAAAAAGTGGAATGAATAGAGAATTA GTCATGTGGTATCACATGGCAGCTCACTCAGGAATCATAAAGCTGGCCTTGG GATAACAGAGCTAAACTTTAGAGTCCTGAGTCCTGTTAATACTCAATCCTCT GTGAATTCAATTAGTGAGCTTCAGTTACCTAAA
PS	CTGCAGTGAGCCGAGATCGTGCCACTGCCATCCAGCCTGGGCGACAGAGCGA GACTCCGTCTCAAAAAAAAAAAAATTACTATCATGTAAGGG

RK *TAAAC*AATGGTCCCCCCACCCTCACCCCGTGGCACTCGGCCAAAGGAAGAGG
AAAAGTTCTCTGTTAAAGAAAATGAATCAGAGAAGAGGCCCAGACCGTGCCT
CCGAAAATTCTCATTGTAGGGACTAAGTCCTCTCACTCTGAACTCACACCCG
TC

SM AGGATCCTAGATCTTGCGATTCTCGTACGA

Note: Letters in bold show a sequence that is neither BCR nor ABL sequence. Letters in italics indicate a sequence that exists in both BCR and ABL.

Table 4-11. Bubble PCR products

Patient	PCR product	Size of BCR sequence	Size of ABL that was sequenced
AE	450 bp	192 bp	49 bp
AH	312 bp	265 bp	47 bp
AJ	1,400 bp	235 bp	81 bp
BA	900 bp	164 bp	185 bp
BM	650 bp	542 bp	161 bp
BO	200 bp	80 bp	49 bp
BY	389 bp	287 bp	102 bp
CC	488 bp	373 bp	115 bp
DM	843 bp	653 bp	190 bp
GL	333 bp	273 bp	60 bp
GM	300 bp	72 bp	244 bp
ML	135 bp	86 bp	61 bp
MT	1,440 bp	766 bp	189 bp
PS	1,200 bp	860 bp	93 bp
RK	720 bp	255 bp	158 bp
SL	780 bp	613 bp	160 bp
SM	700 bp	537 bp	30 bp

4.3. Discussion

4.3.1. Comparison of results from Bubble PCR, Southern blot and RT-PCR

Of the 64 CML patients studied, samples were randomly selected for RT-PCR, Southern blot and Bubble PCR. For 33 patients, localisation of the BCR-ABL breakpoint was carried by both Southern blot and RT-PCR. Twenty five patients were characterised by both Southern blot and Bubble PCR.

4.3.1.1. RT-PCR cannot be used for localisation of genomic BCR-ABL breakpoint in the M-BCR region

Both Southern blotting and RT-PCR analysis were performed on 33 patients. An apparent disparity between the two techniques was found in 4 (40%) of the 10 cases who expressed transcripts b2a2 (Table 4-3). Patients AD, MM and CU all expressed b2a2 BCR-ABL mRNA but their genomic breakpoints were in zone 3, i.e. downstream of exon b3. Patient TL, who also expressed b2a2, had a BCR-ABL breakpoint in zone 4 (Table 4-1).

The breaks in twenty three patients who expressed b3a2 BCR-ABL (including one patient who co-expressed b2a2/b3a2) were located to zones 3, 4 or 5 of the M-BCR (Table 4-1). The thirteen patients with b3a2 RNA type had a genomic break in zone 4. The three patients with b3a2 RNA type has a break in zone 5.

These results demonstrate that it is not possible to localise the BCR-ABL breakpoint in genomic DNA by RT-PCR according to b2a2 or b3a2 RNA type. Southern blot is much better, but the resolution is rather low, and for some patients, the break can not be identified (Table 4-1).

The distribution of the breakpoints within the M-BCR is similar to that described in several publications (Table 4-12). Of the 34 patients for whom I successfully localised the breakpoint by Southern blot, 6 (18%) fell in zone 1 or 2, 11 (32%) fell in zone 3, 14 (41%) in zone 4 and 3 (9%) in zone 5 (Table 4-12). If the frequency of breaks was simply proportional to the length of DNA in each zone (2,607 bp in zone 1/2, 1,107 bp in zone 3, 1,282 bp in zone 4 and 3,224 bp in zone 5) (Figure 4-3), then the frequency of breaks would theoretically be 32% in zone 1 and 2, 13.6% in zone 3, 14.8% in zone 4 and 38% in zone 5. The disparities between the Southern blot results and theoretical

Table 4-12. Distribution of the breakpoints in the M-BCR

Zones				Patients studied	References
1 + 2	3	4	5		
31%	27%	42%	0%	26	Schaefer-Rego, et al., 1987
24%	33%	36%	6%	99	Shtalrid, et al., 1988
25%	34%	37%	4%	80	Mills, et al., 1989
31%	33%	36%	1%	129	Grossman, et al., 1989
35%	23%	35%	8%	66	Jaubert, et al., 1990
20%	44%	33%	2%	81	Morris, et al., 1990
18%	36%	47%	0%	45	Tien, et al., 1990
22%	35%	38%	5%	106	Mills, et al., 1991b
42%	28%	22%	8%	60	Opalka, et al., 1991
18%	32%	41%	9%	38	This study
26%	33%	37%	4%	752	Average percentage / Total

estimate in terms of the length are due to the definition of the boundary of the M-BCR. The great majority of breaks actually fell between exons b2 and b4. It is clear, therefore, that Southern blot can not provide a precise location of the breakpoints. Sequence data, however, would be more informative (see 4.3.4.).

Comparison of the data derived from my study with the results obtained by others (Schaefer Rego *et al* 1987; Shtalrid *et al* 1988; Mills *et al* 1988; Mills *et al* 1989; Grossman *et al* 1989; Jaubert *et al* 1990; Morris *et al* 1990; Tien *et al* 1990; Mills *et al* 1991b; Opalka *et al* 1991), demonstrates that there is no subclustering of breakpoints within the M-BCR (Table 4-12).

The percentages of b2a2 and b3a2 RNA expression types in a population of 52 patients investigated by Mills *et al* were 54% and 46% respectively (Mills *et al* 1991b). In a report of 34 patients studied by Futaki *et al* b2a2 and b3a2 were 35% and 38% respectively. Dual expression b2a2/b3a2 was 24%. The remaining 3% was negative for BCR-ABL expression (Futaki *et al* 1992). These data suggested that b2a2 and b3a2 RNA splicings randomly occurs in a population. For the 48 patients I studied by RT-PCR, 18 (38%) expressed b2a2 BCR-ABL; 27 (56%) expressed b3a2 BCR-ABL; 2 (4%) expressed both b2a2 and b3a2 BCR-ABL and 1 (2%) expressed other types. These proportions are somewhat different from the publication figures; this is probably due to the fact that the sample size was relatively small.

4.3.1.2. The results from Bubble PCR and Southern blot are concordant

Of 17 patients whose BCR-ABL breakpoints amplified by Bubble PCR, ten had Southern blot results (Table 4-1). Three of these patients had breakpoints in zone 2 (BO, BM, SL), three patients (AJ, DM and MT) in zone 3 and four (PS, BA, AE and CC) in zone 4. The genomic rearrangement in all these patients had been localised to the same zone by Southern blot except for one (patient BA). For this patient, no rearrangement had been detected. The reason for this could be: either that the rearranged DNA fragments were, by co-incidence, the same size as the germline bands or very similar to the germline bands in length. The other seven patients characterised by Bubble PCR were not analysed by Southern blot.

Of 42 patients for whom Bubble PCR was attempted, 25 had been analysed by Southern blot (Table 4-1). 16 out these 25 patients were blotted with Rsa I digestion and probed with the 3' probe (Table 4-1). In the Rsa I blot, 10 of the 16 patients showed a rearrangement. Of these 10, BCR-ABL breakpoints were successfully amplified by Bubble PCR for 6 patients (AE, AJ, CC, DM, MT and PS). The sizes of the rearranged bands after Rsa I digestion for those 6 patients were estimated to be 1.5 kb, 1.4 kb, 0.5

kb, 0.8 kb, 1.4 kb and 1.2 kb respectively. The locations of the breakpoints (Table 4-5) and size of the fragments (Table 4-11) obtained by Bubble PCR in the M-BCR were concordant with the ones defined by Southern blot (Table 4-1): for the five patients who had zone 3 or 4 breaks, the Bubble PCR product was, as expected, approximately the same size at the rearranged Rsa I band. For patient AE, the Bubble PCR product was approximately, but smaller than the rearranged Rsa I band, because the primers Z4A and Z4B were used for this patient, rather than primers JGZ3 and JGZ4. The size of the bands in the six patients were all smaller than 2 kb on the Southern blot (Figure 4-6B). Of the remaining 4 patients (IA, DD, SI and JB) who had a rearranged Rsa I band, the length of the fragments was too large to be amplified by Bubble PCR (2.8 kb, 2.6 kb, 3.4 kb and 3.0 kb respectively). Therefore, the Bubble PCR results were completely concordant with those obtained by Southern blotting.

Two patients expressed both b2a2 and b3a2 mRNA. For patient SS, Southern blot showed that there was a single rearrangement in zone 3 rather than two independent genomic BCR-ABL breakpoints in the M-BCR (Table 4-1). The other patient (EK) with dual expression was tested by Bubble PCR but not Southern blot. Unfortunately the breakpoint failed to amplify in this patient. The explanation for the dual expression is that RNAs are presumably due to alternative splicing of exon b3, possibly due to disruption of the normal splicing pattern by its translocation (Lee *et al* 1989; Morgan *et al* 1990).

Patients CS expressed an aberrant size of BCR-ABL mRNAs (Figure 4-2). This irregular size mRNA was characterised by sequencing and found to result in a fusion between BCR exon b3 and ABL exon 3 (b3a3). Southern blot failed to find the location of the breakpoint in the M-BCR. Bubble PCR also failed to clone the breakpoint in this patient. There was a report claiming that patients with BCR-ABL transcript can lack ABL exon a2 (Iwata *et al* 1994). In the two cases reported with b3a3 BCR-ABL, one was found in a patient with CML and the other in a patient with ALL. ABL exon a2 is

found consistently in the majority of CML patients. The 58 amino acids encoded by this exon (174 bp) is part of a stretch of 50 amino acids that form the src homology 3 region (SH3), which is thought to have a negative regulatory effect on the kinase domain (SH1). In BCR-ABL fusions lacking exon a2, this regulatory region is partially absent.

Bubble PCR had been confirmed by Southern blot to efficiently detect the breakpoint if there is a convenient Rsa I site. Subsequently therefore, Bubble PCR was directly performed on patients without Southern blot or RT-PCR data with three combinations of the BCR primers spanning zone 2, 3 and 4 (Table 4-1).

4.3.2. Analysis of the ABL sequences

The ABL sequence of patient GL contained an L1 like repetitive element. The homology between the L1 sequence and a similar elements in the Genbank database is shown in Figure 4-9. The match was not perfect (69% of bases identical) but was significant ($p = 0.011$).

For three patients (patient SM, DM and MT), the breakpoints fell in the Alu element of the M-BCR, but the break in ABL did not occur in an Alu. Conversely there are two patients (CC and PS) whose breakpoints were in an ABL Alu element, but not in the M-BCR Alu. The breakpoint in another patient (GM) is close to an ABL Alu. For the remaining 11 patients there was no Alu repetitive sequence found at the junction of the translocation (Table 4-8). The Alu elements found in the cloned ABL sequences in patients CC, GM and PS were in the same orientation as the one in the M-BCR, from 5' to 3'. The consensus Alu element is approximate 300 bp long and consists of two directly repeating monomer units. The first monomer is about 120 bp followed by an central A-rich stretch and the second monomer is, of approximate 150 bp, followed by a poly A tail (Kariya *et al* 1987). The Alu-like sequence cloned from patient CC ABL was highly homologous to the first unit of the consensus Alu (Kariya *et al* 1987). The

Alu element amplified from the ABL side of the break in patient PS was a partial sequence of the second unit, from position 220 to the poly A tail of consensus Alu (Kariya *et al* 1987). The ABL Alu sequence found in patient GM was from the 5' portion of the first Alu unit. The possible role of Alu elements in the BCR-ABL translocation is discussed in Chapter 6.

4.3.3. Rsa I is a reasonable choice for M-BCR region

I considered a blunt ended Bubble oligonucleotide to be potentially the most useful in this study because it is compatible with all end cutting restriction enzymes. This considerably broadens the range of enzymes that could be used for cleavage of genomic DNA for Bubble PCR. Also, fragments with cohesive ends could be filled with deoxynucleotides and ligated with the blunt ended Bubble oligonucleotide. However, the first choice for the enzyme should be a blunt ended cutter.

For blunt ended fragment generated enzymes in the M-BCR, Alu I (AG/CT) is certainly not the right choice because it has 26 digestion sites in this 5 kb M-BCR region. It is overrepresented in the M-BCR. If an oligonucleotide primer was made for each Alu I site, it would be very expensive. Similarly Hae III (GG/CC) is also over-represented, with 44 recognition sites in the M-BCR. The average length created by Hae III within this region is about 113 bp. Dra I (TTT/AAA), Msc I (TGG/CCA), Pvu II (CAG/CTG), and Stu I (AGG/CCT) and Sma I (CCC/GGG) all have a small number of recognition sites in the M-BCR but they are six base pair cutters, so the probability of a convenient site on the ABL side of this translocation is small.

Rsa I was selected for the cutter finally. It is a blunt ended cutter with 5 cleavage sites in the 5,000 bp M-BCR region. The distribution appeared ideal, at least for 2,541 bp of sequence that was available at the time. Furthermore, I found this enzyme to be reliable: Complete digestion was easily achieved under 20 units of Rsa I (GIBCO) for 5 μg of

genomic DNA at 37°C overnight. No additional enzyme was required and digested fragments appeared to ligate well to the Bubble oligonucleotide.

The sequence recognised by Rsa I is GTAC. The local G-C base composition will affect the frequency of Rsa I sites. If genomic DNA is 40% or 60% G-C rich, then probability of Rsa I site is theoretically once every 278 bp (Table 4-13). If genomic DNA is composed of 10% or 90% G-C, then a Rsa I site will appear once in every 1,976 bp. For a random DNA sequence with 50% G-C composition, Rsa I would be expected to cut on average once every 256 bp.

However, the sequence recognised by Rsa I is underrepresented in the M-BCR. The average length where Rsa I occurs is 856 bp (range: 90 bp - 1702 bp). When I started this study, however, the frequency of Rsa I sites in ABL was totally unknown. If Rsa I sites occurred at the theoretical frequency, one in every 256 bp, then a high proportion of different BCR-ABL breakpoints should be amenable to amplification by Bubble PCR. If the average size of Rsa I fragments was at the frequency as in the M-BCR (one in every 856 bp), then the PCR is still capable of amplifying the breakpoint in at least some patients. However, if Rsa I is also largely underrepresented in ABL, then this enzyme would not be a good choice. Therefore, the frequency of Rsa I sites in the ABL gene is a crucial factor for successful Bubble PCR amplification of BCR-ABL breakpoints.

When I started this study, the full sequence of zone 4 was not available. After Bubble PCR amplification of normal M-BCR with the PCR primers both for zone 3 (JGZ3, JGZ4, Nvamp-1 and Nvamp-2) and zone 4 primers (Z4A, Z4B, Nvamp-1 and Nvamp-2), I found that a Rsa I site was about 900 bp downstream of the annealing site for primers Z4A and Z4B, very close to the exon b4. Later the published M-BCR sequence confirmed this finding (Sowerby *et al* 1993).

Table 4-13. Probability of a Rsa I enzyme site occurs in genomic DNA with a different G-C composition

G-C composition (%)	Probability	Average interval length
0%	—	∞
10%	$\frac{1}{0.45 \times 0.45 \times 0.05 \times 0.05}$	1,975 bp
20%	$\frac{1}{0.40 \times 0.40 \times 0.10 \times 0.10}$	625 bp
30%	$\frac{1}{0.35 \times 0.35 \times 0.15 \times 0.15}$	363 bp
40%	$\frac{1}{0.30 \times 0.30 \times 0.20 \times 0.20}$	278 bp
50%	$\frac{1}{0.25 \times 0.25 \times 0.25 \times 0.25}$	256 bp
60%	$\frac{1}{0.30 \times 0.30 \times 0.20 \times 0.20}$	278 bp
70%	$\frac{1}{0.35 \times 0.35 \times 0.15 \times 0.15}$	363 bp
80%	$\frac{1}{0.40 \times 0.40 \times 0.10 \times 0.10}$	625 bp
90%	$\frac{1}{0.45 \times 0.45 \times 0.05 \times 0.05}$	1,975 bp
100%	—	∞

Note: the table above indicates that probabilities of a Rsa I site occurring in genomic DNA with different G-C composition. The recognition sequence of Rsa I is GTAC. Thereby, different G-C base composition will affect the probability of Rsa I sites. For example, in 40% G-C composition DNA the probability of a G or C appearing at a particular site in the genome would be 0.20, but a base A or T would be 0.30. A Rsa I site would therefore appear on average once in every 278 bp.

To gain a brief general profile of the distribution of Rsa I site in the human genome, two protein coding genes, HLA Class III gene, Bruton's tyrosine kinase (BTK) gene, and non-protein coding genes, Val- and Lys-tRNA (VL-tRNA) genes, were randomly selected from Genbank (Genbank, Z15027, L35265, M33940) and analyzed using the computer program DNA Strider. 20 Rsa I sites were found in 9 kb of the 5' region (BTK1) of BTK gene, 5 sites were found in 4 kb of the first intronic region (BTK2) of BTK gene, 8 sites in 5 kb of VL-tRNA genes and 20 sites in 11 kb of HLA class III

gene. The mean values for the sizes of fragments in Rsa I digestion for each gene are 507 bp (Table 4-14). The central tendency of the fragment sizes in overall four genomic

Table 4-14. Analysis of the frequency of Rsa I site in four randomly selected genes

Gene or region / Stastic values	BTK1	BTK2	VL-tRNA	HLA class III	Overall
Length of DNA	9kb	4kb	5kb	11kb	29kb
Fragments	19	4	7	19	49
Minimum size	6bp	131bp	152bp	13bp	6bp
Maximum size	1675bp	1325bp	1428bp	3010bp	3010bp
Median	357bp	356bp	478bp	168bp	357bp
Mean	504.2bp	542bp	663.3bp	445.4bp	507bp

Note: BTK1: the 5' region of Bruton's tyrosine kinase gene. BTK2: the first intronic region of Bruton's tyrosine kinase gene. VL-tRNA: Val- and Lys-tRNA genes.

DNA regions is 1/2 (Q1 + Q3) = 1/2 (113 + 741) = 427 (base pairs). That means that 50% of the fragments lie in the range 427 bp $\pm$ 314 bp. Therefore, there is 50% probability that a Rsa I site falls into within 750 bp on ABL side. In the M-BCR region primers in zone 2 can cover 776 bp in length, primers for zone 3 can cover 820 bp and zone 4 primers cover 877 bp. Therefore, 50% fragments amplified with BCR primers for Rsa I digested genomic DNA would be within 1.6 kb in size. This figure is reasonable close to the percentage of patients for whom BCR-ABL was successfully amplified.

Currently the known ABL sequences, ABL1, ABL2 and ABL3 (Genbank, U07561, U07562 and U07563) cover most of the whole gene (Chissoe *et al* 1995), about 180 kb in size. However, there are still two gaps totalling about 70 kb within the first intronic region that have not yet been sequenced (Chissoe *et al* 1995). I analysed these sequences for Rsa I sites with the program DNA Strider and found there were 297 Rsa I recognition sites. The fragments generated with this enzyme ranged from 6 bp to 4,800 bp (Mean size is 581 bp). These figures are similar to the ones from the genes randomly selected in Genbank (see above).

4.3.4. The BCR-ABL breakpoints appear to be randomly distributed between exons b2 and b4 within M-BCR

If the breakpoints were subclustered in the M-BCR, this would suggest that there might be some sequence features playing a role in chromosome translocation. Thereby, investigation of subclustering in the M-BCR would help us to know how a reciprocal translocation between chromosomes 9 and 22 occurs.

In the M-BCR region thirty one BCR-ABL breakpoints in total have been characterised so far from a standard translocation t(9;22)(q34;q11), of which 17 (61%) breakpoints were characterised by Bubble PCR in my work and 14 breakpoints were individually identified in other studies (Groffen *et al* 1984; Heisterkamp *et al* 1985; Grosveld *et al* 1986; de Klein *et al* 1986; Chen *et al* 1989a; Chen *et al* 1989b; van der Feltz *et al* 1989; Papadopoulos *et al* 1990; Soekarman *et al* 1990; Mills *et al* 1992; Sowerby *et al* 1993; Litz *et al* 1993) (Figure 4-8). All the BCR-ABL breakpoints appear to be randomly distributed between exons b2 and b4 of the M-BCR (Figure 4-8).

It is likely that the breakpoints that have been characterised to date, are not a completely random sample. For example, in my study patients, were selected principally on the basis of having a conveniently positioned Rsa I site on the ABL side of the translocation. In the study of Mills *et al* (1992), three patients were selected on the basis that the breaks fell within the M-BCR Alu element, as detected by Southern blotting. However, a sufficient number of breakpoints have now been characterised to make it clear that there is no subclustering within the M-BCR, in contrast for example to the mbr of BCL2 (Wyatt *et al* 1992). Specific sequence features that may be associated with the translocation are discussed in the next chapter.

CHAPTER FIVE COMPLETE CHARACTERISATION OF THE RECIPROCAL TRANSLOCATION BETWEEN CHROMOSOMES 9 AND 22

5.1. Introduction

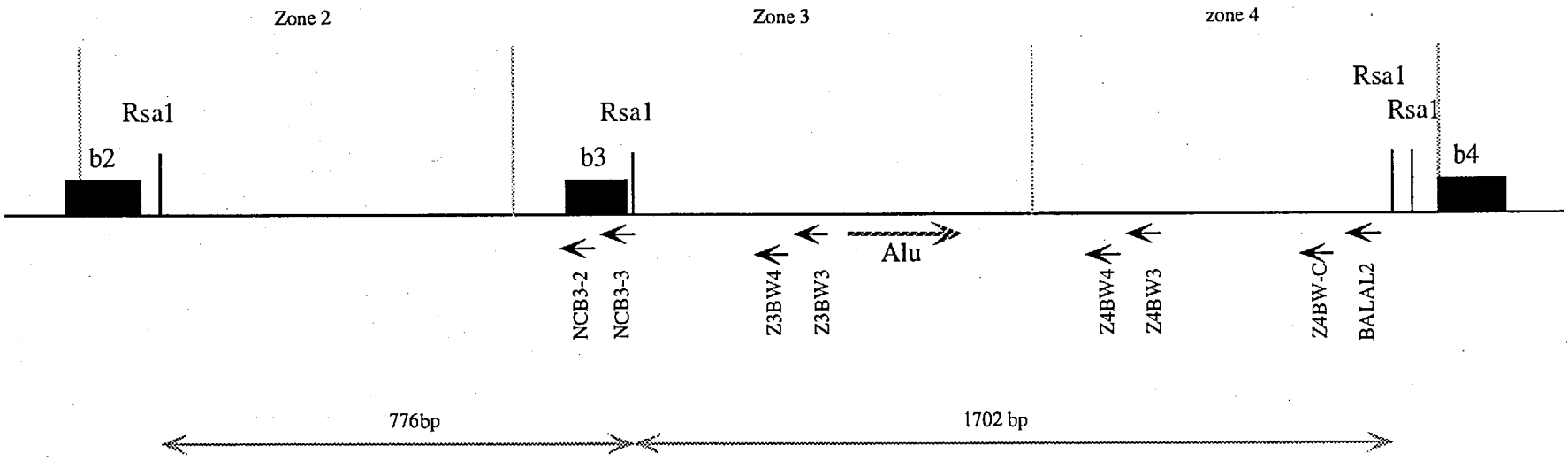
The reciprocal translocation between chromosomes 9 and 22 in CML results in two derivatives: chromosomes 22q- and 9q+. At the molecular level the chromosomal fusions give rise to BCR-ABL chimaeric oncogene on chromosome 22q- and ABL-BCR oncogene on chromosome 9q+. In order to understand how a reciprocal translocation occurs between these two chromosomes in CML, the two derivatives of the translocation have to be cloned and sequenced. This information might indicate what sequence features actually play a role in the translocation.

The position of the BCR-ABL breakpoint within the M-BCR has been characterised in 17 CML patients (Chapter 4). Bubble PCR was subsequently performed in the reverse direction to clone the 9q+ breakpoint in those patients. ABL primers were designed flanking the breakpoint and were used to amplify the normal ABL sequence, which was not known at the time. Then the sequence of the normal ABL, normal BCR, 22q- and 9q+ breakpoints could be compared to give a full picture of the translocation.

5.2. Results

5.2.1. Characterisation of ABL-BCR breakpoints on chromosome 9q+

For amplification of the ABL-BCR breakpoint on chromosome 9q+ by Bubble PCR, four sets of PCR primers were designed to anneal in reverse orientation for each zone in the M-BCR (Figure 5-1). Primers NCB3-3, and NCB3-2 were made for zone 2. Their



Fiure 5-1. Positions of Primers for Bubble PCR Amplification of 9q+ breakpoint

A short solid arrow indicates Bubble PCR primers. The longer arrow shows the position and orientation of Alu element. A black box illustrates as the exons in M-BCR region. The size between the first two Rsa I sites is 776bp and between the second and third Rsa I is 1,702bp.

annealing sites are immediately upstream of the Rsa I site within exon b3. The fragment amplified from normal M-BCR region with primers NCB3-2 and Nvamp-2 is 768 bp in size. Z3BW3 and Z3BW4 were designed to anneal the area between position 3,363 and 3,317 of zone 3 (the positions shown in the text are according to the 5,000 bp of the M-BCR published by Sowerby *et al* 1993; Genbank, L02935). The DNA fragment obtained from normal M-BCR by Bubble PCR with primers Z3BW4 and NVamp-2 is 500 bp in length. Two pairs of primers Z4BW3 and Z4BW4, BALAL2 and Z4BW-C were synthesised for zone 4. Z4BW3 and Z4BW4 were located between positions 3,967 and 3,916, BALAL2 and Z4BW-C were situated between positions 4,170 and 4,113 (Figure 5-1). The normal M-BCR PCR products amplified by Bubble PCR with primers Z4BW4 and Nvamp-2, primers Z4BW-C and Nvamp-2 are 1,099 bp and 1,298 bp respectively. Primers NCB3-3, Z3BW3, Z4BW3 or BALAL2 in combination with primer Nvamp-1 were used for the first round PCR. The primers NCB3-2,

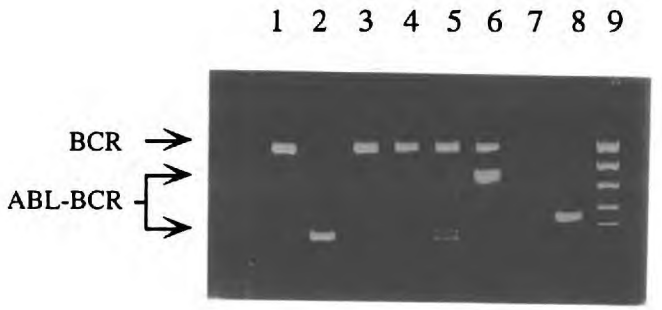
Table 5-1. Primer combination for amplification 9q+ breakpoints

PCR / M-BCR	First step PCR		Second step PCR	
Zone 3	NCB3-3	Nvamp-1	NCB3-2	Nvamp-2
Zone 3	Z3BW3	Nvamp-1	Z3BW4	Nvamp-2
Zone 4	Z4BW3	Nvamp-1	Z4BW4	Nvamp-2
Zone 4	BALAL2	Nvamp-1	Z4BW-C	Nvamp-2

Z3BW4, Z4BW4 or Z4BW-C in combination with primer Nvamp-2 were used for the second round PCR (Table 5-1). Two sets of primers were made for zone 4 in order to reduce the length of BCR portion in the fragment to increase the chance of finding the reciprocal breakpoint in the patients whose BCR-ABL breakpoint in the M-BCR had already been identified.

The seventeen patients, from whom the BCR-ABL breakpoints had been cloned, were screened for amplification of the ABL-BCR breakpoints on chromosome 9q+ by Bubble PCR with a BCR primer annealing at downstream of the BCR-ABL

A



B

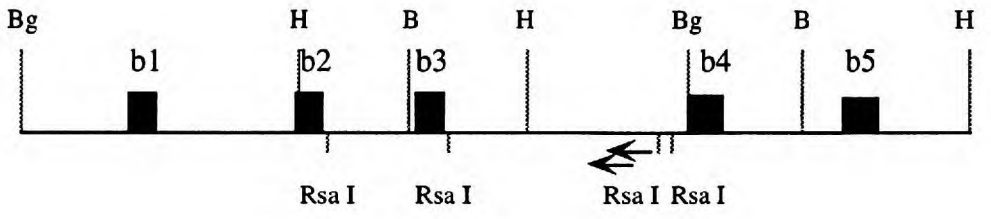


Figure 5-2. Amplification ABL-BCR breakpoints by reverse Bubble PCR

A) A photograph of amplification of ABL-BCR breakpoints with reverse BCR primers on an agarose gel. Lane 1, patient CC; lane 2, patient AE; lane 3, patient BA; lane 4, patient PS; lane 5, patient MT; lane 6, patient DM; lane 7, blank; lane 8, PCR reaction positive control; lane 9, molecular length marker.
B) Schematic representation of the M-BCR region. Black boxes indicate exons in M-BCR. Dot lines show the positions of restriction enzymes. Bg, Bgl II; H, Hind III and B, BamH I. The arrows indicate reverse BCR primers, Z4BW-C and BALAL2.

breakpoint in M-BCR in the 3'->5' direction of the gene (Figure 5-1). For patients BO, ML, BM, SL and GM, primers NCB3-3, NCB3-2 (zone 2) were used. Patients GM, AJ and GL were tried with primers Z3BW3, Z3BW4 (zone 3). Patients SM, DM, MT (zone 3) and patients PS, BA and AE (zone 4) were screened with primers Z4BW3, Z4BW4. Patients BA and AE were also repeated with BCR primers BALABL2, Z4BW-C (zone 4). The ABL-BCR breakpoint in patients RK, AH, BY and CC were screened with primers BALABL2 and Z4BW-C. In all cases, M-BCR oligonucleotides were used in combination with the Bubble oligonucleotides Nvamp-1 and 2 (Table 5-1).

ABL-BCR breakpoints were successfully amplified from only 4 (24%) patients (AE, AH, DM and GM). Two of those four ABL-BCR breakpoints amplified are shown in Figure 5-2. The ABL-BCR breakpoint in patient AE was amplified with two

Table 5-2. ABL sequences obtained from chromosome 9q+

patient	sequence (5'–3')
AE	ACACTTTTAATACAGATCCCAATAG
AH	AATTGCGGCAGGAAGTGATGATACATGTTTGACTGCCTCCTACTC TTTGCACCCTGATGTGAGGGAAGGGCTGGACCCTTCTACATCTTT CTGTCTGGATGAAGCTCCTCTGCCGTCATTTCCTCCTCCTTCCCT AGTGCGGCAAGCAGCTCTGCCAGC
DM	TGACCCAAGTTTGAAGAGACTGGTAGGGGTAAATTTAGAGCCTGG GGCTCCAAAGTTTGTCTACCC
GM	ACTGGGGTGTCATGTTCGTTTCCTTAAGTGAAGCTCTAGGGCCAG GAGGGCTTCACTTTTGCGTTCTTTCTACCTACTCTCCTTCTCTAA TTACATCTGTTTTATTTTATTTTTCTTTCCTGTT

different sets of BCR nested primers in the zone 4 of M-BCR region. The one in patient DM was located in the zone 3, amplified with zone 4 primers, Z4BW3 and Z4BW4. The breakpoint in patient AH was amplified with primers BALABL2 and Z4BW-C. The remaining breakpoint GM was amplified with both zone 3 primers, NCB3-3 and NCB3-2, and zone 4 primers, Z4BW3 and Z4BW4. The size of the ABL sequences obtained from the chromosome 9q+ was 25 bp in patient AE, 159 bp in patient AH, 66 bp in patient DM and 124 bp in patient GM (Table 5-2) from the corresponding amplification products (Table 5-3). The results for all four patients were confirmed in a second independent Bubble PCR analysis.

5.2.2. Amplification of the normal ABL sequence for complete characterisation of the reciprocal translocation

For each of the 4 patients for whom reciprocal breakpoints on chromosomes 9q+ and 22q- were sequenced, two ABL primers were made, one for the ABL sequence cloned from chromosome 9q+ and the other for the ABL sequence cloned from the chromosome 22q-. These primers were used to amplify the ABL sequence flanking the breakpoints from genomic DNA of a normal individual (Figure 5-3). The sequence of ABL primers were designed a few base pairs away from the breakpoint and included a restriction enzyme recognition site (BamH I or EcoR I) to facilitate cloning. The oligonucleotide derived from the ABL sequence on chromosome 22q- was called the 3' ABL primer. The oligonucleotide derived from the ABL sequence on chromosome 9q+ was known as the 5' ABL primer. PCR was attempted by one step conventional PCR with the corresponding pairs of primers.

Table 5-3. Size of the fragments amplified from chromosome 9q+

Patients	PCR product	BCR size	Sequenced ABL
AE	313 bp*	288 bp	25 bp
AH	400 bp	216 bp	159 bp
DM	900 bp	419 bp	66 bp
GM	206 bp*	82 bp	124 bp

* For patient AE, PCR amplification and sequencing by two sets of zone 4 primers which are annealed in zone 4. The sizes indicated in the table above are generated by primers BALABL2 and Z4BW-C. For patient GM, amplification performed with two sets of primers in zone 3. The sizes in the table are derived from primers NCB3-3 and NCB3-2.

Amplifications were carried out on both a normal and patient genomic DNA samples. In patient AH the 3' ABL primer was tested from both patient and normal individuals in combination with a forward BCR primer upstream of the BCR-ABL breakpoint (Figure 5-4, lanes 1 and 2). The 5' ABL primer was tested for amplification of ABL-BCR breakpoint with a reverse BCR primer downstream of the breakpoint (Figure 5-4,

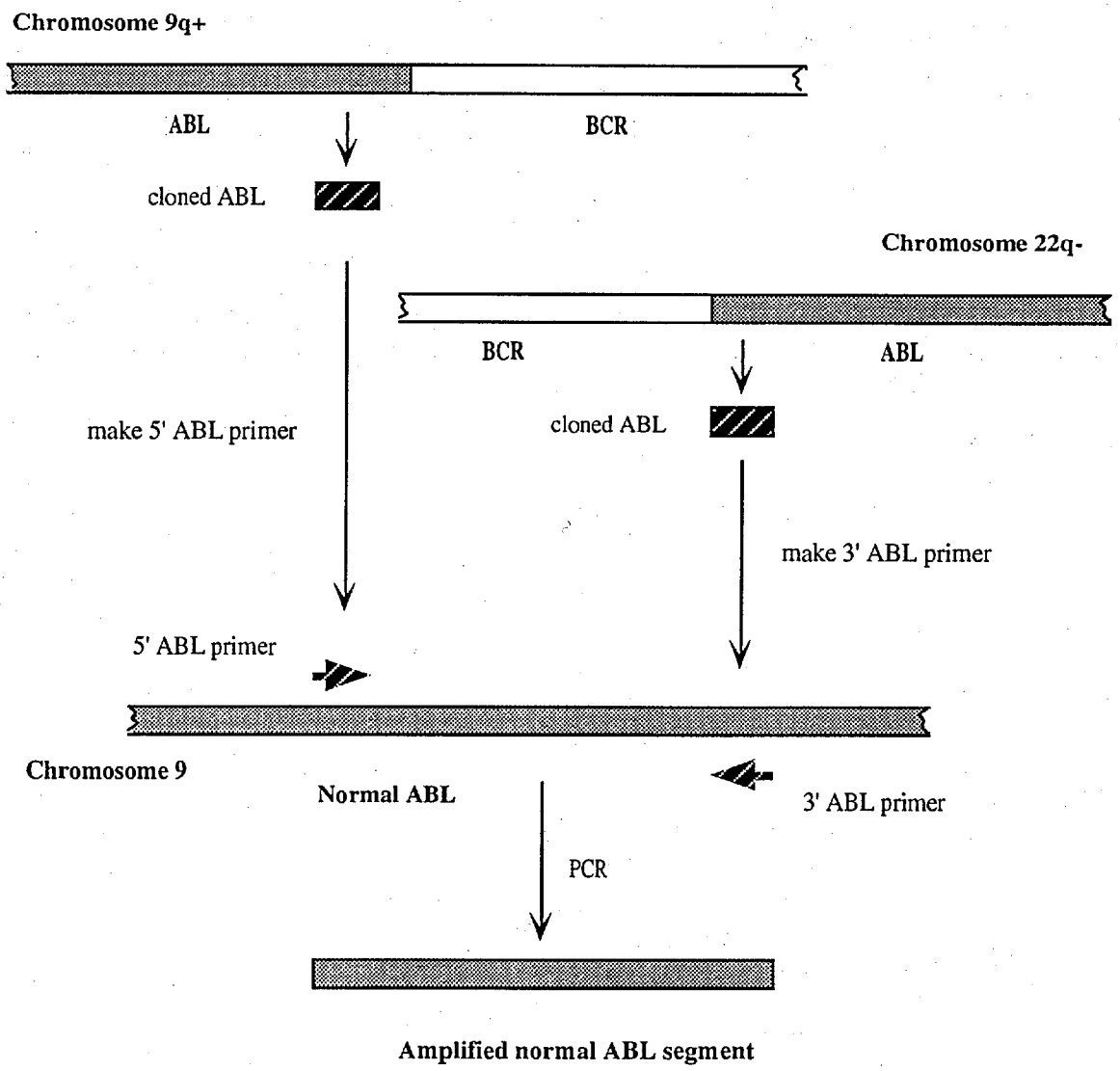


Figure 5-3. Diagram for amplification of normal ABL sequence around the junctions

ABL segments were amplified by Bubble PCR from both chromosome 9q+ and chromosome 22q-. Two ABL PCR primers were synthesised complementary to the cloned ABL sequences and the normal ABL sequence around the breakpoints were then amplified from a normal individual.

lanes 3 and 4). Both PCRs were positive for genomic DNA extracted from patient AH, but not for a normal genomic DNA, indicating specificity of PCR primers for the reciprocal translocation. When the 5' and 3' ABL primers were used together, both the patient and normal DNA was positive, yielding a band of the same size (Figure 5-4, lanes 5 and 6), indicating amplification from the normal ABL gene in both individuals. Similarly, amplification of part of the normal ABL gene was successfully achieved in

patients DM and GM (not shown). In one case (patient AE), no normal ABL product was obtained, despite trying different 5' and 3' primers and a variety of PCR conditions. The sizes of amplified normal ABL products for the three successful patients were 199 bp, 270 bp and 405 bp respectively (Table 5-4).

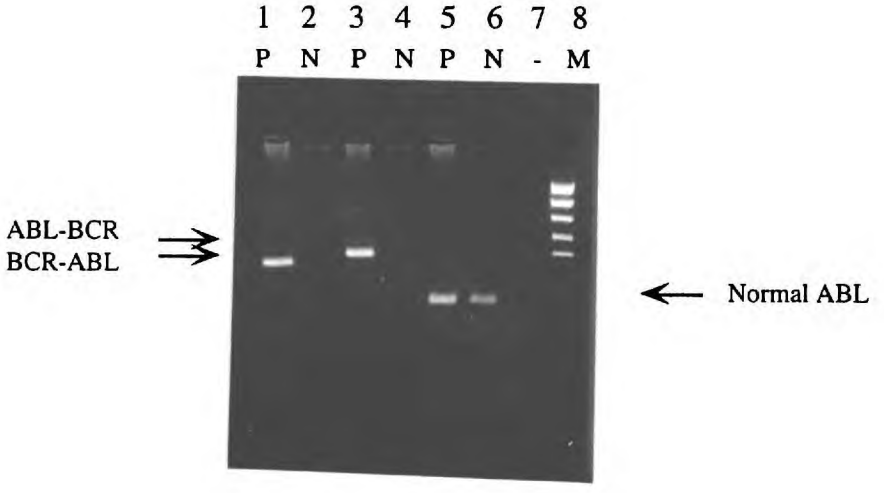


Figure 5-4. Amplification of normal ABL sequence from patient AH

In patient AH the 3' ABL primer was tested from both patient and normal individuals in combination with a forward BCR primer upstream of the BCR-ABL breakpoint (lanes 1 and 2). The 5' ABL primer was tested for amplification of ABL-BCR breakpoint with a reverse BCR primer downstream of the breakpoint (lanes 3 and 4). PCR products were amplified from the normal ABL gene with the 5' and 3' ABL primers from DNA of both patient AH and a normal individual (lanes 5 and 6). P: genomic DNA from patient AH, N: genomic DNA from a normal individual, −: PCR reaction negative control.

5.2.3. Sequence analysis of the reciprocal translocation in the fully characterised patients

5.2.3.1. Analysis of the amplified normal ABL sequences for sequence features

The normal ABL sequences derived from those three patients were compared with the known sequences released in Genbank to search for repetitive sequences. They were also compared with short repetitive sequences, for example, heptamer/nonamer sequences (CACAGTG/ACAAAAACC) or consensus topoisomerase II recognition

sites (GTN[ATC]A[CT]TN[GA][GT]NN[AG]) (Darby *et al* 1986; Dong *et al* 1993), chi-like element (GC[A/T}GG{A/T]GG) (Krowczynska *et al* 1990). No significant repetitive sequences were found in the ABL sequences amplified from patients AH and DM. There is an about 80 bp Alu sequence in the 3' end of the sequence from patient GM 158 bp downstream of the breakpoint. The orientation of this Alu is from 5' to 3' according to the consensus Alu (Kariya *et al* 1987).

Table 5-4. ABL sequences amplified between the breakpoints of BCR-ABL and ABL-BCR

patient	sequence (5'-3')
AH	GGCAGGAAGTGATGATACATGTTTGACTGCCTCCTACTCTTTGCACCCTGA TGTGAGGGAAGGGCTGGACCCTTCTACATCTTTCTGTCTGGATGAAGCTCC TCTGCTGTCATTTCCTCCTCCTTCCCTAGTGCGGCAAGCAGCTCTGCCAGC TTGTCATAGAAGGGCATTTAAGTGACTTTGCCAAGAGAAACAGTAA
DM	TGACCCAAGTTTGAAGAGACTGGTAGGGGTAAATTTAGAGCCTGGGGCTCC AAAGTTTGTCTACCCAGTTTTAAATCCTGGCTTTCCCCTTAACTTGGGTGG GATCACACCATTAACCGTGTTACCCCTCTGAAGCTTTGGCTCCCACACCTG TGAGATGTGAATGTTGATAACACACACCCACCTCATAGGATTAATGAGAAC ATGCCAAGATATAATGCGCTGAAGGTCTTAGCATGCCTGCGCAGAGCCCCA GCATTCTTGTTAGCT
GM	ACTGGGGTGTCATGTTCGTTTCCTTAAGTGAAGCTCTAGGGCCGGAGGGCT TCACTTTCTACCTACTCTCCTTCTCTAATTACATCTGTTTTATTTTATTTT TCTTTCCTGTGTAGTCTTTAGTTTTATAAATGAAAAAAAAAAGTAAATTAA GGGTTATGGGTCTTCACTTTCGTAGCTTCTAGGTGGGTATGAAATTGATCT GATTTGAGACTGGTGCTCCCAGACCATTGCCGTCAGCAGAAAGCCTATCTG TGCTTCAGAGATGGATGGCACTTGGACTTCTCTGTTGTTTTCAACTAAGAA TATAAGAGTAAAAATGTGGCCGGGCGCGGTGGCTCAAGCCTGTAATCCCAA CTCTTTGGAGGCCGAGACGGTGGATTATTTGAGGTCAGGATTTCGAGA

5.2.3.2. Defining accurate location of the breakpoints in both BCR and ABL genes

The BCR and ABL sequences around the junctions derived from Bubble PCR were compared with normal BCR and ABL genes for the three patients AH, DM and GM (Figure 5-5; Figure 5-6).

The translocation between chromosomes 9 and 22 in patient AH is illustrated in Figure 5-7. A GCT trinucleotide present in the normal ABL sequence had been duplicated and

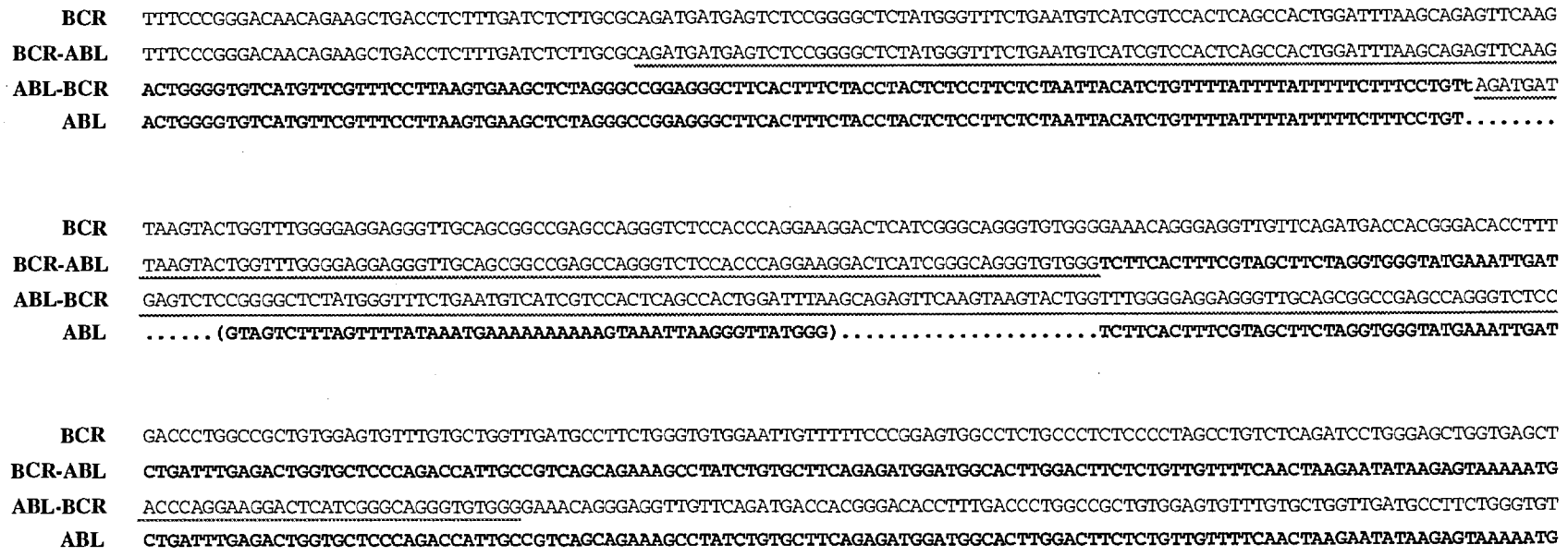


Figure 5-5. Sequences around the junctions in patient GM

Sequence in plain text is BCR and sequence in bold text is ABL. Sequences (159bp) of BCR underlined with a dotted line are duplicated in the chimaeric genes. The sequence (50bp) of ABL in brackets is deleted in the chimaeric genes. The lowercase letter indicates that the base is derived neither from BCR nor from ABL.

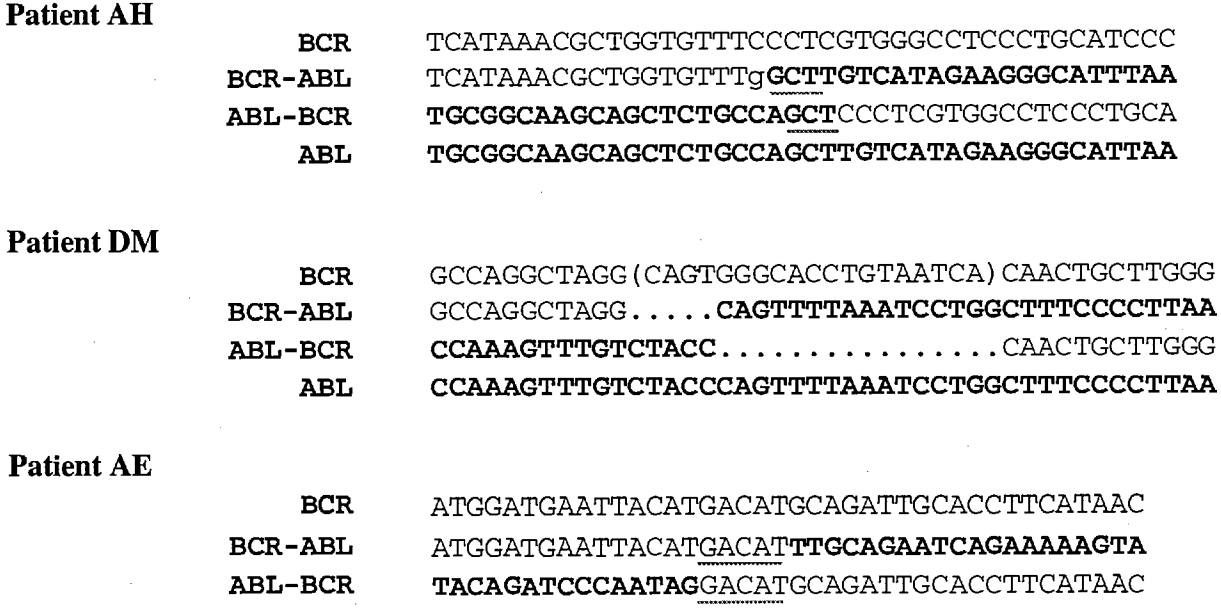


Figure 5-6. Sequences around the junctions in patients AH, DM and AE

Plain text and bold text indicate BCR and ABL sequences respectively. Sequences underlined with a dotted line are duplicated in the chimaeric genes. The sequence in brackets is deleted in the chimaeric genes. The lowercase letter indicates that the base is derived from neither BCR nor ABL.

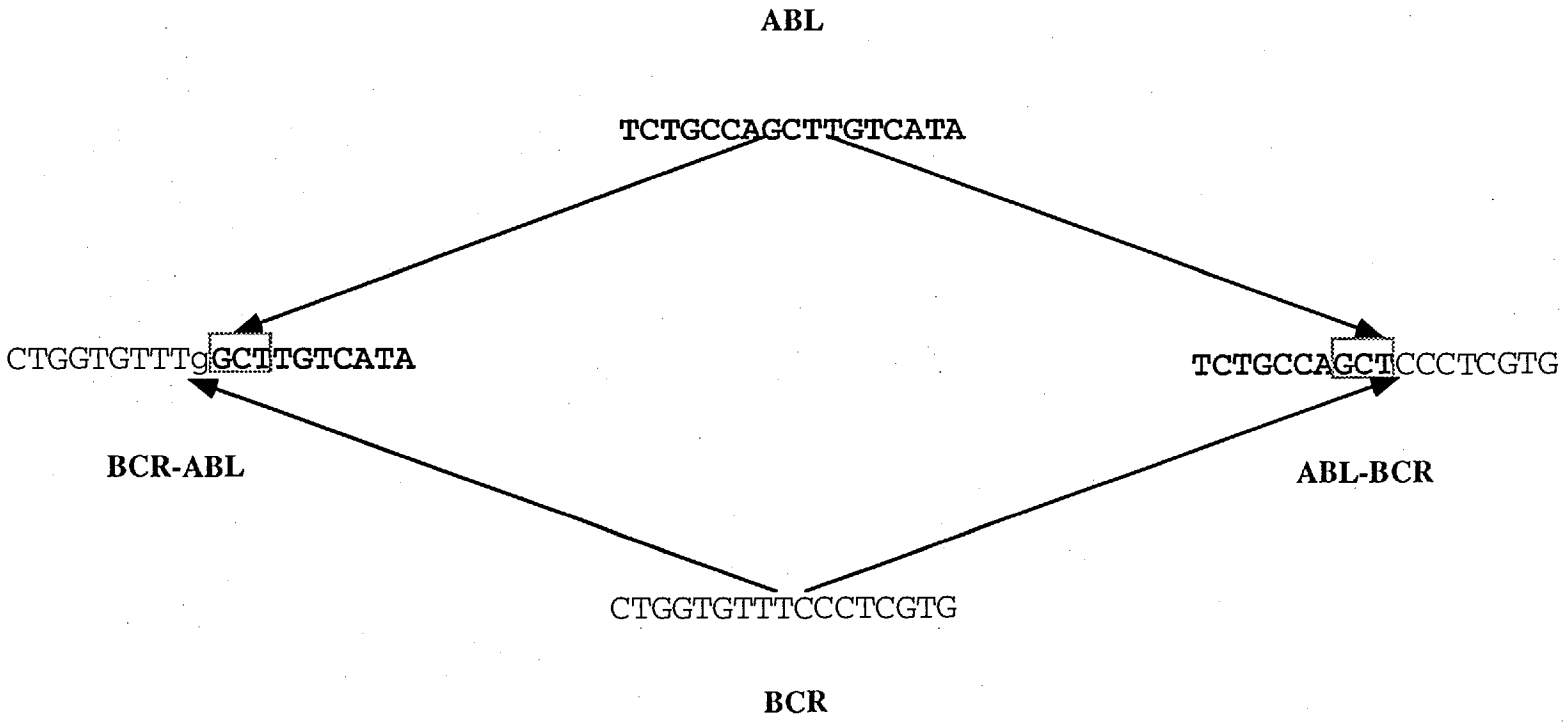


Figure 5-7. The reciprocal translocation between chromomsomes 9 and 22 in patient AH

Plain text and bold text indicate BCR and ABL sequences respectively. The letter in lower case indicates a nucleotide belongs neither BCR nor ABL. The arrows point out where breaks occur in both BCR and ABL. The trinucleotide GCT from ABL is duplicated in both the BCR-ABL and ABL-BCR derivatives.

was present on both BCR-ABL and ABL-BCR chimaeric genes. The precise positions of the breakpoint in ABL and BCR are unclear but two explanations are possible:

i) There was a clean break in the BCR sequence. Three base pairs (GCT) of ABL were duplicated and an extra base (G) was inserted at the 22q- break.

ii) There was a clean break in the BCR sequence. Four base pairs (AGCT) of ABL was duplicated but the A mutated to G in the BCR-ABL.

The precise position of the chromosomal translocations in patient DM is ambiguous since there is a homology (CAGT and CA) between the BCR and ABL genes at the critical sites (Figure 5-8). However, it is clear that a total of 19 bp of sequence has been deleted by the translocation of which between 1 and 5 bp has been lost from ABL and between 14 and 18 bp from BCR.

The reciprocal translocation between chromosomes 9 and 22 in patient GM is quite clear. 159 bp of BCR sequence including exon b3 has been duplicated and 50 bp of ABL sequence has been deleted at the junctions (Figure 5-9). One nucleotide insertion or mutation can be seen at the junction of ABL-BCR (Figure 5-5). Previously we had found that the patient GM expressed both b3a2 BCR-ABL and Ib-b3 ABL-BCR transcripts (Melo *et al* 1993a), i.e. that exon b3 appears to be included in both reciprocal fusion mRNAs. Here we have confirmed at the genomic DNA level that this sequence is indeed present on both chromosome derivatives. Interestingly the breakpoint on the 9q+ chromosome was only 2 bp upstream of exon b3. The polypyrimidine component of the splice recognition site (Green, 1986) has therefore been provided by the ABL sequence.

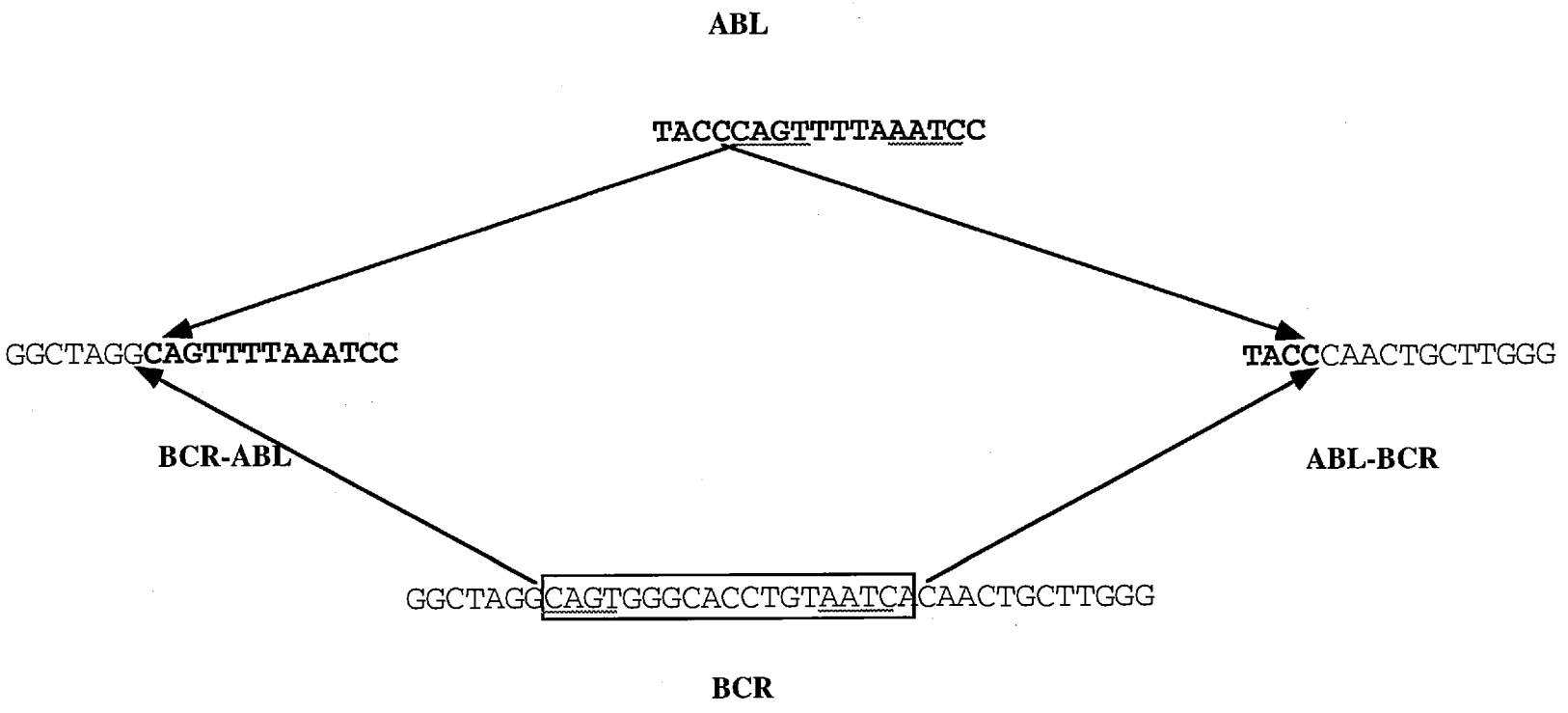


Figure 5-8. The reciprocal translocation between chromomsomes 9 and 22 in patient DM

Plain text and bold text indicate BCR and ABL sequence respectively. The arrows point where the breaks occur in both BCR and ABL. There are CAGT and AATC base homologies between BCR and ABL genes. These translocations could be explained by a clean cut having occurred in ABL but a short stretch of nucleotides (19bp) from BCR were deleted in the chimaeric derivatives.

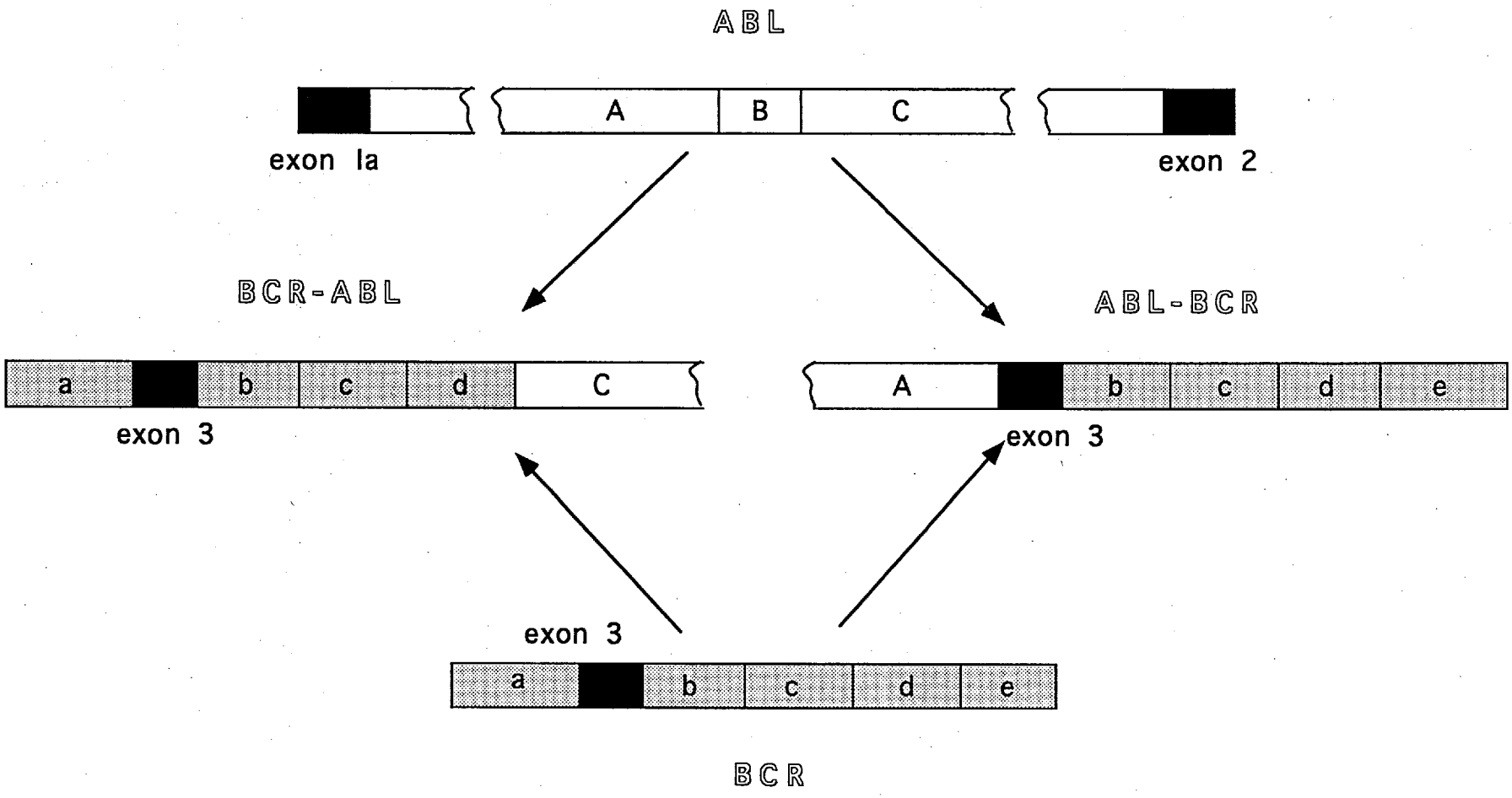


Figure 5-9. Diagrammatic representation of the translocation in patient GM.

159bp of BCR including the whole of exon b3 (stippled boxes b, c, d and the black box in both BCR-ABL and ABL-BCR) is duplicated; whilst 50bp of ABL (white box B) is deleted during the translocation.

Although the normal ABL sequence around the breakpoints failed to be amplified in patient AE, it is clear that 5 bp (GACAT) of BCR sequence was duplicated on the translocated chromosomes (Figure 5-6).

5.3. Discussion: how does a chromosomal translocation occur ?

The mechanism by which the 9;22 translocation in CML is formed is unclear. Broadly there are two possibilities: either there are specific sequence or topological features in BCR and ABL that lead to an increased aberrant recombination frequency, or the propensity for translocation of these two genes is no greater than background but there is selection for the BCR-ABL gene by virtue of the growth advantage it confers. These possibilities are not mutually exclusive. Evidence for the former includes the apparent subclustering of breakpoints within ABL intron 1 (Jiang *et al* 1990) in CML and BCR intron 1 in Ph positive ALL (Chen *et al* 1989b), suggesting preferred sites of illegitimate recombination. On the other hand irradiation of HL60 cells produced a variety of BCR-ABL fusion genes which are not normally found in CML or ALL (Ito *et al* 1993), emphasising the probable role of biological selection.

5.3.1. Possible staggered cut or gene conversion involved in the BCR-ABL translocation

Duplication and/or deletion of short sequences at translocation breakpoints can be explained as a result of a staggered cut in one or both of the parent sequences. Also, there may be a possibility for modification of the cohesive ends of the cleaved fragments after breakage, for example, single stranded portion of the end may be partially digested off by an exonuclease. Alternatively the end could be filled with nucleotides, changing it into a blunt end, or one or more nucleotides could be directly

added by terminal deoxynucleotidyl transferase (TdT). Furthermore, single stranded breaks could initiate gene conversion events (see below).

5.3.1.1. The reciprocal translocation in patient AH

Due to homologies or mutations at the breakpoints, different interpretations of the events are possible as above. One possibility is that an ABL allele was cleaved into two parts with a cohesive end (with 5' AGCT overhang or protruding). At some stage in the process, one of the overhanging A residue mutated to a G. BCR was cut cleanly between the triple Ts and triple Cs (Figure 5-10). After filling in the cohesive ABL ends, the fragment with the mutated G at the 5' end would ligate with the 3' end of the blunt ended BCR fragment, leading to the BCR-ABL chimaera. The other ABL fragment could have ligated with the 5' blunt end of BCR fragment, giving rise to the ABL-BCR.

5.3.1.2. The reciprocal translocation in patient DM

In patient DM the exact positions of the breakpoints are ambiguous due to short stretches of homology (CAGT & CA) between BCR and ABL at the critical sites. However, it is clear that a short stretch of BCR sequence was deleted on formation of the chimaeric chromosomes. One explanation is: a staggered cut occurred around the CAGT sequence within ABL. In the BCR gene a 21 bp staggered cut occurred (Figure 5-11). Subsequently the BCR staggered ends were partially or completely digested by a exonuclease prior to ligation to ABL. This translocation could also be explained by gene conversion (see below).

5.3.1.3. The reciprocal translocation in patient GM

In patient GM, 159 bp of BCR including the whole of exon b3 was present on both the 22q- and 9q+ derivatives, and had therefore been duplicated during the translocation, whilst 50 bp of ABL had been deleted (Figure 5-9). This could perhaps have resulted in

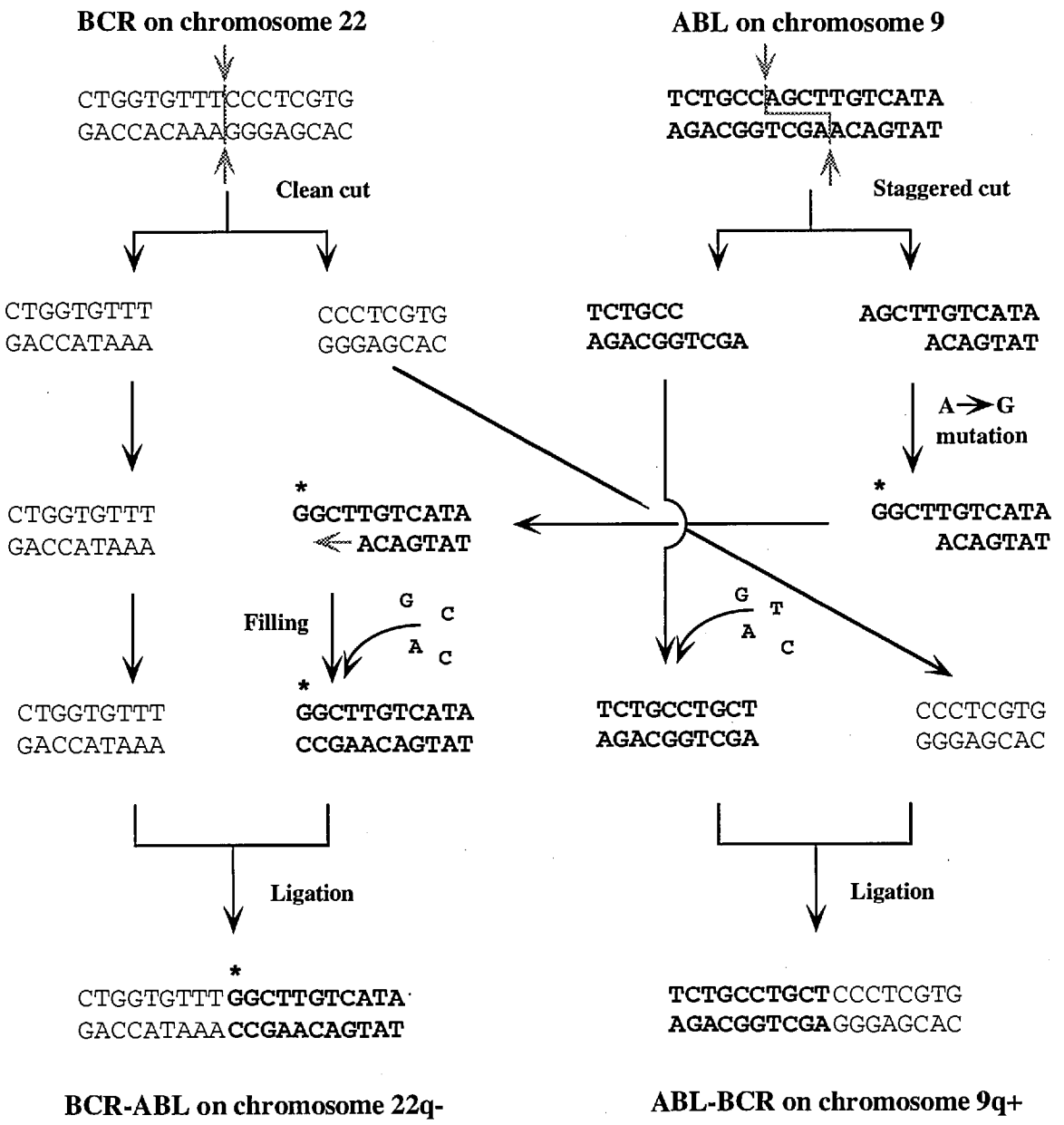


Figure 5-10. The reciprocal translocation by a staggered cut between chromosomes 9 and 22 in patient AH

The translocation in this patient could involve process of a staggered cut, filling in and fragment ligation.

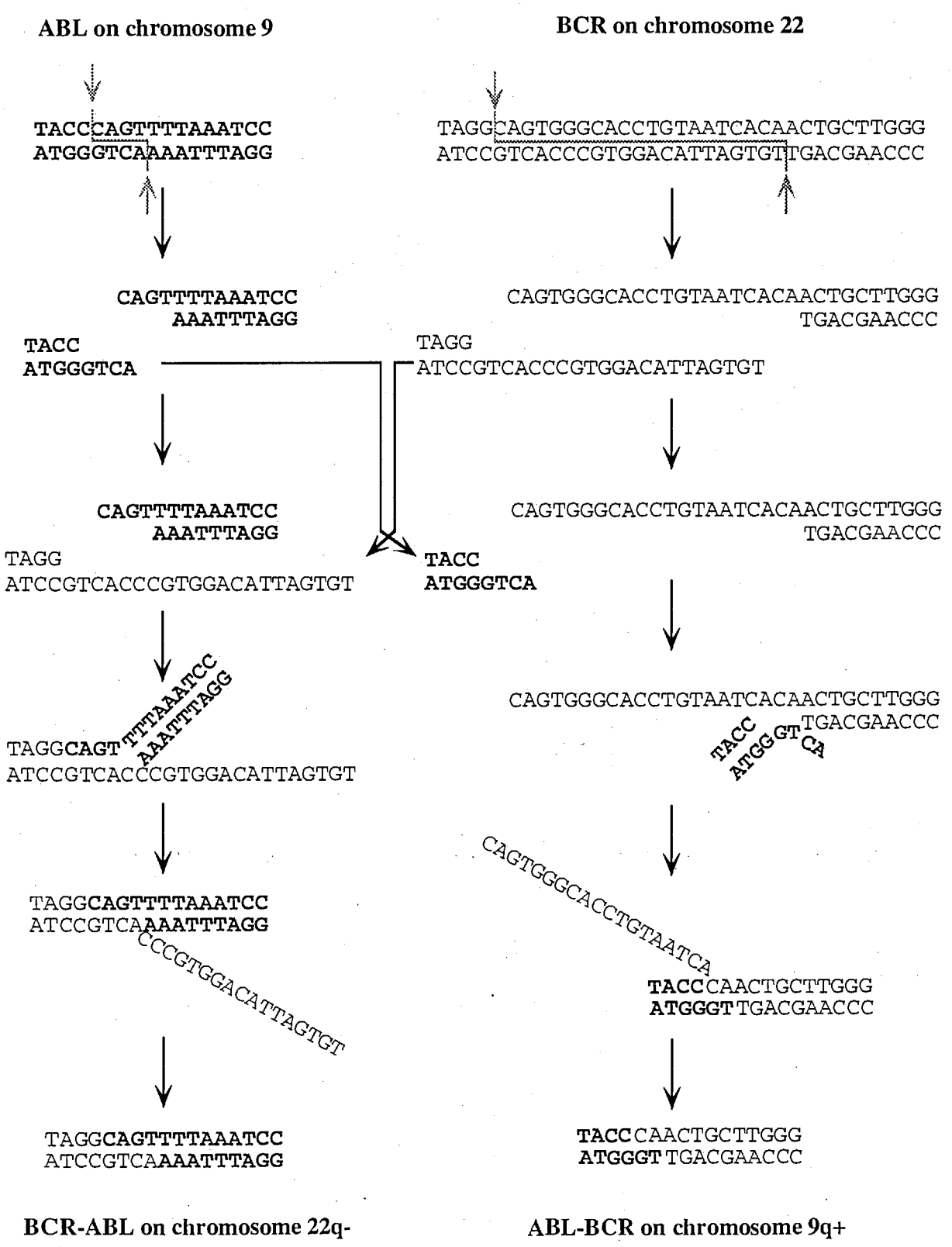


Figure 5-11. The reciprocal translocation by a staggered cut between chromosomes 9 and 22 in patient DM

The translocations of the genes in patient DM might involve a process with a staggered cut and ligation.

part from a very large staggered cut. But another explanation could be gene conversion. Gene conversions is a mismatch repair process associated with DNA recombination. The precise mechanism by which gene conversion occurs is unclear, but the process certainly involves either a single stranded (Meselson & Radding, 1975; Brisebois & Bubow, 1993) or double stranded cut (Szostak *et al* 1983; Haber, 1995), followed by strand invasion, mismatch correction or new synthesis followed by segregation of the DNA strands. In patient GM the translocation could have occurred within the framework of the single stranded DNA break model (Meselson & Radding, 1975; Brisebois & Bubow, 1993). For example, a break was formed in one strand of the BCR gene and this strand invaded the ABL gene, forming a heteroduplex. After cleavage, the two chimaeric DNA molecules segregated. The mismatched base pairs in heteroduplex regions were subject to enzymatic repair. After the recombination events, ABL sequences were linked to the BCR genes but 50 bp is lost. However, a portion of BCR was duplicated due to gene conversion in the recombined chromosomes (Figure 5-12a). This translocation could be explained by either single strand breaks model (Figure 5-12a) or double strand breaks model (Figure 5-12b). The translocations in patient DM could be explained in similar way, but on a smaller scale.

Another possibility is that there were two independent recombination events involving independent pairs of chromatids (Figure 5-13, Dr K. I. Mills, personal communication). One event occurred between exons b2 and b3; the other occurred between exons b3 and b4. The breaks in ABL were between exons Ia and a2. Therefore, for any double recombination event there is a small chance that exon b3 will be present on both derivatives of the chromosomal translocation and thereby the corresponding mRNA molecules both contain exon b3. The explanation that the incidence of this large duplication on the translocation is rare may be due to the low probability of the double recombination events occurring between the two chromosomes or small probability of gene conversion in this region. In practice, it is not possible to distinguish between

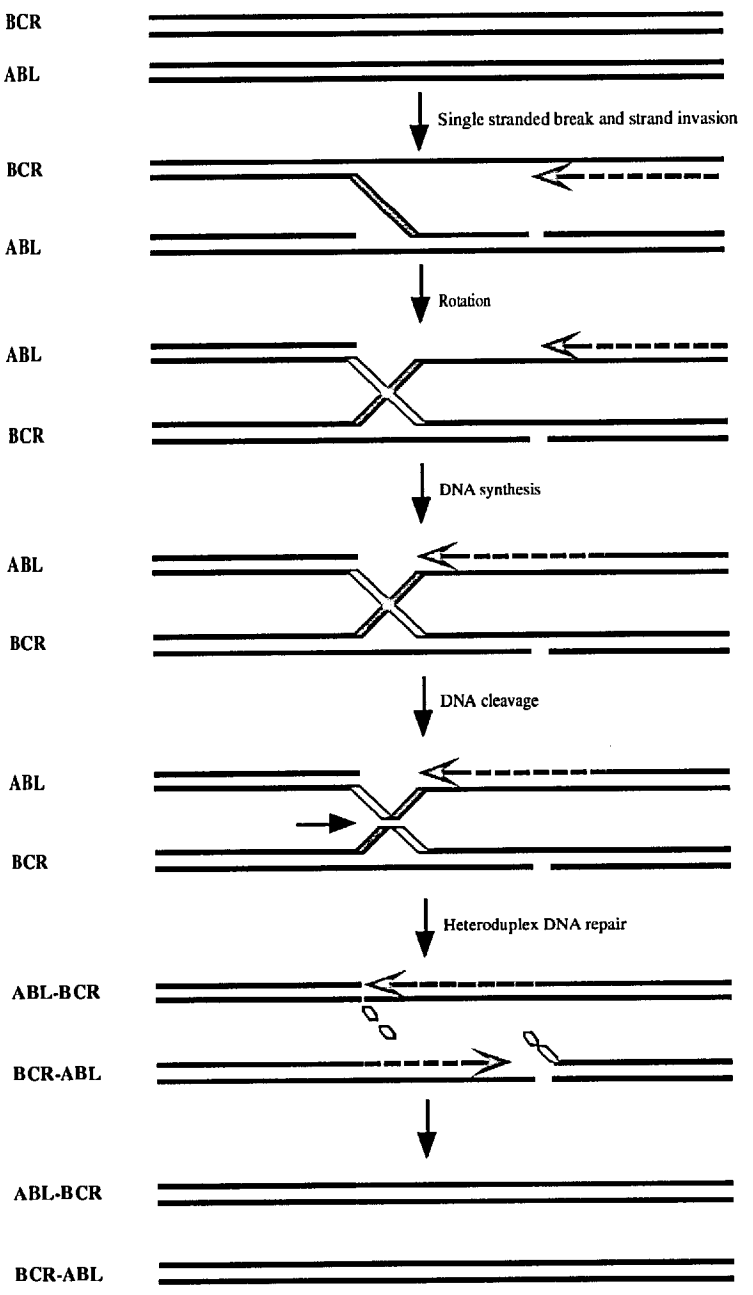


Figure 5-12a. The translocation in patient GM according to the single stranded gene conversion model

Gene conversion by single strand breaks could result in a deletion of part of ABL and duplication of part of BCR in patient GM. The line in red indicates the ABL gene and the one in blue illustrates the BCR gene. The broken lines in red indicate degradation of ABL genomic DNA. The black horizontal arrow indicates the position where DNA molecules are cut off.

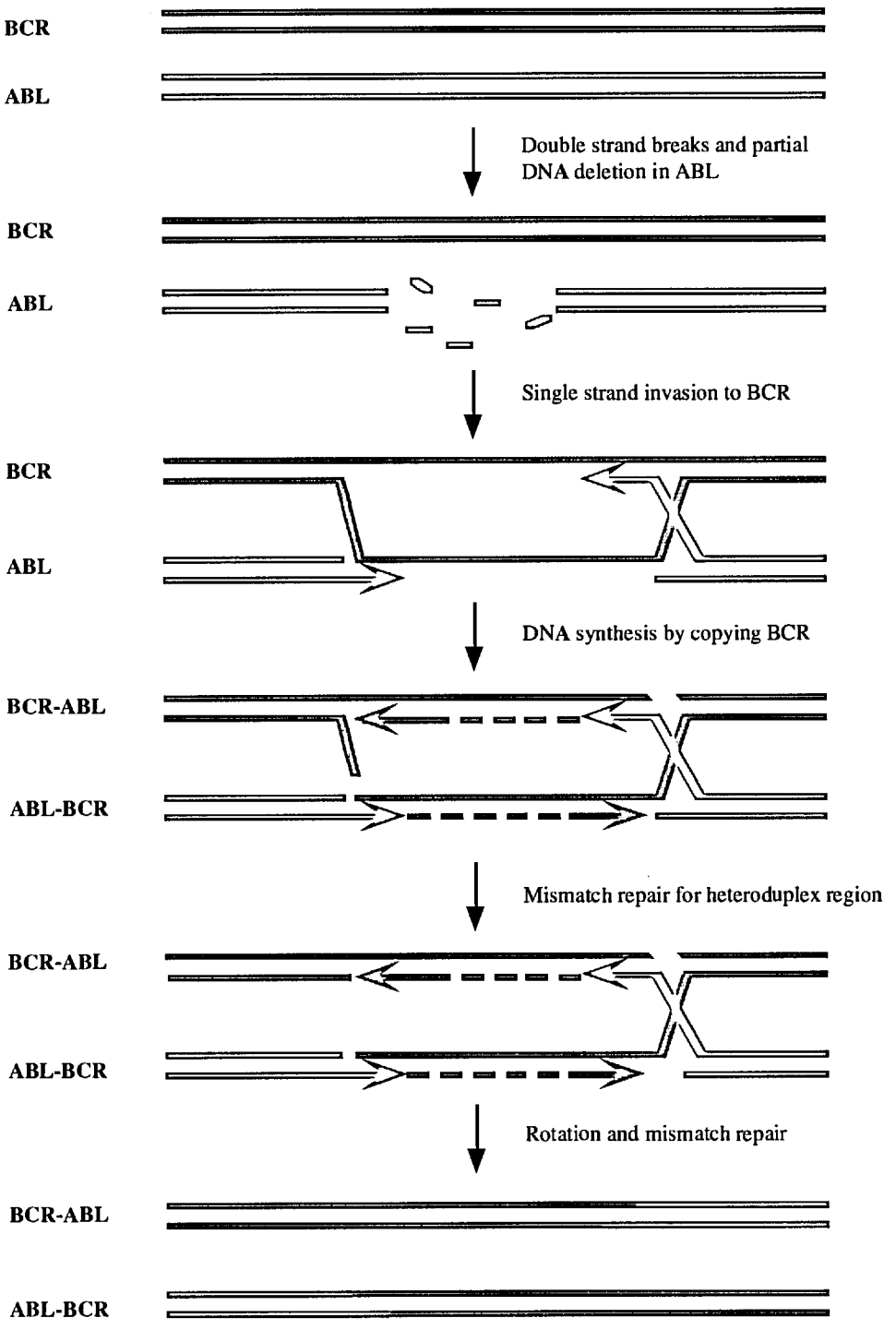


Figure 5-12b. The translocation in patient GM according to the double strand gene conversion model

Gene conversion by double strand breaks could result in a deletion of part of ABL and duplication of part of BCR in patient GM. The line in red indicates the ABL gene and the one in blue illustrates the BCR gene. The short lines in red indicate degradation of ABL genomic DNA. The broken lines in blue indicate DNA synthesis by copying BCR sequences.

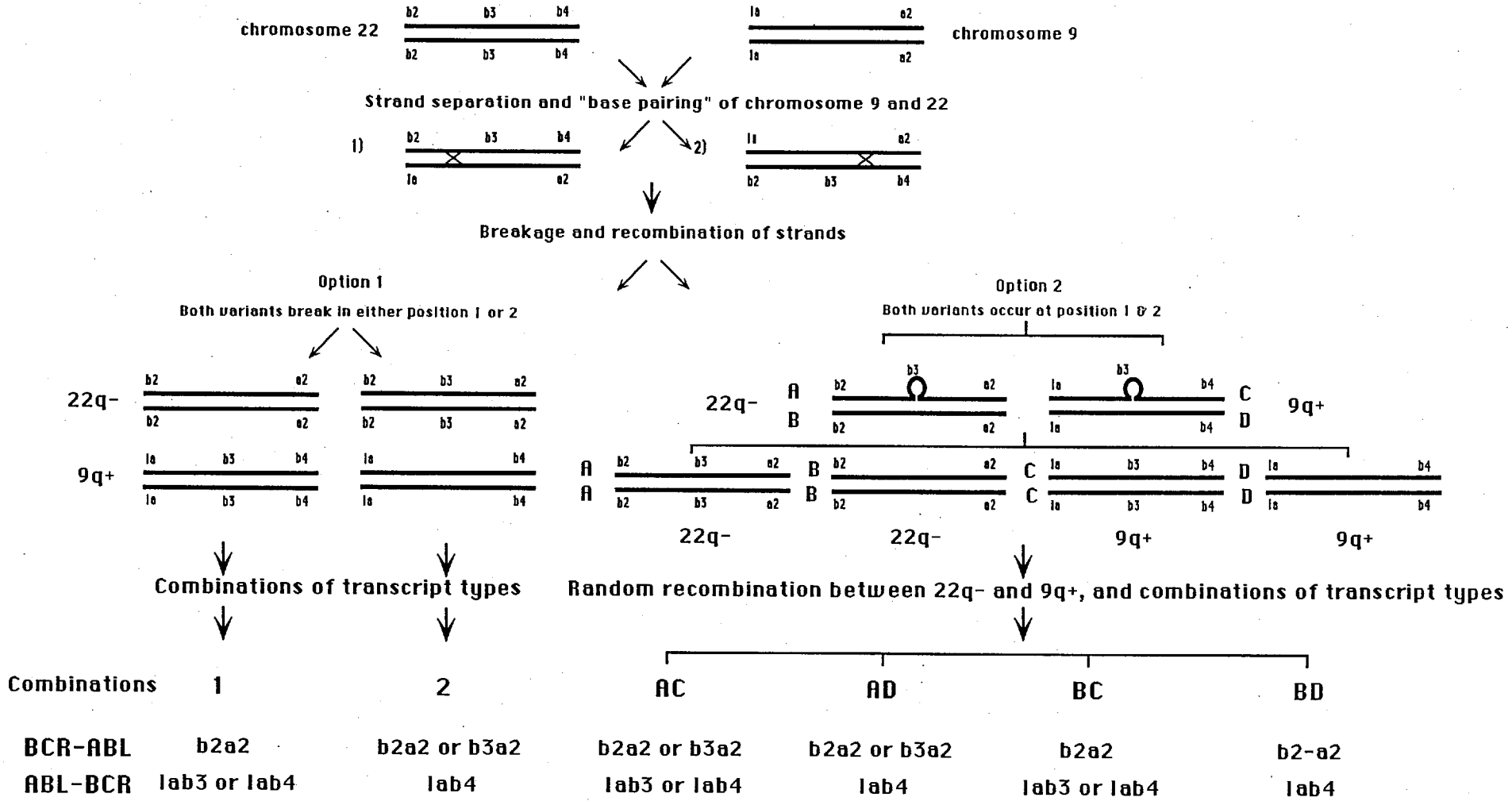


Figure 5-13. A model of the reciprocal translocation between chromosomes 9 and 22

these various models of the translocation (large staggered cut, gene conversion or double recombination).

5.3.1.4. The translocation in patient AE

The normal ABL sequence failed to amplify using PCR primers designed from the 9q+ and 22q- sequences of patient AE. The ABL sequences derived from both the 9q+ and 22q- chromosomes of patient AE were searched in Genbank (released in August, 96). No significant homology was found between the ABL sequence derived from the BCR-ABL and ABL1, ABL2 or ABL3. In contrast, the ABL sequence generated from the ABL-BCR breakpoint of this patient matched ABL2 (Genbank, U07562) (Figure 5-14). There are two possible explanations for this finding: i) There was a large deletion of ABL sequence on translocation, such that the BCR-ABL breakpoint fell into one of the gaps which remain non-sequenced but the ABL-BCR breakpoint fell into ABL2, or ii) an unknown amount of extraneous DNA from another location had been inserted between the BCR and ABL sequences on the chromosome 22q-, as for example has been described elsewhere (Heisterkamp *et al* 1985; Gregor & Morrison, 1986).

Gregor and Morrison reported previously a mouse myeloma mutant with a deletion of at least 4 kb immediately 3' of the alpha gene and introduction of at least 5 kb of novel (non-immunoglobulin) sequence in its place. A short direct repeat, eight copies of the 17-mer GCCTATAGAAGTAAGGA, is located at the junction of the alpha and novel sequences (Gregor & Morrison, 1986). Heisterkamp *et al* found that there was a 27 bp sequence inserted at the junction of ABL-BCR chimaeric gene, which did not originate from the normal chromosomes 9 and 22 in a CML patient, 02120185 (Heisterkamp *et al* 1985). It could be speculated that the "ABL" sequence obtained from chromosome 22q- in patient AE might be a similar sequence insertion.

To determine if there had been a mistake, I repeated the BCR-ABL Bubble PCR in this patient, but obtained exactly the same result after sequencing. Subsequently, this aberrant and unexplained breakpoint was confirmed by amplification of BCR-ABL from patient AE using a M-BCR primer (Z4A or Z4B) and a primer (1489ABL) made to the unknown sequence. As shown in Chapter 4 (see Table 4-9), these primers resulted in a product from AE, but not from any other patient.

Nevertheless this patient illustrates that in some cases the process of translocation may result in significant loss (or gain) of sequence.

```
Database:  genbank
           1,082,118 sequences; 744,272,137 total letters.

Sequences producing High-scoring Segment Pairs:
..
GB:HSABLGR2 ! U07562 Human ABL gene, intron 1b, partial s...
\\End of List


>GB:HSABLGR2 U07562 Human ABL gene, intron 1b, partial sequence.
  Length = 59,012

  Minus Strand HSPs:

 Score = 125 (34.5 bits), Expect = 0.021, P = 0.020
 Identities = 25/25 (100%), Positives = 25/25 (100%), Strand = Minus / Plus

Query:     25 CTATTGGGATCTGTATTAAAAGTGT 1
              |||||||||||||||||||||||||
Sbjct:  39303 CTATTGGGATCTGTATTAAAAGTGT 39327
```

Figure 5-14. Localisation of the ABL sequence amplified from chromosome 9q+ in patient AE

ABL sequence from chromosome 9q+ in patient AE matched the sequence of ABL2.

5.3.1.5. Amplification of ABL-BCR breakpoints in other patients

The ABL-BCR breakpoints were successfully amplified from only 4 out of 17 patients (24%). The reasons for this are probably due to the lack of a convenient Rsa I site on the ABL side of the 9q+. It is also possible that not enough combinations of primers were used. When I screened ABL-BCR breakpoint in the patients, I did not make a BCR primer for each patient individually. Instead, 4 sets of PCR primers were used. If a

reverse BCR primer had been specifically designed to anneal at a position close to the BCR-ABL breakpoint identified previously for each patient, I believe that more ABL-BCR breakpoints would have been identified.

From those patients, whose 9q+ breakpoints were not amplified by Bubble PCR, it is not clear whether a staggered cut or a gene conversion-like mechanism was involved in the translocation. However, the sequence flanking the breakpoint identified in patient RK shows that there is a small homology between BCR and ABL genes in this case (see Chapter 6).

5.3.2. Sequence features involved in chromosomal translocations

Recent data suggest that there are several sequence features are involved into chromosomal translocations, for example, heptamer and nonamer sequences, Alu element, L1 repeats, AT-rich sequences and MER elements. Perhaps the best understood example of a translocation is the signal sequences involving the translocation between BCL2 and IgH genes in lymphoid malignancy (see below).

5.3.2.1. Signal sequences in the chromosomal translocation between the BCL2 and IgH genes

A reciprocal translocation t(14;18)(q32;q21) is found in 90% of follicular lymphomas (Tsujimoto *et al* 1985). In this translocation, the BCL2 gene on chromosome 18 is joined to one of the six JH segments by mechanism similar to the normal VDJ joining of the IgH gene on chromosome 14 (Tsujimoto *et al* 1988). The breakpoints on chromosome 18 are largely clustered in the 3' UTR of the BCL2 gene, within the 150 bp of the mbr (Tsujimoto *et al* 1984; Cleary *et al* 1986; Kneba *et al* 1991). The normal DJ intrachromosomal assembly of the IgH gene is mediated by heptamer-spacer-nonamer sequence signals flanking the D and J segments (Figure 5-15). A heptamer

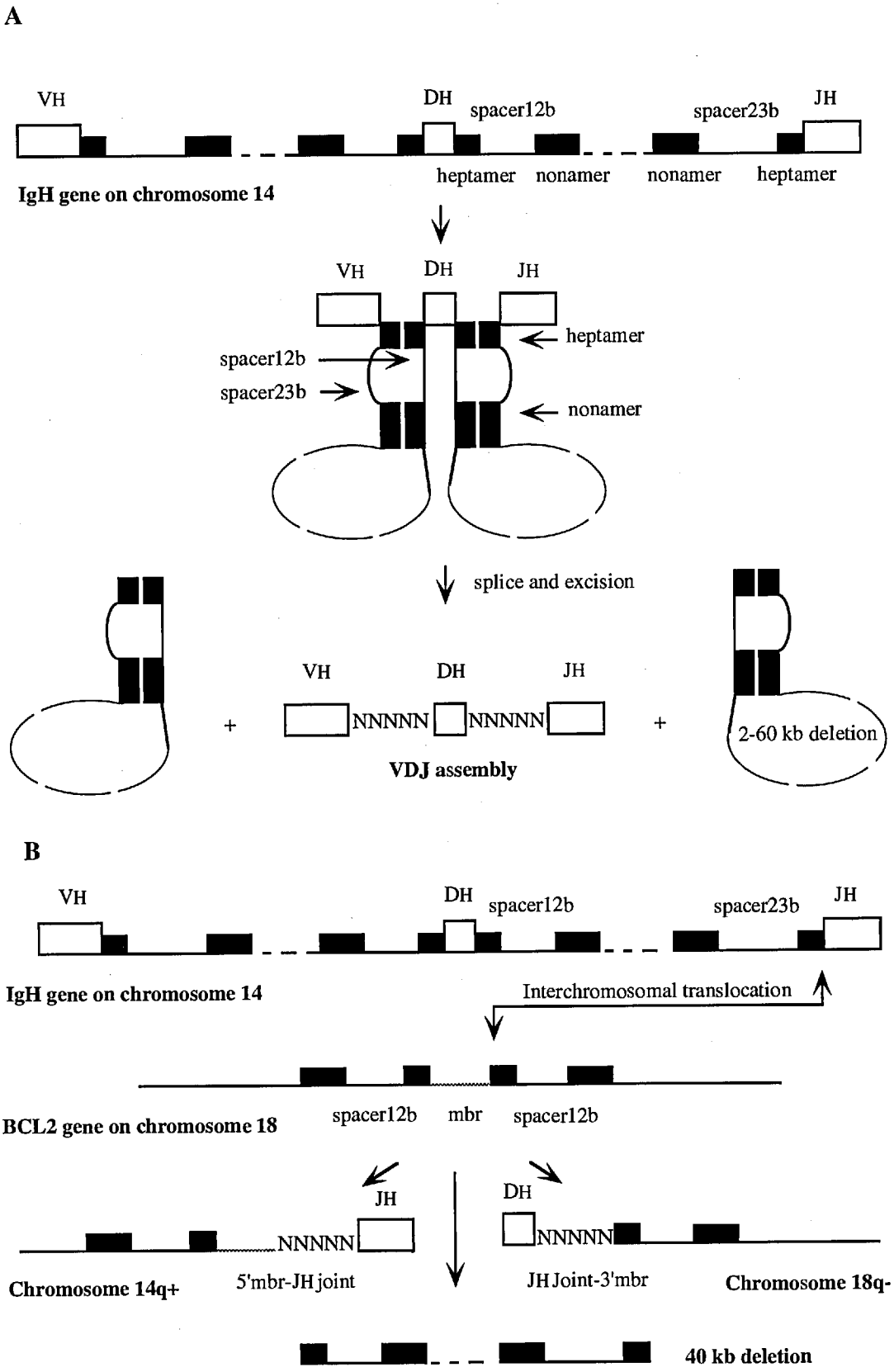


Figure 5-15. Rearrangements of the IgH gene and BCL2 gene

A. Normal VDJ assembly of the IgH gene. B. The reciprocal translocation between BCL2 and IgH genes. ▬ represents nonamer signal; ■ indicates heptamer signal; NNNNN: multiple nucleotide added at the junction.

(CACAGTG) and a nonamer (ACAAAAACC) signal sequences are represented as small and big solid boxes. The V, D and J segments are shown as open boxes. The signals direct the VDJ recombinase complex, and result in the recombination and rejoining between one of 30 human DH segments and one of six JH segments in the first step of IgH gene assembly. After double stranded cleavage adjacent to each heptamer, the heptamer DNA ends are ligated to form a signal joint, deleting up to 60 kb of intervening DNA. The DH and JH ends are modified by exonucleolytic nibbling and multiple nucleotide addition, forming the coding joint (Figure 5-15A).

One model for interchromosomal translocation between BCL2 major breakpoint cluster region (mbr) and JH may be described as follows: The BCL2 mbr region functions as a pseudo D segment, including the signal sequences, and is cross-ligated by the VDJ recombinase, as in normal DJ assembly. Two chimaeric genes are predicted in this model: one is BCL2-IgH fusion gene with junction 5'mbr-JH, the other is IgH-BCL2 gene with a breakpoint JH-3'mbr. If the reciprocal translocation is really balanced, chromosome 18q- should be the joint of the immediate upstream of the JH segment with the 3'mbr junction. However, data from some analyses demonstrated a 40 kb deletion upstream of the JH (Bakhshi *et al* 1987; Wyatt *et al* 1992). The joining on the IgH-BCL2 actually found was a DH-3'mbr. Nevertheless, this model indicates that lymphoid translocations can arise by aberrant recombinase activity (Figure 5-15B).

In addition to the heptamer/nonamer sequences, three short segments (14, 16, and 18 bp) were identified within the mbr near to the breakpoints at which translocations between BCL2 and IgH genes occurred with very high frequency (Wyatt *et al* 1992). These segments were homologous and, in particular, shared an octamer core element, GC[A/T]GG[A/T]GG, which is strongly similar to chi, the prokaryotic activator of recombination. This sequence feature could be related to the 40 kb deletion in IgH gene during chromosomal translocation mentioned above.

This deletion during translocation could be explained by the model illustrated in Figure 5-16 (Wyatt *et al* 1992). The initial crossover occurs between one of the chi-like sequences in the mbr (e.g. cluster 2) are a homologous sequence in the IgH gene (hom 2). Recombination is mediated by a specific, but unknown, protein complex bound to DNA at the chi-like motifs. This complex is responsible for DNA cleavage, either by single stranded breakage analogous to chi, or staggered double stranded breaks. Recombination then proceeds through homologous strand invasion and heteroduplex formation. The heteroduplex configuration may transiently resolve, generating the chromosome 14q+ and 18q- reciprocal derivatives. These intermediates recruit the VDJ recombinase complex, or some other associated enzymes (chi sequence-associated recombinase). Rearrangement continues, deleting the intervening DNA of IgH gene, and juxtaposing the observed mbr cluster sequences with DH and JH coding sequence (Figure 5-16). Based on the analysis of 64 IgH-BCL2 translocations, Wyatt *et al* (1992) have proposed that chi-like signals represent a distinct class of recognition sites for the recombinase complex during the translocation.

A 45 Kd nuclear protein (bp45) was isolated from early B cell extracts. This protein binds to a homopurine-homopyrimidine stretch (GGG AGG ACG GGA GGA AGG CG) in the mbr of BCL2, part of which is similar to the *E. coli* chi element. The protein also binds to homologous sequences in the IgH locus (Jaeger *et al* 1993). This suggests that VDJ-recombinase, heptamer-spacer-nonamer, and chi minisatellite-core mediated recombination may all contribute to t(14;18) reciprocal translocation (Jaeger *et al* 1994).

In the T-cell receptor (TCR) gene, the mechanism of recombination is remarkably similar to that described for the IgH chain both for normal intrachromosomal assembly and malignant interchromosomal translocation. The genomic organisation of the TCR gene is similar to that of the IgH gene (Griesser, 1995). VDJ combinations are also mediated by a set of conserved nucleotides, heptamers and nonamers. Those sequence

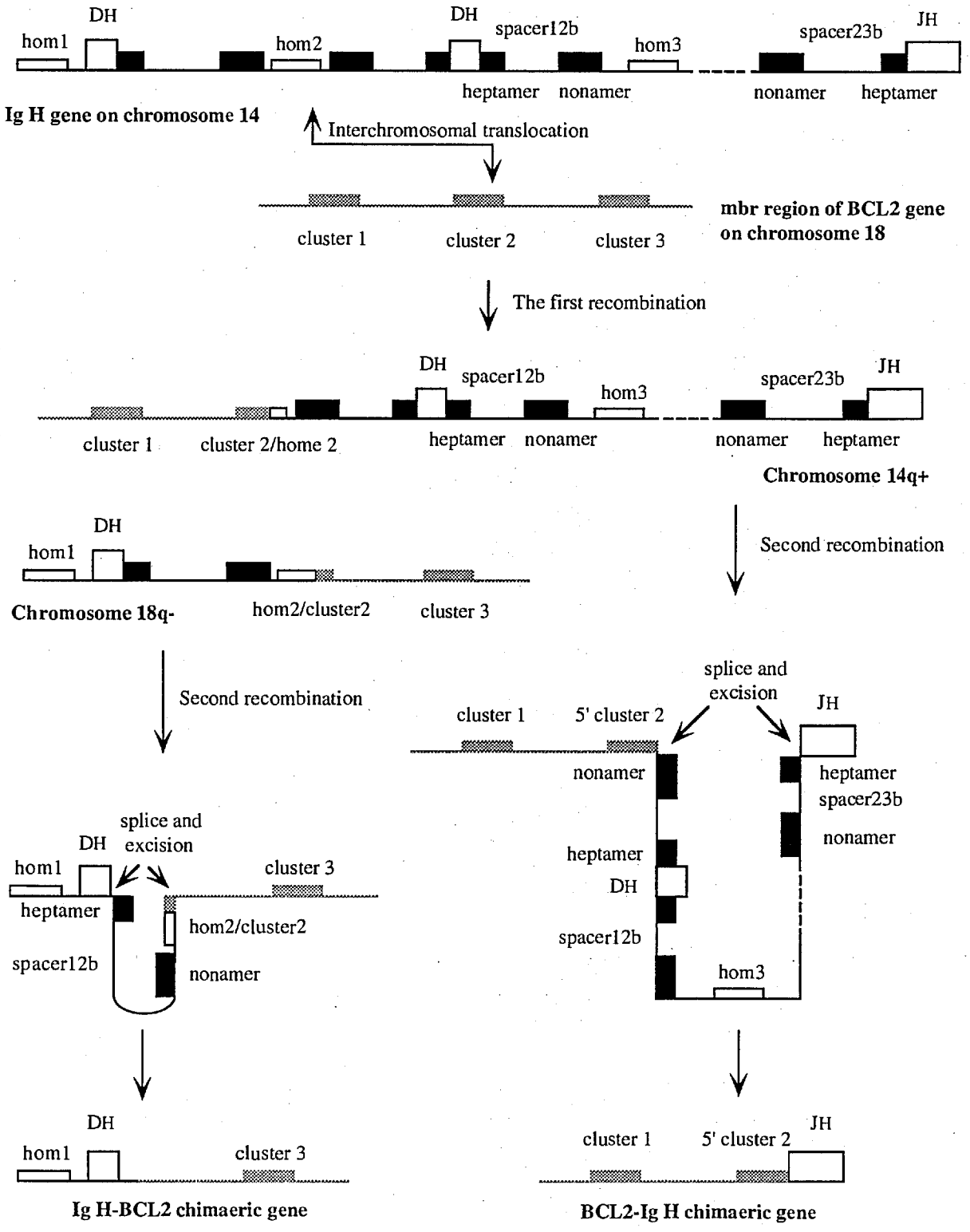


Figure 5-16. Chi-like elements involved in the rearrangements of the IgH and BCL2 genes

Chi-like sequences in the break clusters in the mbr region of BCL2 rcombine with a homologous sequence in the IgH gene in the first step. A transient heteroduplex structure is formed after strand invasion at the junction and then rearrangement continues on both transient substrates through the chi-like sequences and through heptamer/ nonamer sequences.

■ represents nonamer sequence; ■ indicates heptamer signal; □ represents DH or JH segment; ▭ homologous sequences; ▨ indicates chi-like sequence containing breakpoint cluster region; ▭▨ or ▨▭ indicates a transient heteroduplex structure.

features function as recombination signal sequences for V-D, D-V or V-J joining, and are recognised by a recombinase enzyme system (Stenzel Poore *et al* 1987). These signal sequences are separated by a nonconserved spacer. Usually one V and one J can recombine, but more than one D segment can join (O'Connor *et al* 1986). Most commonly, the coding joint stays in the chromosome, and a circular DNA molecule containing signal sequences and intervening sequences is excised (Okazaki & Sakano, 1988).

Chromosome translocations have been characterised predominantly for T cell leukaemias. The most frequently affected chromosomal site in these neoplasms is the TCR α or δ gene locus on chromosome 14q11. Involvement of the TCR β chain genes on chromosome 7q35 occurs more rarely. Participation of the VDJ recombinase system is likely in the rearrangements at TCR α or δ gene locus since the breaks often occur at heptamer recognition sequences of TCR D or J segments (Baer *et al* 1987). Numerous other examples of lymphoid chromosomal translocations mediated by aberrant recombinase activity have been described [Brown *et al* 1990 for t(1;14)(p34;q11); Duro *et al* 1996 for t(9;14)(p21-22;q11); Cheng *et al* 1990 for t(11;14)(p13;q13)]. However, in some cases, no evidence for association with heptamer or nonamer signal sequences in T-ALL has been found (Dong *et al* 1995 for t(11;14)(p13;q11).

5.3.2.2. Analysis of the sequences flanking the breakpoints

The ABL sequences generated from Bubble PCR were compared pairwise with each other to see if in any case the breakpoint involved an identical or related region of the ABL gene, but no significant cross homologies were found. Moreover the sequences did not contain any chi-like minisatellite recombination signals, GC[A/T]GG[A/T]GG (Krowczynska *et al* 1990), lymphoid heptamer/nonamer sequences (CACAGTG/ ACAAAAACC) or consensus topoisomerase II cleavage sites, GTN(ATC)A(CT)A (CT)TN(GA)(GT)NN(AG) (Darby *et al* 1986; Dong *et al* 1993) and MER elements

(Toth *et al* 1994; Rubin *et al* 1993; Iris *et al* 1993; Jurka *et al* 1993; Edwards & Gibbs, 1992; Kaplan *et al* 1991; Kaplan & Duncan, 1990). At the time I searched these ABL sequences in Genbank (release 81, February 1994) for any significant sequence features, including repetitive sequences. The ABL sequence in patient GL has a homology with a L1 element. The ABL sequence cloned from the 22q- in patient AE is AT-rich. An Alu-like sequence was found in patients CC, GM and PS (see Chapter 4), but no other significant sequence homologies were detected.

Topoisomerase II may play a role in the formation of translocations since this enzyme catalyses both the strand breakage and ligation reactions that are necessary for their formation. A t(15;17) translocation has been reported to have consensus topoisomerase II cleavage sites close to the breakpoints (Dong *et al* 1993) and topoisomerase II consensus-binding sequences have been identified near the chromosomal breakpoint of a secondary leukaemia with the t(4;11) (Domer *et al* 1995). I failed to find any similar homologies in our sequences. However, this does not rule out the involvement of topoisomerase II in BCR-ABL translocations since a general cleavage site for this enzyme is poorly defined and differs considerably depending on the drug used for induction of breaks (Cummings & Smyth, 1993). The 17 BCR-ABL sequences in the cases reveal no apparent homologies at the site of breakage. Likewise features of translocations in lymphoid cells, eg. heptamer/nonamer sequences (Finger *et al* 1986), polypyrimidine tracts (Boehm *et al* 1988) or chi-like elements (Krowczynska *et al* 1990), were not found at BCR-ABL breakpoints.

5.3.3. Translocation may be associated with chromosome structure

5.3.3.1. Complex Ph rearrangements

Apart from the standard reciprocal translocation between chromosomes 9 and 22, i.e. t(9;22)(q34;q11), a numer of complex translocations have been described that typically involve chromosomes 9, 22 and one, two or even three other chromosome bands. In

many cases the complex translocations are not random, but involve restricted regions of the genome (Mitelman, 1993). Typically, these regions are not only Alu-rich, but also GC-rich, CpG-island dense and gene rich (Holmquist, 1992; Craig & Bickmore, 1993; Craig & Bickmore, 1994). Sixty one percentage of CML patients with complex translocations involving chromosome 11 in addition to chromosome bands 9q34 and 22q11 showed disruption within band 11q13 (Mitelman, 1993). One t(9;22;11) (q34; q11; q13) has been cloned and shown to result in translocation of the 3' part of the BCR gene to the glutathione S-transferase Pi (GST-Pi) gene of 11q13 (Koduru *et al* 1993). Although no GST-Pi/BCR fusion expression was identified, it may be relevant that this gene was actively transcribed at least blast crisis.

Transcriptionally active DNA has an open chromatin structure which enhances the efficiency of transcription and replication, but is accessible to nucleases and may also make genome more vulnerable to breakage with consequential errors of rejoining (Craig & Bickmore, 1993). It is likely that both the BCR and ABL genes are transcriptively active in early haematopoietic progenitor cells. The M-BCR region of the BCR gene and the first intron of the ABL gene could be in an open chromatin structure, which may predispose them to breaking and joining. An alternative hypothesis is that the BCR and ABL gene are in close proximity during interphase, increasing the chance of aberrant recombination. Whether there is a defined or favoured structure during interphase is currently unknown, but this question would theoretically be addressed by a combination of FISH and confocal microscopy.

5.3.3.2. Ph-negative CML

Approximately 5% CML patients have no typical Ph chromosome, and are designated Ph-negative CML (Hagemeijer, 1987). One third of those Ph-negative CML patients are BCR-ABL positive on molecular analysis (Cortes *et al* 1995). These patients can be cytogenetically normal or have other aberrant chromosomes in chronic phase (Nishigaki *et al* 1992). Some of these BCR-ABL positive, Ph-negative CML patients

have an insertion of ABL-containing DNA sequences into a normal-appearing chromosome 22 (Mohamed *et al* 1995; Lazaridou *et al* 1994; Nishigaki *et al* 1992; Morris *et al* 1990; Rassool *et al* 1990; Kuriyama *et al* 1989). The translocation with this insertion seems to be different from either the standard reciprocal translocation t(9;22)(q34;q11) or the complex masked Ph rearrangement t(9; 22; 11) mentioned above (Morris *et al* 1996; Koduru *et al* 1993). However, Ph-negative BCR-ABL positive CML has an indistinguishable phenotype from standard Ph-positive CML (Cortes *et al* 1995; Dreazen *et al* 1987; Morris *et al* 1986), and the ABL insertion into chromosome 22 may be an example of another type of complex chromosomal translocation in CML.

A model has been proposed to explain the mechanism of ABL transposition in Ph negative BCR-ABL positive CML (Morris *et al* 1990; Fitzgerald & Morris, 1991). It is unlikely that the insertion of part of chromosome 9 into BCR on chromosome 22 and complex chromosome rearrangements result from stepwise serial translocations (Fitzgerald & Morris, 1991; Nishigaki *et al* 1992), because clones carrying the first translocation between chromosomes 9 and 22 are rarely seen in patients whose leukaemic cells have either the ABL insertion or complex translocations (Fitzgerald & Morris, 1991). Similarly there no documented cases of loss of the Ph-chromosome but retention of BCR-ABL during progression of CML. The formation of the BCR-ABL gene is the crucial step in the aetiology of CML and leukaemia would develop without the second translocation. It is likely that the second translocation occurs at the same time as, or soon after, the first translocation. If a leukaemic phase was present between the two translocations, the second, which does not involve BCR-ABL fusion, would need to confer a stronger leukaemic advantage in order to become the predominant cell type. The serial translocation model also requires at least two separate recombination events, involving chromosome breakage and rejoining, at precise sites in a leukaemic cell. The complex chromosome rearrangements are more likely to occur as a combinational and simultaneous events than by stepwise serial translocations

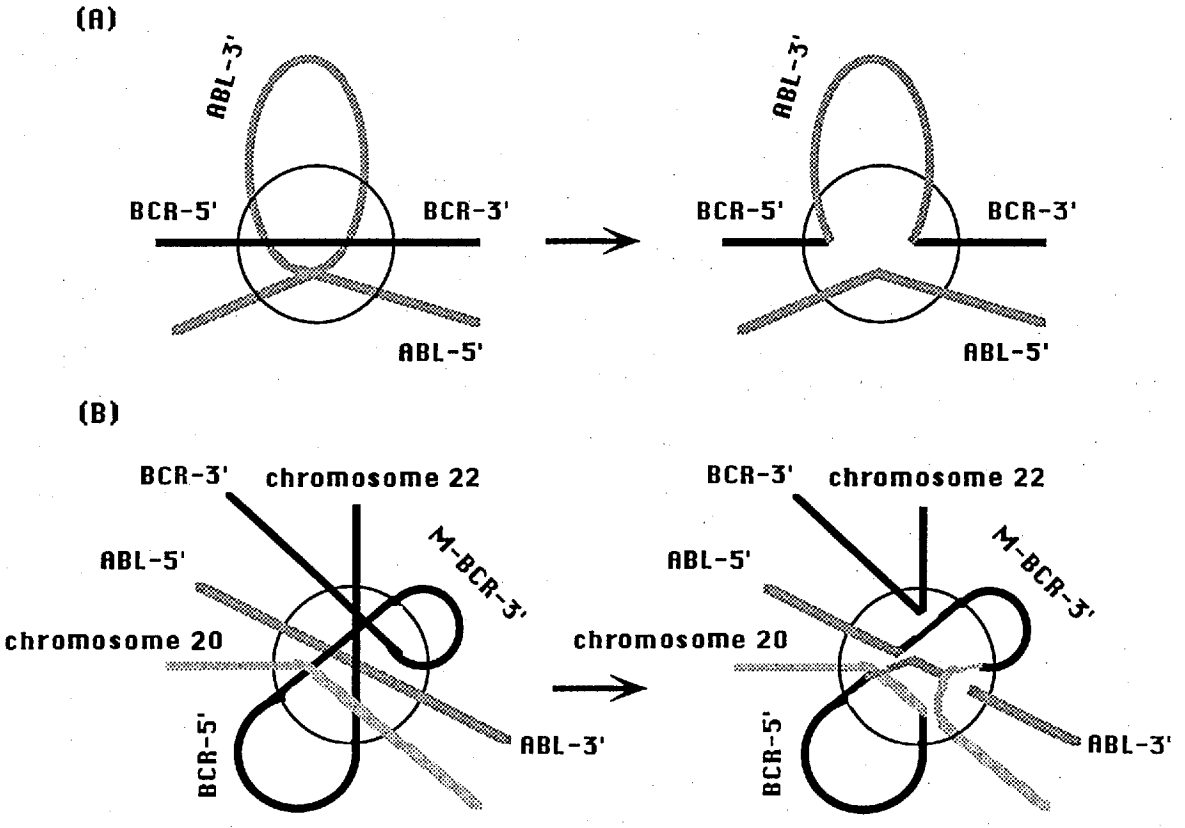


Figure 5-17. Fitzgerald & Morris chromosome translocation model.

A) Diagram of chromosome breakage and repair causing the insertion of part of chromosome 9 carrying ABL sequences between 5' and 3' sequences of the BCR gene of chromosome 22. B) Diagram showing the origin of the t(9;22;20)(q34; q11;13) from a single concerted event which breaks all three chromosomes. The Figures above were modified from a paper published by Fitzgerald and Morris in 1992.

(Fitzgerald & Morris, 1991) (Figure 5-17). This concerted mechanism may reflect an association between chromatin structure and the translocation events.

5.3.3.3. Mobile genetic elements

Mobile elements have been reported to exist in a number of organisms, including maize, yeast, mice and humans (McClintock, 1951; Shapiro, 1983; Shapiro, 1995; Eibel *et al* 1981; Monson *et al* 1982; Murphy *et al* 1984). Genetic material can be moved by transposition in mammals mediated with a mobile element (Sharp *et al* 1983; Weiner *et*

al 1986). Genetic aberrations, including translocations, can result from insertion and excision of a mobile element (Rubin *et al* 1982). However, it is generally considered implausible that the 3' part of ABL gene move from chromosome 9 into chromosome 22 by retroviral or transposon mechanisms. Current evidence fails to show that there is a mobile genetic element structure associated with either the BCR or ABL gene on the translocated derivatives. For BCR-ABL positive, Ph-negative CML, the chromosome 9 segment carrying ABL was shown to be more than 300 kb long in a patient characterised by Morris *et al* (1990), a greater size than has been considered possible for DNA sequences to be moved by transposon mechanisms (Sharp *et al* 1983; Weiner *et al* 1986). Although at least a subset of Alu and L1 elements may be mobile (Brookfield, 1995), there is no evidence to support the idea that transposition of these elements mediates the transfer of ABL from chromosome 9 to chromosome 22.

5.3.3.4. Transcription silencer

A 427 bp region that can function as a transcriptional suppressor of the BCR promoter has been found in the region between exons b2 and b4 in the M-BCR (Stewart *et al* 1994). It is conceivable that this element and the proteins that bind to it may result in a spatial structure that could, for example, regulate BCR gene transcription and make the M-BCR vulnerable to breakage. However, this binding site has no obvious association with the sites of BCR-ABL breakpoints and it remains unclear whether they really play a role in transcriptional regulation or not.

5.3.3.5. Chromosomal scaffolds

Recent studies have demonstrated that scaffold attachment regions (SARs) are key elements in the dynamics of chromosomes (Strick & Laemmli, 1995). Recruitment of topoisomerase II to SAR sequences may be one of the mechanisms responsible for gene regulation and expression (Kas & Laemmli, 1992). Therefore, topoisomerase II and other proteins (Jaeger *et al* 1993) in the nuclear matrix are likely candidates for the

processes of DNA breakage, strand invasion and religation that are necessary for chromosome translocation.

Chromosomal scaffolds are the structures that remain after the removal of histones and soluble proteins with low concentrations of the detergent-like chaotrope, lithium-3',5'-diiodosalicylate (Mirkovitch *et al* 1984). The SARs are the control elements maintaining independent realms of gene activity, participating in the control of gene transcription, replication, repair, and recombination (Boulikas, 1995). In the mouse, the Igκ light-chain intronic SAR has been mapped to translocation breakpoint junctions (Sperry *et al* 1989). A SAR was also mapped close to the junction on a human ring chromosome 21 (Sperry *et al* 1989) and deletions occur at the human β globin IVS2 SAR (Anand *et al* 1988). Furthermore, two human glioma breakpoint junctions have been mapped to DNA scaffold sites in a case with a 9p deletion (Pomykala *et al* 1994). It is possible therefore that SARs are one of the important factors involved in chromosomal rearrangements.

Further evidence relating chromosomal translocations to chromosome structure is provided studies defining the distribution of 11q23 breakpoints within the MLL breakpoint cluster region. This gene is involved in multiple translocations in *de novo* leukaemias, but is also significantly involved in therapy related leukaemias, particularly after prior treatment with etoposide. This drug is a topoisomerase II inhibitor and several studies have demonstrated that topoisomerase II cleavage site are associated with the MLL translocation breakpoints in secondary leukaemias, but not in the primary MLL leukaemias. A weak-affinity SAR mapping just centromeric to the MLL breakpoint cluster region and a high-affinity SAR mapping within the telomeric half of the breakpoint cluster region were identified (Broeker *et al* 1996). A topoisomerase II mapped adjacent to the telomeric SAR, whereas 6 sites mapped within the same SAR. They found that 74% of *de novo* leukaemia and 25% of therapy related AML (tAML) breakpoints mapped to the centromeric half of the breakpoint cluster region located

between the two SARs; in contrast, 26% of the *de novo* leukaemia and 75% of the tAML patient breakpoints mapped to the telomeric half of the breakpoint cluster region that contains both the telomeric SAR and the topoisomerase II sites (Figure 5-18). It is possible that the SARs confer a unique three-dimensional chromatin structure protecting the 3' portion of the breakpoint cluster region from breakage in *de novo* leukaemias. The breaks that occur in the SAR in tAML, may reflect an etoposide related change in the SAR structure (Berrios *et al* 1985; Earnshaw & Heck, 1985; Gasser *et al* 1986).

The translocation between chromosomes 9 and 22 may also be associated with SARs and associated scaffold proteins. The scaffold structure of both the BCR and ABL genes has not been defined yet. The sequences of different SARs are not closely related (Gasser & Laemmli, 1986; Gasser & Laemmli, 1987); they do not cross-hybridise with each other and they do not have a strict consensus sequences, although they share asymmetric AT-rich sequences. Even their organised loop structures are not strictly conserved, regularly repeating units (Gasser & Laemmli, 1987). The scaffold proteins appear to recognise characteristic DNA structures and do not act strictly as sequence-specific DNA-binding proteins (Kas *et al* 1989). Therefore, the characterisation of the scaffolds in the BCR and ABL genes would entail the analysis of both the nonscaffold bound DNA and the scaffold bound DNA fractions. The distance between two adjacent SARs varies between 4.5 and 112 kb (Gasser & Laemmli, 1987). That means that the genomic region within which breakage occurs might be quite variable, if there is indeed an association between susceptibility to breakage and chromatin scaffold structure in both the BCR and ABL genes.

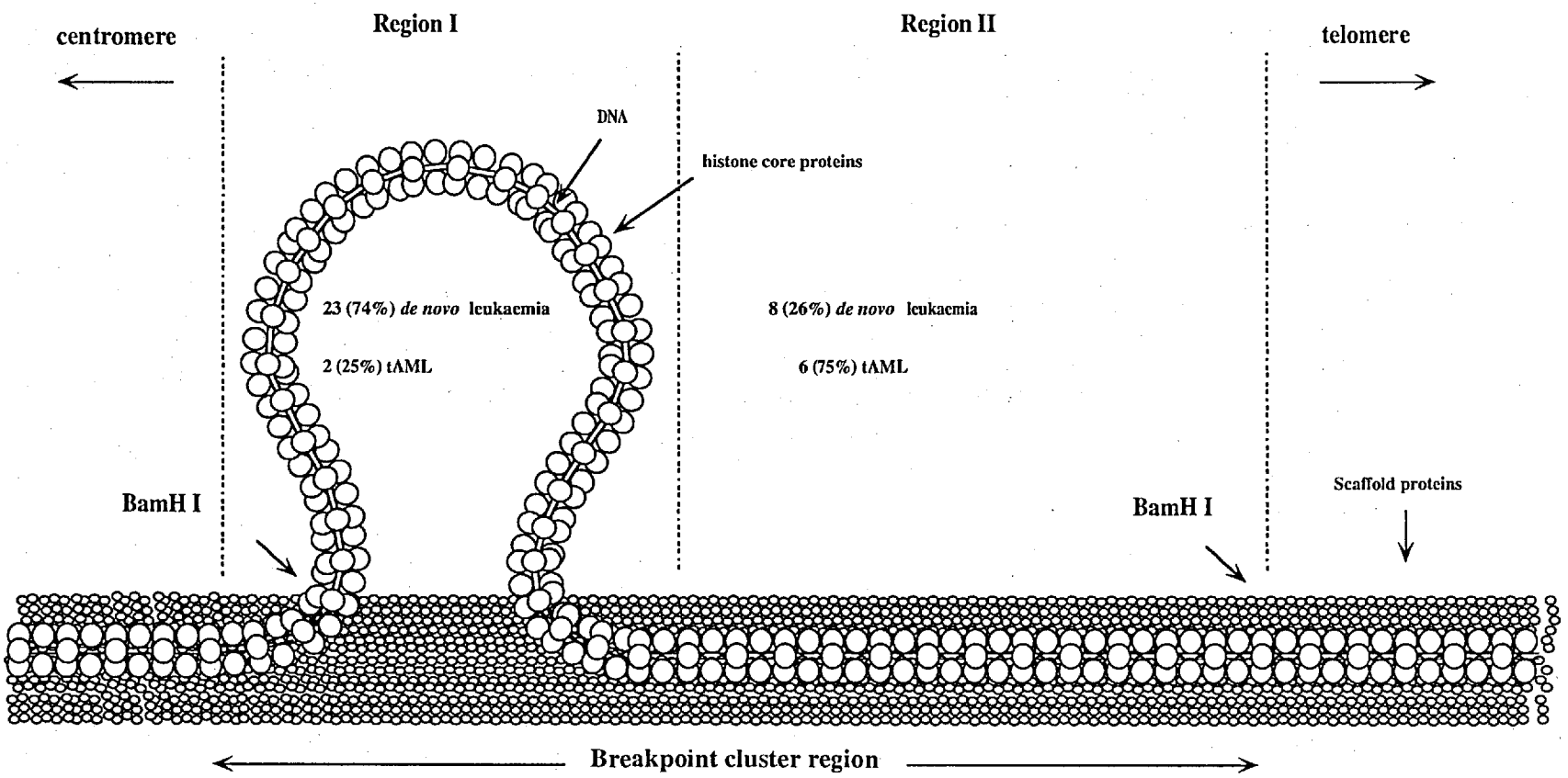


Figure 5-18. Proposed model of the chromatin structure of the MLL breakpoint cluster region.

5.3.4. Selection of a population of cells bearing a chromosome translocation in haemopoiesis could be one of the mechanisms involved into expansion of tumour cells

There is much evidence to suggest that there is a selection mechanism during oncogenesis that favours one specific clone over other clones. In 1993, Ito *et al* found that five BCR-ABL fusion gene transcripts in 10^8 HL60 cells after irradiation with 100 Gy. These fusion genes contained not only CML-specific BCR-ABL rearrangements (b2a2 or b3a2), but also other forms of BCR-ABL fusions such as a b4a2 junction with an unknown DNA sequence in between, b5a2 and b4a2 (Ito *et al* 1993). The fact that fusion genes which are not found naturally can be produced by irradiation suggests that only a fraction of the possible translocation products actually result in a biologically active, disease producing fusion gene. One reason for this is that only a subset of possible BCR-ABL fusions would be in frame. The irradiation data is consistent with the hypothesis that chromosomal translocations are largely random but only those changes leading to a selective advantage actually predominate. However, it still can not be ruled out that DNA rearrangements occur by sequence dependent mechanisms and that there really are preferred sites of illegitimate recombination.

CHAPTER SIX DISTRIBUTION OF THE BCR-ABL BREAKPOINTS IN THE ABL GENE

6.1. Introduction

As discussed above, the mechanism by which BCR-ABL breakpoints occur is unclear. However, if breakage and rejoining occurred completely at random, then a random distribution of breakpoints would be expected, although constrained of course by the reading frame of each individual exon and the biological activity of the fusion protein. On the other hand, if specific sequence elements were instrumental in the translocation, the breakpoints would be expected to occur at defined sites or clusters. The first intron of the ABL gene may be particularly informative for distinguishing between these two models since it is very large (about 170 kb) and the position of the breakpoints would not be restricted by coding constraints. Furthermore, a large portion of this intron has recently been sequenced (Chissoe *et al* 1995).

Subclustering of BCR-ABL breakpoints at three sites between exons Ib and Ia in the first intron of ABL has been reported on basis of data from pulsed field gel electrophoresis (PFGE) (Jiang *et al* 1990). This finding implies that there are indeed some sequence features in the ABL gene that leads to clustering of the breakpoint.

In this chapter I have analysed the distribution of breakpoints within ABL intron 1, using the data derived from Bubble PCR and also the sequence of breakpoints that have been published by other groups.

6.2. Results

6.2.1. Localisation of the BCR-ABL breakpoints in ABL gene on the basis of sequencing

A total of 179,512 bases from the ABL gene has been sequenced recently (Chissoe *et al* 1995). The sequence consists of three non-contiguous pieces, called ABL1, ABL2, and ABL3 that are 35,962 bp, 59,012 bp, and 84,538 bp long respectively (Chissoe *et al* 1995; Genbank, U07561, U07562 and U07563). Together, these sequences cover 72% of whole ABL gene. There are, however, still two gaps in the sequence of intron 1 (Figure 6-1). The size of each gap is unknown, but the sum of the gaps is estimated to be about 70 kb (Chissoe *et al* 1995). Nevertheless, the known sequences provided a base for me to localise the breakpoints within the ABL gene.

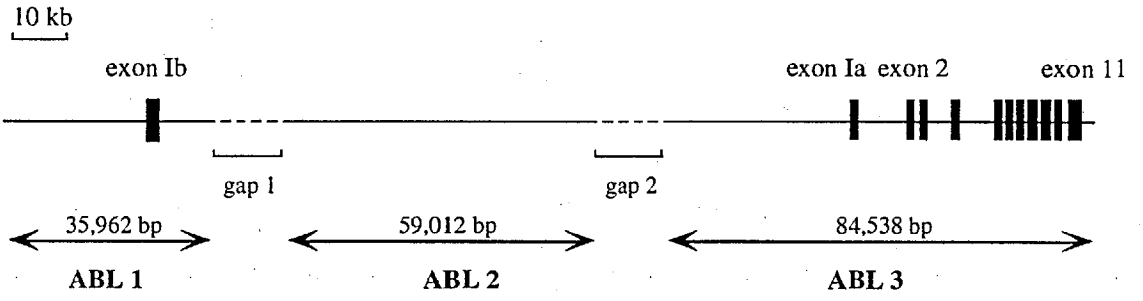


Figure 6-1. Regions of the ABL gene that have been sequenced

Solid rectangles indicate exons. The lines represent intronic regions. The broken lines indicate unknown sequence gaps,which together total about 70 kb. The scale is indicated.

As described in Chapter 4 and 5, I used Bubble PCR to amplify the breakpoints (BCR-ABL or ABL-BCR) from a total 17 patients with CML. A median of 117 bp (range 30 - 244 bp) of ABL sequence was determined for each patient. ABL sequences were compared to the Genbank database to look for repeats and to localise the breakpoints within the ABL gene. The breakpoints in 10 out of the 17 patients matched ABL sequences (Genbank, U07561, U07562 or U07563). Of these 10 patients, breakpoints were localised for 6 patients from the BCR-ABL junction sequence; for 3 patients from

both BCR-ABL and ABL-BCR sequences and for 1 patient from ABL-BCR sequence only. Six breaks were within ABL2 and four breaks within ABL3.

```
Database:  genbank
           1,082,118 sequences; 744,272,137 total letters.

                                                                    Smallest
                                                                      Sum
                                                          High      Probability
Sequences producing High-scoring Segment Pairs:           Score     P(N)        N
..
GB:HSABLGR2 ! U07562 Human ABL gene, intron 1b, partial s...  212   1.2e-09     1
GB:T83034 ! T83034 yd43b02.r1 Homo sapiens cDNA clone 110...  110   0.26        1
GB:YSPNMT1A ! J05493 S.pombe no message in thiamine prote...  103   0.75        1
GB:EVU67875 ! U67875 pESP-I yeast expression vector, comp...  103   0.75        1
GB:CELB0280 ! U10438 Caenorhabditis elegans cosmid B0280.     102   0.82        1
GB:R09189 ! R09189 yf22f04.s1 Homo sapiens cDNA clone 127...   98   0.94        1
GB:T97756 ! T97756 ye58d03.s1 Homo sapiens cDNA clone 121...   98   0.95        1
GB:H75470 ! H75470 yu07h08.s1 Homo sapiens cDNA clone 233...   98   0.95        1
GB:H65622 ! H65622 yr61f09.s1 Homo sapiens cDNA clone 209...   98   0.96        1
GB:W01750 ! W01750 za37h03.r1 Soares fetal liver spleen 1...   98   0.96        1
GB:T99973 ! T99973 ye69a11.r1 Homo sapiens cDNA clone 122...   98   0.96        1
GB:N54297 ! N54297 yv35a04.s1 Homo sapiens cDNA clone 244...   98   0.96        1
GB:N70346 ! N70346 za60g09.s1 Homo sapiens cDNA clone 296...   98   0.96        1
GB:MUSMHCAB1 ! M11800 Mouse MHC class II A-beta-2 gene, s...   97   0.99        1
.. ......... . ...... ..... ... ..... .. ..............        ..   ....        .


>GB:HSABLGR2 U07562 Human ABL gene, intron 1b, partial sequence.
  Length = 59,012

  Plus Strand HSPs:

 Score = 212 (58.6 bits), Expect = 1.2e-09, P = 1.2e-09
 Identities = 44/46 (95%), Positives = 44/46 (95%), Strand = Plus / Plus

Query:      2 GCTTGTCATAGAAGGGCATTTAAGTGACTTTGCCAAGAGAAACAGT 47
              |||||||||||||||||||||||||||||||| | |||||||
Sbjct:  54877 GCTTGTCATAGAAGGGCATTTAAGTGACTTTGCCAACACAAACAGT 54922
```

Figure 6-2. Localisation of the ABL sequence cloned from Philadelphia chromosome in patient AH

The ABL sequence amplified from patient AH was localised to the sequence of ABL2 (Genbank: HSABLGR2).

Figure 6-2 shows an example of a BLAST comparison between the 46 bp of ABL sequence amplified from Philadelphia chromosome of patient AH and the Genbank database [Release 99.0 (02/97)]. As shown, the 46 bp matches to the sequence between positions 54,877 and 54,922 in ABL2. The match is highly significant (probability of the match happening at random = 1.2×10^{-9}). All the other matches on the list are not significant. Two mismatches between the sequence of AH and ABL2 are seen. These could be due to sequencing errors, polymorphisms or misincorporation errors of

nucleotides during Bubble PCR amplification. For all patients, significant matches were detected only against ABL, apart from three individuals who had repeated sequences at or near the breakpoint. In two patients (CC and PS) the sequence distal to BCR consisted entirely of Alu sequence (See Chapter 4, Table 4-11) and therefore it was not possible to determine their origins. Neither, however, matched precisely any of the sequenced Alu elements within ABL intron 1 (see below). The ABL sequence in patient GL had homology to a L1 repetitive element but did not match any of the known ABL sequences. Indeed, the published sequence of ABL does not appear to contain any L1 elements (Chissoe *et al* 1995). Although one patient (patient GM) had an Alu element close to the breakpoint, once this sequence was removed from the BLAST search it was possible to localise the breakpoint precisely within ABL3. The reciprocal breakpoint (ABL-BCR) of patient GM was also localised to the same area of ABL3 (see Chapter 5). The sequences of the remaining four patients did not match anything in the database. It is likely that they, and patient GL, have breakpoints that fall into the 70 kb of sequence that has not yet been determined. Alternatively, the breakpoints could have been upstream of ABL1.

Of the 19 BCR-ABL breakpoints that have been characterised by other groups, I was able to localise the positions of the breakpoint within the ABL gene for 14 cases (Groffen *et al* 1984; Heisterkamp *et al* 1985; Grosveld *et al* 1986; de Klein *et al* 1986; Chen *et al* 1989a; Chen *et al* 1989b; van der Feltz *et al* 1989; Papadopoulos *et al* 1990; Soekarman *et al* 1990; Mills *et al* 1992; Litz *et al* 1993; Sowerby *et al* 1993) (Table 6-1). Nine (64%) of the 14 breakpoints were located in ABL3; 4 (31%) in ABL2 and 1 (8%) in ABL1. In combination with those characterised in my study, a total of 24 breakpoints can be localised within the known sequence of ABL. The distribution of these breakpoints is shown in Figure 6-3.

Inspection of the positions of the breakpoints suggests that there may be a significant subclustering in the vicinity of ABL exon a2. I have called this region the putative subcluster region (PSR), defined here as the 31 kb region between the Hind III site at

Table 6-1. Philadelphia chromosome translocation breakpoints localised within the ABL gene

Breakpoint	Region	Patient	References
22,910-22,913	ABL1	CML02120185	Heisterkamp, *et al.*, 1983; Groffen, *et al.*, 1984; Heisterkamp, *et al.*, 1985
1	ABL2	CML K562	Heisterkamp, *et al.*, 1983; Groffen, *et al.*, 1984
7,915-7,916	ABL2	SL	
18,919-18,922	ABL2	AML SA	Chen, *et al.*, 1989b
21,334-21,335	ABL2	CML ICB1	Groffen, *et al.*, 1984
21,372-21,373	ABL2	CML 0311068	Heisterkamp, *et al.*, 1983; Groffen, *et al.*, 1984; Grosveld, *et al.*, 1986
23,194-23,195	ABL2	BY	
39,328-39,329	ABL2	AE	Breakpoint was characterised from chromosome 9q+.
44,334-44,335	ABL2	MT	
50,658-50,659	ABL2	DM	
54,876-54,877	ABL2	AH	
24,844-24,845	ABL3	CML 83-84H	De Klein, *et al.*, 1986
30,142-30,143	ABL3	CML 7701C	Groffen, *et al.*, 1984
32,613-32,936	ABL3	CML A	Litz, *et al.*, 1993
32,623-32,624	ABL3	BA	
34,958-34,965	ABL3	ALL NA	Chen, *et al.*, 1989b
36,892-36,893	ABL3	RK	
38,758-38,759	ABL3	GM	
41,333-41,335	ABL3	CML 0319129	Heisterkamp, *et al.*, 1983; Groffen, *et al.*, 1984; Heisterkamp, *et al.*, 1985
43,617-43,618	ABL3	ALL FY	Van der Feltz, *et al.*, 1989
43,804-43,805	ABL3	ALL SUP-B13	Papadopoulos, *et al.*, 1990
43,902-43,904	ABL3	ALL BON	Chen, *et al.*, 1989a
47,946-47,947	ABL3	AJ	
50,083-50,086	ABL3	ALL1	Soekarman, *et al.*, 1990

Note: the breakpoints without references were characterised in my study.

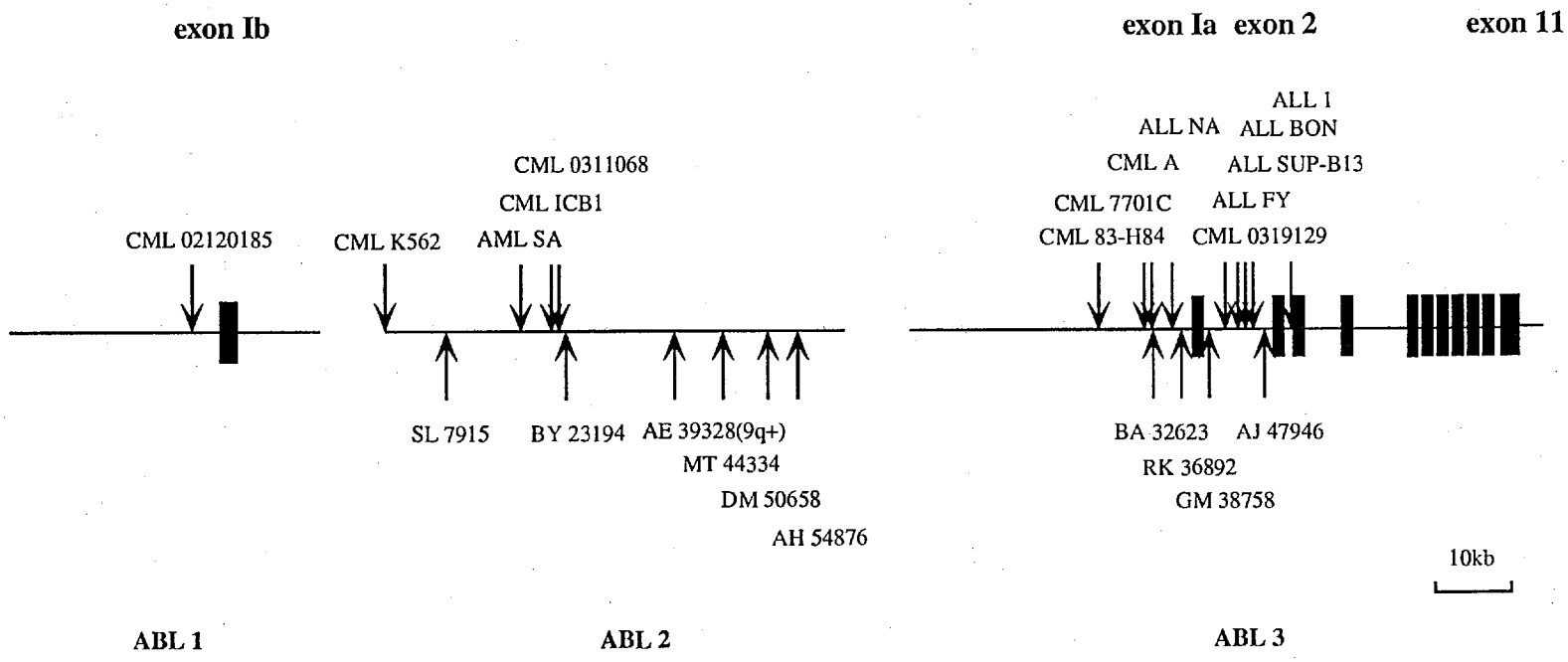


Figure 6-3. Distribution of the BCR-ABL breakpoints in ABL1, ABL2 and ABL3

Arrows indicate the positions of each breakpoint. Those indicated by an arrow under the line are the breakpoints characterised in my studies. The ones indicated by an arrow above the line are the breakpoints identified by others (Groffen et al., 1984; Heisterkamp et al., 1985; Grosveld et al., 1986; de Klein et al., 1986; Chen et al., 1989a; Chen et al., 1989b; Van der Feltz et al., 1989; Soekarman et al., 1990; Papadopoulos, et al., 1990; Litz et al., 1993 and Chissoe et al 1995).

position 19,504 to the EcoR I site at 50,464 of ABL3. Of the 10 patients in my study for whom the ABL breakpoint was successfully localised, 4 fell into the PSR. Of the 14 breakpoints published elsewhere, nine fell into the PSR. In total 13 (54%) out of 24 patients had a breakpoint in the PSR.

6.2.2. Analysis of sequence features flanking the breakpoints

6.2.2.1. A search for regions of homology between BCR and ABL

The ABL sequences within 2 kb of each breakpoint were compared using the DNA Strider computer program in order to find whether there are any common sequence features prone to genomic breakage. Comparison was made by looking for perfect matches in window of 3 bp. However, no clear common sequence features were identified apart from some Alu elements (see below).

The ABL sequences around the breakpoints were also compared with BCR sequences flanking each corresponding breakpoint in the M-BCR. For patients AH and BY there was a short sequence at the BCR-ABL junction which did not appear to be derived from either the BCR or ABL genes. A single base pair difference at the junction was found in patient AH. Five base pairs of unknown sequence were identified at the junction in patient BY (Figure 6-4). For two patients, there was a short stretch sequence homology between BCR and ABL at the junction. In patient SL, 4 bp sequence (ATGG) occurs at the breaks in both BCR and ABL. Also, for patient RK, 5 bp sequence (TAAAC) appears at the junction which may have come from BCR or ABL. A similar situation was found for patient DM (see Chapter 5). This may indicate that the illegitimate recombination of BCR and ABL DNA is assisted by short region of sequence homology.

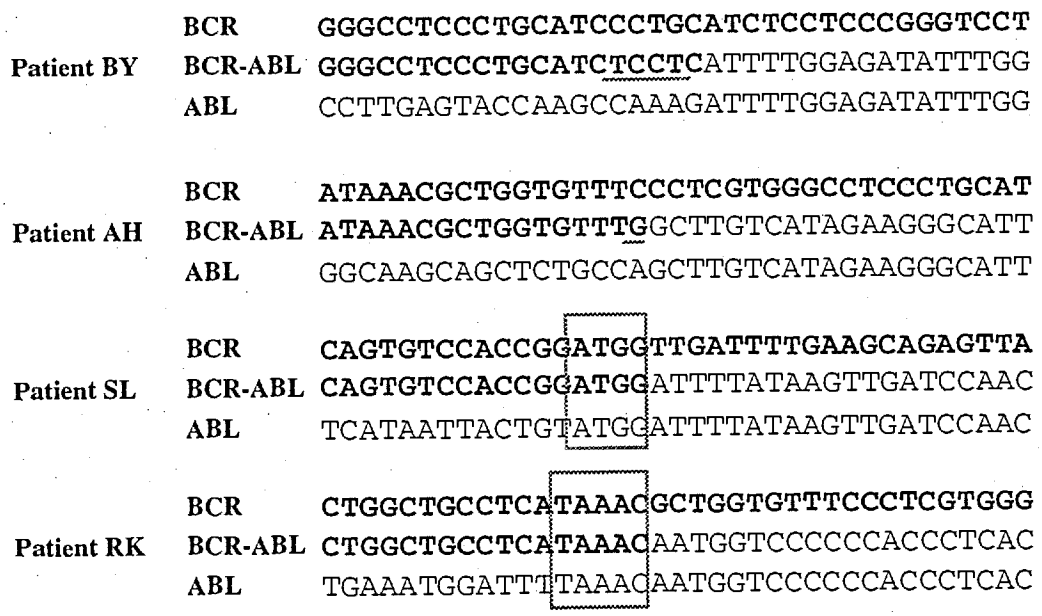


Figure 6-4. Comparison of the sequences at the junction between the BCR and ABL genes

BCR sequence is shown in bold text and ABL in plain text. Sequences of BCR-ABL underlined are not found in either BCR or ABL gene. The sequences of BCR-ABL in the boxes are found in both the BCR and ABL genes.

I also compared the sequences of the M-BCR and the PSR by using DNA Strider to determine if there was any sequence similarity in the vicinity of the breaks. No gross homology was found between the PSR and M-BCR regions except for the Alu elements. One breakpoint, CML 7701C, is situated in the one of the 28 Alu elements in the PSR but not in the Alu of the M-BCR. No MER elements (medium reiteration frequency interspersed repetitive elements; Jurka *et al* 1993) are present in the M-BCR whereas one copy of MER30 within the PSR was present approximately 100 bp upstream of the breakpoint in patient ALL FY.

6.2.2.2. The relationship of breakpoints to Alu elements

Three patients (SM, DM, and MT), had a breakpoint in the Alu element within the M-BCR. However, a further 12 patients had a breakpoint within 750 bp of the M-BCR Alu: patients, BM, SL, GM, AJ and GL, had a break just upstream of the M-BCR Alu (739 bp, 669 bp, 429 bp, 266 bp, 228 bp upstream respectively); patients, PS, BA, AE,

RK, AH, BY and CC had a breakpoint 61 bp, 184 bp, 212 bp, 275 bp, 285 bp, 307 bp and 392 bp respectively downstream of the Alu.

For 3 patients (patients DM, GM and MT) the BCR-ABL breakpoint was in or near an Alu sequence on both the 9q+ and 22q- derivative chromosomes (Figure 6-5). The breakpoint in patient DM fell into the Alu of the M-BCR at position 3,532 (Genbank, L02935) which corresponds to position 151 of the consensus bipartite Alu sequence (Kariya *et al* 1987). In ABL, the breakpoint was at position 50,658 in ABL2, 158 bp downstream of an Alu (located at positions 50,201 to 50,500; Genbank, U07562); this ABL Alu is in the opposite orientation (3' to 5') to the one in the M-BCR (Figure 6-5).

Patient GM had a break at position 2,952 of the M-BCR (Genbank, L02935) and at position 38,758 in ABL3. The breakpoint was located 429 bp upstream of the Alu in the M-BCR and 153 bp upstream of an Alu in ABL3. The Alu located in ABL is the same orientation as the one in the M-BCR. This Alu also contains two directly repeating monomers, from position 38,911 to 39,224 (Figure 6-5).

Patient MT is the only one whose breakpoint on the Ph chromosome was found to be flanked by Alus originating from both chromosomes 9 and 22 respectively. In the M-BCR the break was located within the Alu at position 3,646 (Genbank, L02935) corresponding to position 266 of the consensus Alu (Kariya *et al* 1987). However, it is 207 bp the upstream of a ABL2 Alu element. The Alu is in 5' to 3' orientation and located from position 44,541 to 44,814 of ABL2. Interestingly, there is a truncated Alu almost immediately downstream of the ABL2 Alu. The truncated Alu is lying the reverse direction to the former one and is 138 bp in length (from position 44,972 to 45,110). The distance between the two poly A tails of these Alu elements is 158 bp (Figure 6-5).

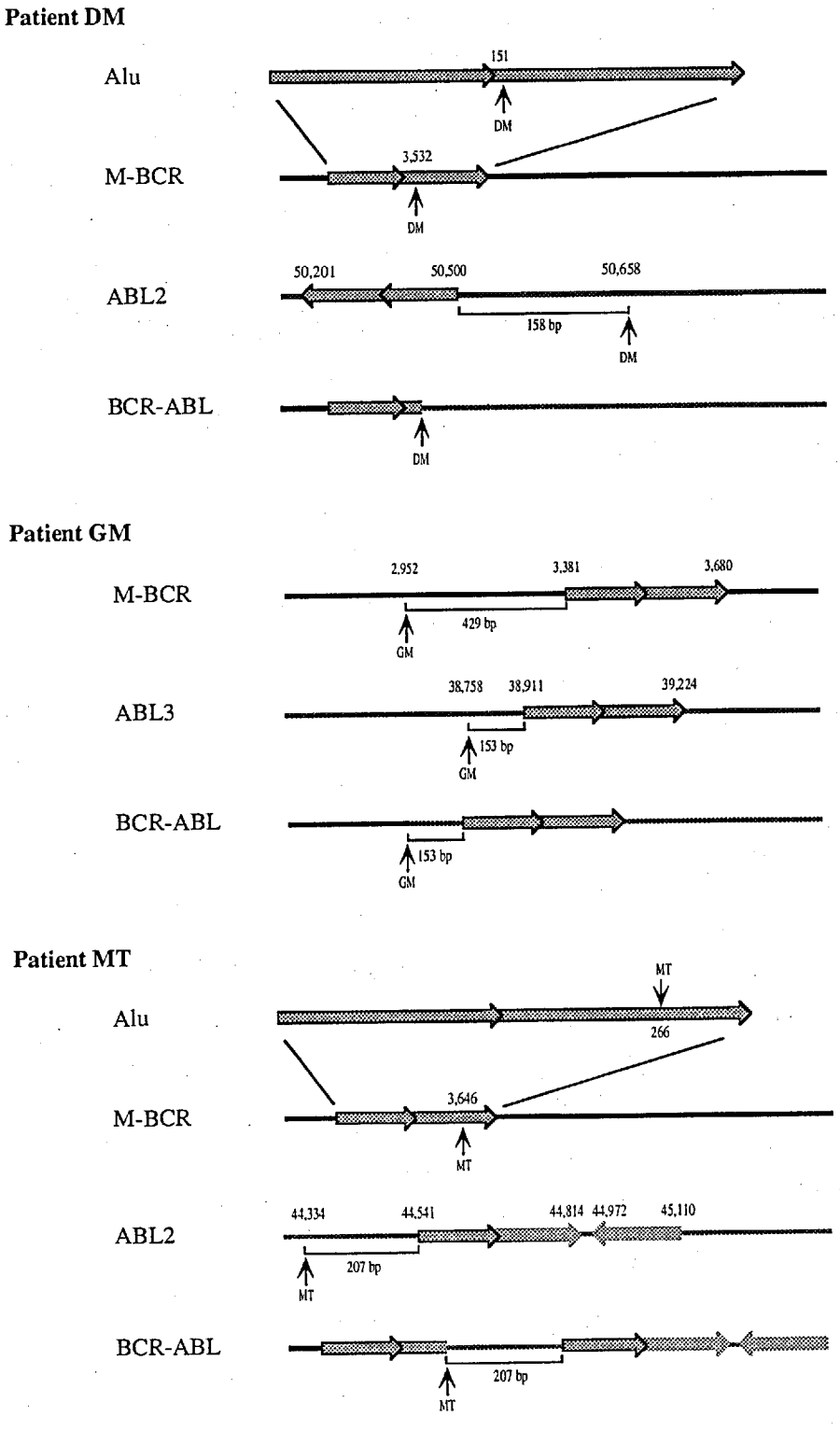


Figure 6-5. The positions of BCR-ABL breakpoints relative to Alu elements in the M-BCR and ABL for patients DM, GM and MT

The position of breakpoints is indicated by the nucleotide number. The consensus Alu element consists of two repetitive units (Kariya, et al., 1987). The position of the M-BCR breakpoint relative to the consensus Alu is also shown. An Alu arrow with solid edge indicates that the sequence has strong homology with the consensus Alu element and the one without solid edge illustrate that the sequence has weak homology with the consensus Alu. The orientation of all the sequences is from 5' to 3'.

6.2.2.3. Search for heptamer/nonamer sequences in ALL patients

The flanking sequences of the five ALL breakpoints (NA, FY, SUP-B13, BON and ALL1) were searched by me for any homology to heptamer/nonamer signal sequences in both the BCR and ABL genes. In only one (patient FY) of those patients, a typical heptamer signal sequence (CACAGTG) and a nonamer signal like sequence (ACAACAAAC) was found immediately upstream of the BCR breakpoint. No similar sequence features, however, were found in the flanking region of ABL. The flanking ABL sequence in SUP-B13 cell line contains nonamer like sequence (CCAAAAACT) located 13 bp upstream of the breakpoint and no similar sequence features were found in BCR. There were no clear heptamer/nonamer sequence features found in the close flanking area in the remaining patients with ALL (van der Feltz *et al*, 1989; Papadopoulos *et al* 1990; Chen *et al* 1989a; Chen *et al* 1989b; Soekarman *et al* 1990).

6.2.3. Localisation of the breakpoints in the vicinity of ABL exon a2 by Southern blotting

To determine if the ABL breakpoints are really clustered around ABL exon a2, I performed Southern blotting with a randomly selected sample of 64 CML patients. Samples from 62 (96.8%) patients were in chronic phase. The other two (3.2%) were from patients in blast crisis. All these patients were either Philadelphia chromosome positive or BCR-ABL positive by RT-PCR.

The 31 kb region between the Hind III site at position 19,504 to the EcoR I site at position 50,464 within ABL3 was analysed (Figure 6-6). This region encompasses the positions of all 13 PSR breakpoints (from the 5' breakpoint CML 83-H84 to the 3' ALL1) described above (Figure 6-3). The physical map of the PSR was determined by analysis of the sequence of ABL3. Four enzymes, BamH I, Hind III, Bgl II and EcoR I

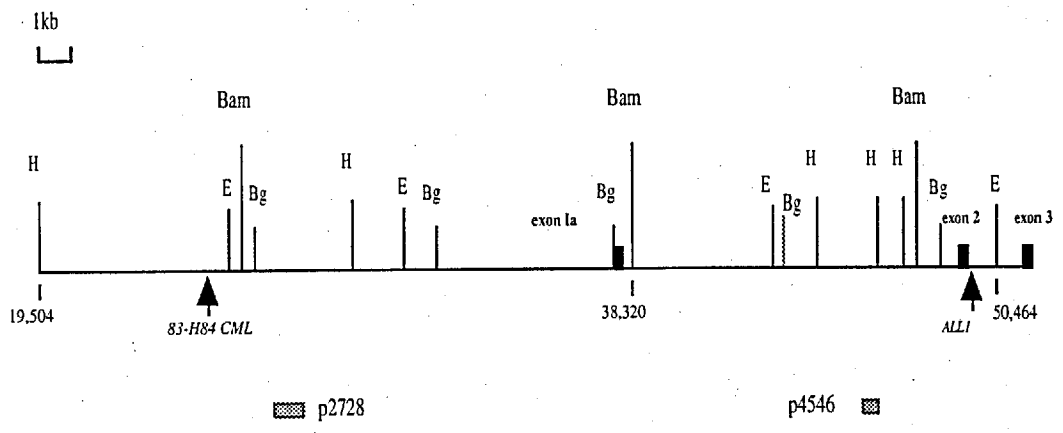


Figure 6-6. Putative Subcluster Region (PSR) in ABL3

Schematic organisation of the PSR, covering a region between the Hind III site (H) at position 19,504 and the EcoR I site (E) at position 50,464 of ABL3. The positions of the probes are shown as stippled boxes. Probe p2728 is located between positions 27,005 and 27,840; probe p4546 is situated between positions 45,647 and 46,329. The sites of the breakpoints identified at the 5' (83-H84 CML) and 3' (ALL1) ends of the PSR are illustrated by arrows. All restriction sites illustrated with a solid line are derived from the sequence of ABL3. A further possible Bgl II site (Bg) as suggested by my analysis is indicated by a dotted line. Bam represents BamH I. The scale is indicated. Black boxes indicate exons. Exon 2 represents the exon a2.

were selected to digest genomic DNA from the CML patients. Two probes, called p2728 and p4546, complementary to the 5' part and 3' part of the PSR respectively (Figure 6-6) were constructed as follows: first, the sequence of the PSR was compared to the Genbank database to identify regions of > 500 bp that were free of repetitive sequences and which may, therefore, make suitable probes. Two regions were selected, from position 27,005 to position 27,840 (835 bp) and from position 45,647 to 46,329 for probe 4546 (682 bp, Figure 6-6). Two sets of PCR primers (PSR1 and PSR2; PSR3 and PSR4) were designed with a few base mismatches to create restriction enzyme sites for EcoR I and BamH I (Table 6-2). PSR1 and PSR2 were used to amplify p2728, and PSR3 and PSR4 to amplify p4546 from normal human genomic DNA. PCR products were then digested with EcoR I and BamH I, gel purified and cloned into plasmid vector pEMBL8(+). The plasmid DNA was grown up in large scale culture, DNA extracted and the insert purified by gel electrophoresis after digestion with EcoR I and BamH I. Both probes were used simultaneously to probe DNA from 64 patients

digested independently with 4 restriction enzymes. An example of one of the Southern blots is shown on Figure 6-7.

Of the 64 patients studied, 12 (17%) had a rearrangement within this region (Table 6-3). Rearrangements were seen for 7 patients with just a single enzyme digest (2 patients with BamH I; 4 patients with Hind III; 1 patient with Bgl II). PSR rearrangement was seen with two enzyme digests for 4 patients (1 patient with BamH I and Bgl II; 1 patient with Hind III and EcoR I; 1 patient with Bgl II and EcoR I; and 1 patient with

Table 6-2. Primers for Making PSR Probes

Name	Sequence
PSR1	GGCACgAatTCGTAGGAGGGGTGGT
PSR2	TGACAgGaTCCTTTTGTTTAAAATGTTG
PSR3	GTCAAGAATtCTCTGCCTCACAGT
PSR4	AATGTGGAtccGTGATTAGAGCTTGA

Note: the letters in lower case denote base changes that were introduced in order to generate restriction enzyme recognition sites for cloning.

BamH I and EcoR I). The remaining one patient had a rearrangement with three enzyme digests (BamH I, Bgl II and EcoR I). Due to the relatively large size of the PSR and the fact that only two probes were used, rearrangements with just a single restriction enzyme are not surprising (see below). However, the appearance of an extra band could be due to a polymorphism, rather than a rearrangement. Of the 7 patients who had an apparent rearrangement with just one enzyme, the size of the novel band was different in five. These are unlikely, therefore, to be due to polymorphisms. The remaining two patients had similar size rearranged bands after digestion with Hind III. It is possible that there were polymorphisms, although it is also possible that there were genuine rearrangements that happened by chance to result in similar size Hind III bands. The results for all patients are summarised on Table 6-3. Figure 6-8 shows an example of a rearrangement detected in the PSR with two restriction enzymes for patient 1310.

Table 6-3. Southern blot results for the PSR

Patients		Phase	results	PSR	Patients		Phase	results	PSR
1.	1527	CP	Bm- H- Bg- Ec-		33	p335	CP	Bm- H- Bg- Ec-	
2.	1653	CP	Bm- H- Bg- Ec-		34.	p229	CP	Bm- H- Bg- Ec-	
3.	1839	CP	Bm- H- Bg- Ec-		35.	1268	CP	Bm+ H- Bg- Ec-	R
4.	1513	CP	Bm- H- Bg- Ec-		36.	1828	CP	Bm- H- Bg- Ec-	
5.	1440	CP	Bm- H- Bg- Ec-		37.	1739	CP	Bm- H- Bg- Ec-	
6.	1479	CP	Bm- H- Bg- Ec-		38.	1858	CP	Bm- H- Bg- Ec-	
7.	1695	CP	Bm- H- Bg- Ec-		39.	1545	CP	Bm- H- Bg- Ec-	
8.	1709	CP	Bm- H- Bg- Ec-		40.	p14	BC	Bm+ H- Bg+ Ec+	R
9.	1460	CP	Bm- H- Bg- Ec-		41.	1103	CP	Bm- H- Bg- Ec-	
10.	1349	CP	Bm- H- Bg- Ec-		42.	1310	CP	Bm- H- Bg+ Ec+	R
11.	1396	CP	Bm- H- Bg- Ec-		43.	1380	CP	Bm- H- Bg- Ec-	
12.	1494	CP	Bm- H- Bg- Ec-		44.	1685	CP	Bm- H- Bg- Ec-	
13.	1572	CP	Bm- H- Bg- Ec-		45.	p200	CP	Bm- H- Bg- Ec-	
14.	1538	CP	Bm- H- Bg- Ec-		46.	p163	CP	Bm- H+ Bg- Ec-	R
15.	1578	CP	Bm- H+ Bg- Ec-	R	47.	1810	CP	Bm- H- Bg- Ec-	
16.	1716	CP	Bm- H+ Bg- Ec+	R	48.	1344	CP	Bm- H- Bg- Ec-	
17.	1633	CP	Bm- H- Bg- Ec-		49.	768	CP	Bm+ H- Bg+ Ec-	R
18.	1629	CP	Bm- H+ Bg- Ec-	R	50.	d576	CP	Bm- H- Bg- Ec-	
19.	1563	CP	Bm- H- Bg- Ec-		51.	d560	CP	Bm- H- Bg- Ec-	
20.	2028	CP	Bm- H- Bg- Ec-		52.	d584	CP	Bm+ H- Bg- Ec-	R
21.	1625	CP	Bm+ H- Bg- Ec+	R	53.	d565	CP	Bm- H- Bg- Ec-	
22.	1798	CP	Bm- H- Bg- Ec-		54.	d593	CP	Bm- H- Bg- Ec-	
23.	p109	CP	Bm- H- Bg- Ec-		55.	d595	CP	Bm- H- Bg- Ec-	
24.	p111	CP	Bm- H- Bg- Ec-		56.	d587	CP	Bm- H- Bg- Ec-	
25.	p332	BC	Bm- H- Bg- Ec-		57.	783	CP	Bm- H- Bg- Ec-	
26.	p189	CP	Bm- H+ Bg- Ec-	R	58.	d757	CP	Bm- H- Bg- Ec-	
27.	1718	CP	Bm- H- Bg- Ec-		59.	746	CP	Bm- H- Bg- Ec-	
28.	1728	CP	Bm- H- Bg+ Ec-	R	60.	p37	CP	Bm- H- Bg- Ec-	
29.	1457	CP	Bm- H- Bg- Ec-		61.	746	CP	Bm- H- Bg- Ec-	
30.	1445	CP	Bm- H- Bg- Ec-		62.	p37	CP	Bm- H- Bg- Ec-	
31.	p191	CP	Bm- H- Bg- Ec-		63.	1502	CP	Bm- H- Bg- Ec-	
32.	p340	CP	Bm- H- Bg- Ec-		64.	p169	CP	Bm- H- Bg- Ec-	

Note: CR: chronic phase; BC: blast crisis; R: rearranged PSR. All patients listed in the table are either Philadelphia chromosome positive or BCR-ABL positive. Bm: BamH I; H: Hind III; Bg: Bgl II; Ec: EcoR I. A '-' indicates no rearranged band was detected for that enzyme; a '+' indictes that a rearranged band was detected.

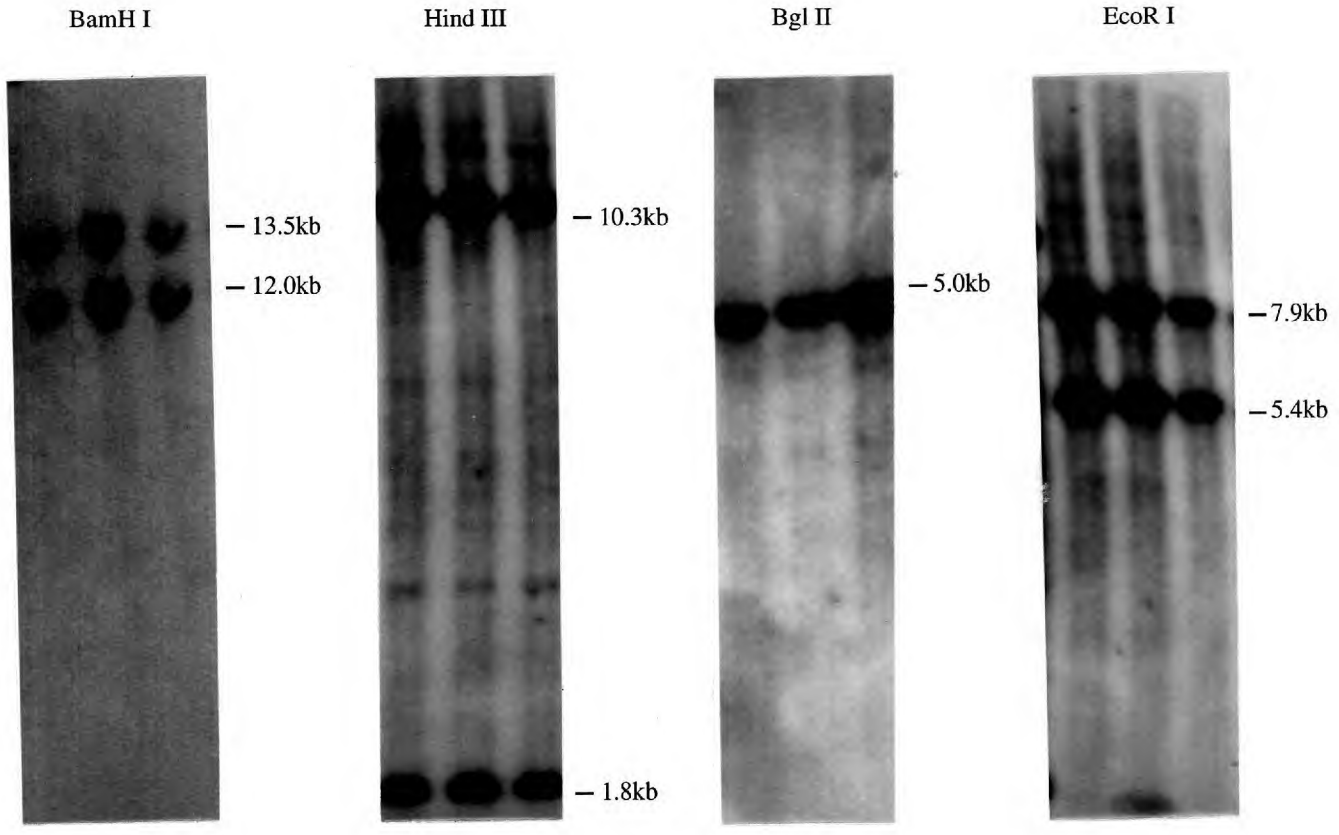


Figure 6-7. Southern blotting for the PSR

Four restriction enzymes (BamH I, Hind III, Bgl II and EcoR I) were used to digest patient genomic DNA. Probes p2728 and p4546 were simultaneously used for hybridisation. The size of the fragments is indicated.

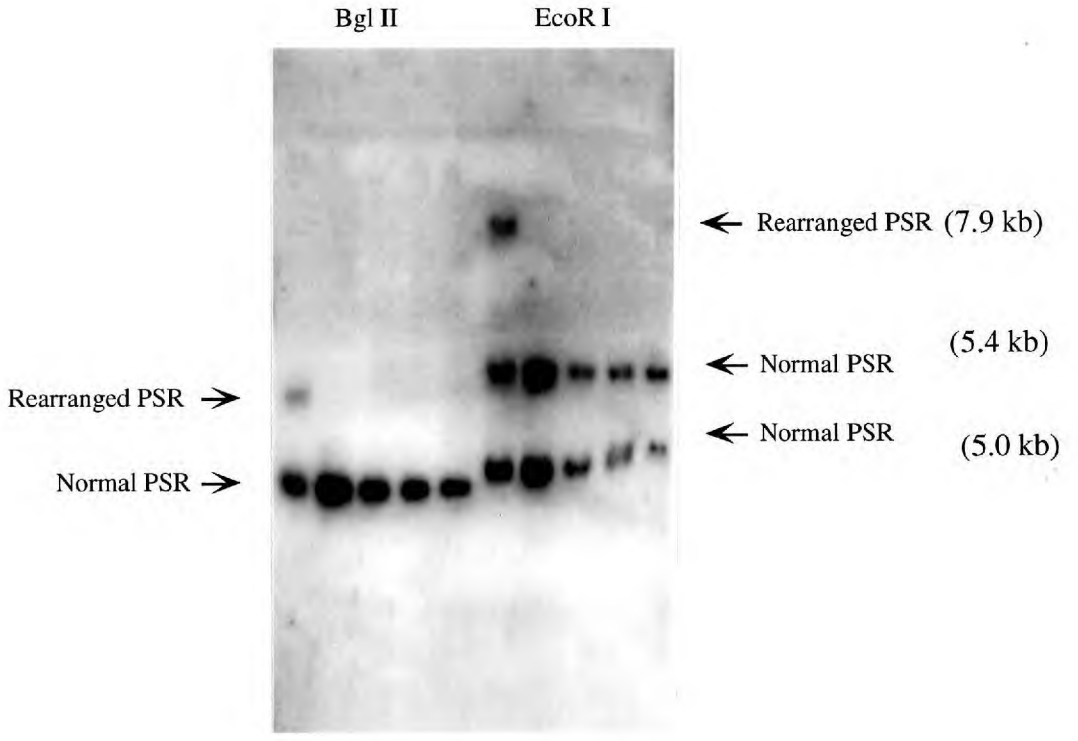


Figure 6-8. Genomic rearrangement in the PSR of patient 1310

The germline bands after Bgl II digestion and probing with p2728 and p4546, from normal individuals and CML patients, were similar in size, about 5 kb. The fragments co-migrated on the gels, indicated by the fact that the intensity of the signal was consistently stronger than that found for other bands (Figure 6-9). The predicted sizes of the two bands from the sequence of ABL3, however, was 5 kb and 10 kb respectively. The size of the fragments after BamH I digestion were also somewhat different from that expected (13.5 kb and 12 kb vs 12 kb and 9 kb). It is possible, therefore, that the sequence of ABL3 may not be entirely representative of the normal allele.

Seven patients (BM, BO, CC, GL, ML, PS and SM) with BCR-ABL breakpoints that were not localised to known ABL sequence were blotted and hybridised with the probes p2728 and p4546. As expected, no rearranged bands were detected. Similarly all five

patients (SL, BY, MT, DM and AH) who had BCR-ABL breakpoints in ABL2 showed no rearranged bands in the PSR. Patient BA, whose BCR-ABL breakpoint had been localised to the PSR, showed a rearrangement when probed by p4546 after BamH I digestion but not after digestion any other enzyme. The other three patients who had a break in the PSR, as determined by Bubble PCR, were not tested by Southern blotting.

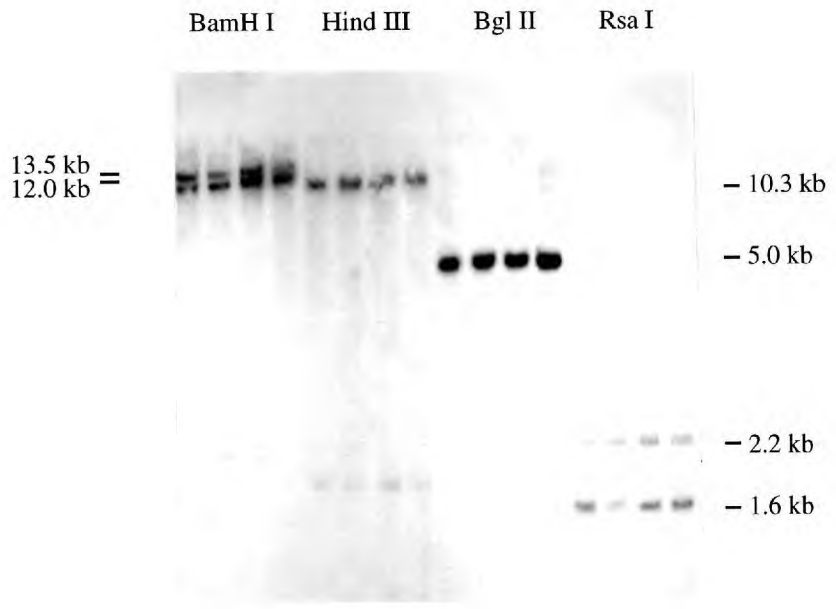


Figure 6-9. The 5' and 3' Bgl II fragments comigrate on the gel

Patient genomic DNA was digested and hybridised with probes p2728 and p4546. The intensity of the Bgl II bands were stronger than the other bands digested by BamH I, Hind III and Rsa I, suggesting that the 5' and 3' Bgl II fragments were comigrating on the gel.

6.2.4. Statistical analysis

6.2.4.1. Analysis of distribution of the known breakpoints in the PSR

Statistical analysis was performed to determine if there was a genuine clustering of breakpoints in the PSR. Initially, a random distribution throughout the relevant region of the ABL gene was assumed. The ABL breakpoint cluster region (ABCR), i.e. the

total region within which the chromosome 9 breakpoints fall, is defined here as the region from the most 5' known breakpoint (patient 02120185 at position 22,910 of the ABL1) to the most 3' breakpoint (patient ALL1 at position 50,083 of ABL3). The size of the ABCR is 192 kb if the length of the gaps between the known ABL sequences is considered to be 70 kb (Chissoe *et al* 1995). Since only a relatively small number of breakpoints have been determined accurately, this figure is likely to be an underestimate of the size of the ABCR, indeed it is known that some breaks may occur upstream of ABL exon Ib (Morris *et al* 1991).

In the sample of 24 breakpoints which has been sequenced, the probability of each breakpoint falling into the PSR can be calculated according to the Binomial distribution, The probability of a breakpoint randomly falling into the PSR is p = 31 kb/192 kb = 0.16. So the probability of a break falling into outside of the PSR is q = 1 - p = 1 - 0.16 = 0.84. The mean and standard deviation of the distribution of the breakpoints are given by:

$$\mu = Np = 24 \times 0.16 = 3.84$$
$$\sigma = \sqrt{Npq} = \sqrt{24 \times 0.16 \times 0.84} = \sqrt{3.2256} = 1.80$$

under the hypothesis that the break is randomly distributed, then

$$Z = (x - \mu) / \sigma = (x - 3.84) / 1.80$$

At Z = 1.96 (0.05 significance level), then $(x - 3.84)/1.80 = 1.96$ and $x = 7.37$

At Z = -1.96 (0.05 significant level), then $(x - 3.84)/1.80 = -1.96$ and $x = 0.31$

Therefore, if the breakpoints in the PSR are randomly distributed, number of the breaks from the 24 patients is expected to be between 0.31 and 7.37. However, in fact, the breakpoints of 13 patients are located in the PSR, suggesting that the breakpoints are significantly clustered in this region.

6.2.4.2. Analysis of distribution of the breakpoints localised by Southern blotting

In the Southern blotting analysis of the 64 randomly selected patients, 12 (19%) fell in the PSR. According to the Binomial distribution, the mean, standard deviation of the distribution of the breakpoints for the population studied by Southern blotting are given by:

$$\mu = Np = 64 \times 0.16 = 10.24$$
$$\sigma = \sqrt{Npq} = \sqrt{64 \times 0.16 \times 0.84} = \sqrt{8.6016} = 2.93$$

under the hypothesis that the break is randomly distributed, then

$$Z = (x - \mu) / \sigma = (x - 10.24) / 2.93$$

At $Z = 1.96$, then $(x - 10.24) / 2.93 = 1.96$ and $x = 15.98$

At $Z = -1.96$, then $(x - 10.24) / 2.93 = -1.96$ and $x = 4.50$

In other words, in a population of 64 patients between 4.5 and 15.98 patients would be expected to fall in the PSR if the distribution of breaks was random. I found that breakpoints in twelve patients fell in the PSR by Southern analysis (Table 6-3) suggesting that there is not a real subclustering in this region.

6.2.5. Comparison of the breakpoint positions in BCR and ABL

A comparison of the positions of the breaks in BCR and ABL was carried out. In CML, of the 13 breakpoints within the M-BCR identified by other groups (Table 6-4), 8 were also localised to ABL (Heisterkamp *et al* 1983; Groffen *et al* 1984; Heisterkamp *et al* 1985; Grosveld *et al* 1986; de Klein *et al* 1986; Litz *et al* 1993). Of the 17 patients for whom the position of the breakpoint was localised within the M-BCR in my studies

(Table 6-4), 10 were also localised to ABL. Therefore, the position of the breakpoints can be compared between the M-BCR and ABL for a total of 18 CML patients. In Ph chromosome positive ALL, the breakpoints within BCR and ABL have been determined for five cases, four of whom had a break in the m-BCR and one in the M-BCR (van der Feltz *et al* 1989; Chen *et al* 1989a; Chen *et al* 1989b; Soekarman *et al* 1990; Papadopoulos *et al* 1990). The BCR-ABL breakpoint in one patient with AML expressing p190 BCR-ABL has also been defined (Chen *et al* 1989b).

Table 6-4. BCR-ABL breakpoints localised in the BCR gene

Breakpoint	Region	Patient	References
55,654-55657	m-BCR	ALL NA	Chen, *et al.*, 1989b
69,807-69,812	m-BCR	ALL FY	Van der Feltz, *et al* ., 1989
70,639-70,640	m-BCR	ALL SUP-B13	Papadopoulos, *et al.*, 1990
73,993-73,994	m-BCR	AML SA	Chen, *et al.*, 1989b
74,656-74,657	m-BCR	ALL BON	Chen, *et al.*, 1989a
123,801-123,802	M-BCR	BO	
123,807-123,808	M-BCR	ML	
123,873-123,874	M-BCR	CML 0319129	Heisterkamp, *et al.*, 1983; Groffen, *et al.*,1984; Heisterkamp, et al., 1985
124,033-124034	M-BCR	CML 0311068	Heisterkamp, *et al.*, 1983; Groffen, *et al.*, 1984; Grosveld, *et al.*, 1986
124,243-124,246	M-BCR	ALL1	Soekarman, *et al.*, 1990
124,256-124,257	M-BCR	CML 7701C	Groffen, *et al.*, 1984
124,263-124,264	M-BCR	BM	
124,334-124,335	M-BCR	SL	
124,384-124,385	M-BCR	CML 02120185	Heisterkamp, *et al.*, 1983; Groffen, *et al* ., 1984; Heisterkamp, *et al.*, 1985
124,471-124,472	M-BCR	CMLA	Litz, *et al.*, 1993
124,573-124,574	M-BCR	GM	
124,586-124,587	M-BCR	ASS 3542	Sowerby, *et al.*, 1993
124,633-124,257	M-BCR	CML K562	Heisterkamp, *et al.*, 1983; Groffen, *et al* ., 1984
124,736-124,737	M-BCR	AJ	

124,774-124,775	M-BCR	GL	
124,960-125,047	M-BCR	CML 83-H84	De Klein, *et al* ., 1986
125,039-125,040	M-BCR	SM	
125,149-125,150	M-BCR	CML1	Mills, *et al.*, 1992
125,154-125,155	M-BCR	DM	
125,212-125,213	M-BCR	CML2	Mills, *et al.*, 1992
125,226-125,227	M-BCR	CML3	Mills, *et al.*, 1992
125,268-125,269	M-BCR	MT	
125,363-125,364	M-BCR	PS	
125,486-125,487	M-BCR	BA	
125,514-125,515	M-BCR	AE	
125,535-125,536	M-BCR	AFU 8766	Sowerby, *et al.*, 1993
125,577-125,578	M-BCR	RK	
125,587-125,588	M-BCR	AH	
125,609-125,610	M-BCR	BY	
125,694-125,695	M-BCR	CC	
126,049-126,050	M-BCR	CML ICB1	Groffen, *et al.*, 1984

Note: the breakpoints without references were characterised in my study.

The position of BCR-ABL breakpoints in the BCR gene was individually compared to the corresponding position of the breakpoints in the ABL gene (Table 6-1; Table 6-4). The relationship between the breakpoint distributions is shown in Figure 6-10. Inspection of Figure 6-10 reveals a possible association between the positions of the breakpoints within BCR and ABL. Many of the breakpoints appear to cluster together and, to simplify the discussion below, I define here six relative positions (A-F), as shown on Figure 6-10.

The breakpoint in patient 02120185 is located in position A. The breakpoints in patients SL, 0311068 and cell line K562 are grouped as position B. Position C includes six patients (patients ICB1, BY, AE, MT, DM and AH). The largest group in this population is in position D, which contains 9 patients (patients 83-H84, 7701C, CML

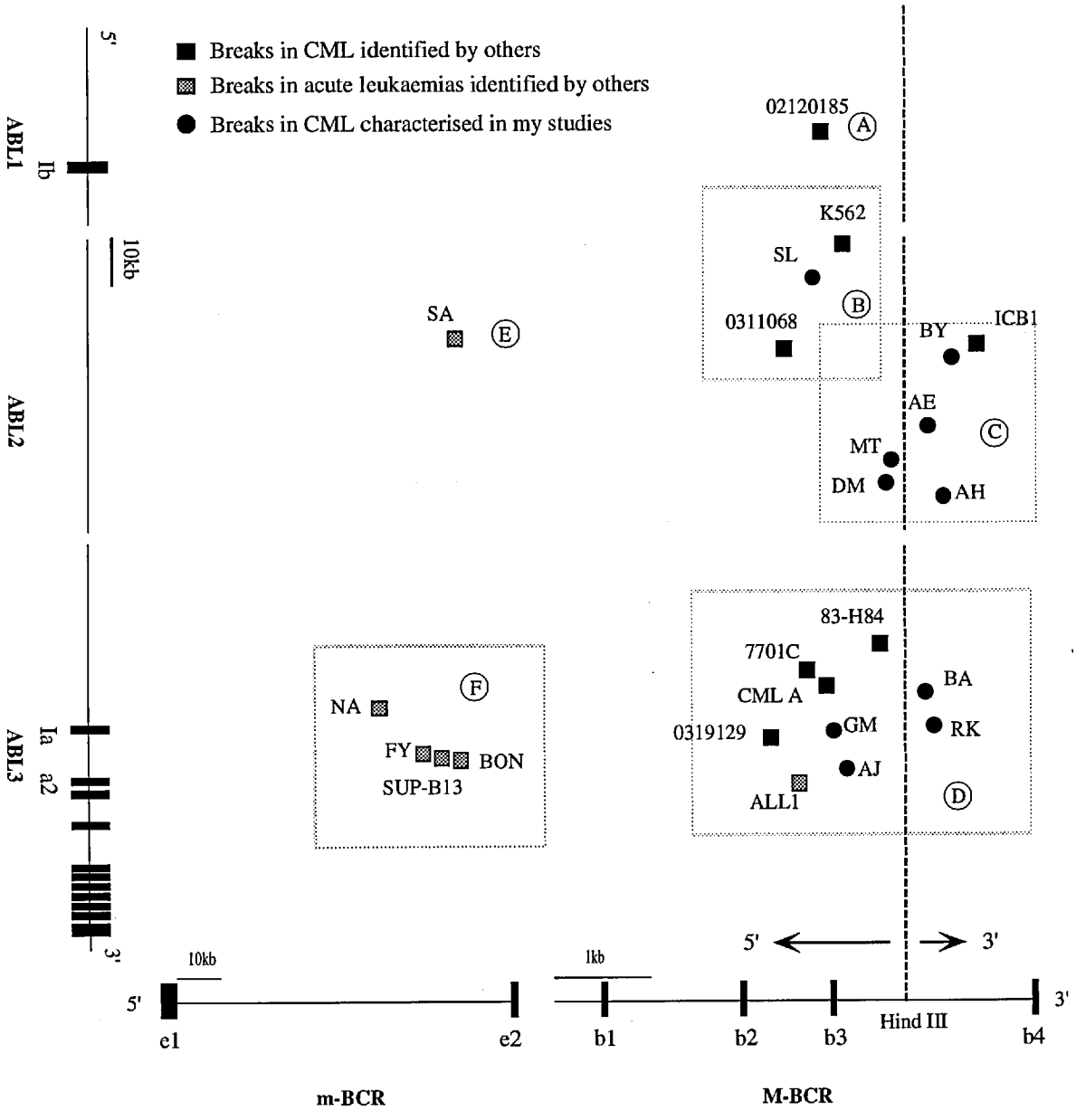


Figure 6-10. Scatter diagram of the BCR-ABL breakpoints in the Philadelphia chromosome.

The horizontal lines represent the m-BCR and M-BCR; the vertical lines are ABL. The filled circles indicate the breaks characterised in my study; the filled squares are the breakpoints published by other groups in CML and the stippled squares are breakpoints found in acute leukaemias. The solid rectangles represent the exons in the BCR and the ABL genes. The scale for each segment is indicated. The positions of breakpoints are indicated by the circled letters A-F.

A, GM, 0319129, AJ, ALL1, BA and RK). In this group one patient had ALL (patient ALL1). The breakpoint in patients SA and patient 02120185 were relatively isolated (positions E and A respectively). The remaining breakpoints from ALL patients (patients NA, FY and BON) are located in position F; this last group also includes the ALL cell line SUP-B13 (Rubin *et al* 1988; Papadopoulos *et al* 1990).

6.3. Discussion

6.3.1. Analysis of breakpoint subclusters by Southern blotting and sequencing

6.3.1.1. Subclustering of breakpoints within ABL determined by sequencing but not Southern blotting

Analysis of the breakpoints within the ABL gene suggested a significant cluster around ABL exon a2, a region I have termed the putative subcluster region or PSR. From the literature, 14 BCR-ABL breakpoints have been identified by other groups. Nine (64%) out of those breakpoints were located a region around exon a2 in ABL, about 25 kb in length. If the breakpoints characterised in my study are included, then a total of 13 (54%) out of 24 breaks are situated in this region (Figure 6-3). This number is significantly larger than the maximum that would have been expected to occur if the frequency of breaks in the PSR was random and simply related to the length of this region.

However, according to data from Southern blot in my study 12 out of 64 patients (Table 6-3) were found to have a rearrangement within the 31 kb PSR (encompassing the 25 kb region) around exon a2. The size of the PSR analysed by Southern blotting was larger due to the restriction imposed by convenient enzyme recognition sites. The Southern blot data suggests that there is not, in fact, a significant subclustering within this region, i.e., the opposite to the result obtained from sequencing. There are two

possible explanations for this discrepancy: i) the Southern blot analysis failed to detect some patients who did in fact have a breakpoint in this region, or ii) the number of patients analysed by sequencing was relatively small and may not be a representative population of patients.

6.3.1.2. Potential false negative results by Southern analysis

Several possibilities could lead to false negativity in detection or rearrangements by Southern blotting. Southern blotting can fail to detect rearranged bands if they are so large that they do not transfer efficiently, if they are so small that they run off the gel, or if they are co-incidentally the same size as the germline band. In a recent analysis of 94 CML patients at or close to diagnosis (Reiter *et al* 1997), 93% had a rearrangement detectable after Bgl II digestion; the remaining patients had rearrangements detected with other restriction enzymes. This means that if a single restriction enzyme is used, approximately 7% of breakpoints will be missed. Although I used four enzymes and two probes, some parts of the PSR were only covered by one enzyme and one probe (Figure 6-11). The length of the regions covered by only one enzyme was expected to be 8,634 bp, according to the ABL3 sequence (Genbank, U07563). In comparison to the whole PSR (30,960 bp), 28% should be covered by a single enzyme. One of these regions is at the 5' end of the PSR and the other located at the 3' end (Figure 6-11). The entire region between the breakpoints 83-H84 and ALL1, i.e. the region containing all the known PSR breakpoints should have been covered by at least two enzymes.

However, in fact, there was a gap located in the central region of the PSR between the two Bgl II fragments recognised by p2728 and p4546. This gap was not predicted from sequence analysis of the PSR. After hybridisation with probe p4546 I found that the 3' Bgl II fragment, expected to be 10 kb in size, was in fact only 5 kb. This band actually co-migrated with the 5' Bgl II fragment, predicted to be 5,027 bp in length (Figure 6-9). If the most 3' Bgl II site (position 48,076 of ABL3) was correct, then the unexpected

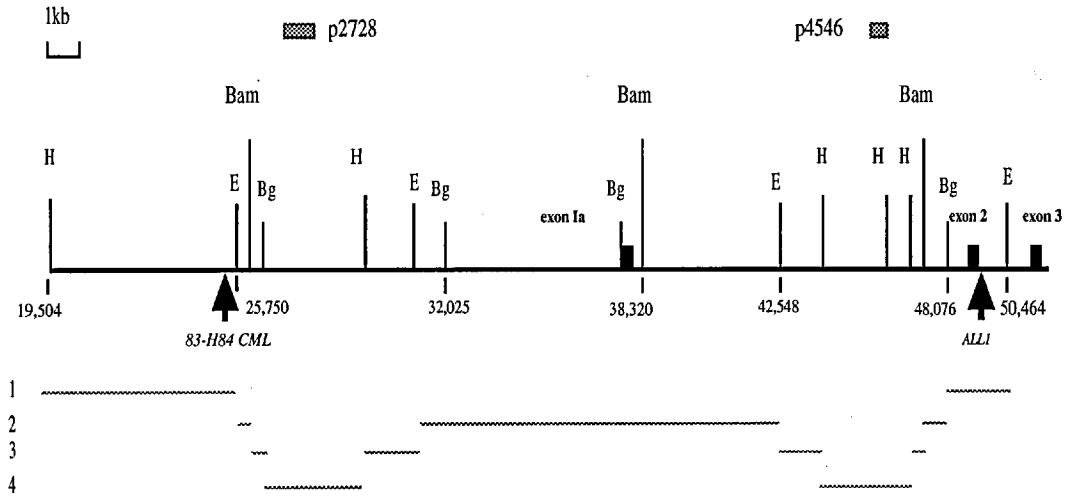


Figure 6-11. Expected coverage of the PSR by Southern analysis

The position and length of regions expected to be covered with one, two, three and four enzymes are indicated by a horizontal dotted lines. 1, 2, 3 and 4 represent number of the enzymes covering each segment. The size of each fragment represented in this map is derived from the BamH I, Hind III, Bgl II and EcoR I sites in the sequence of ABL3. The other symbols indicated are the same as Figure 6-6.

Bgl II site must have been situated somewhere around position 43,000 (Figure 6-12). This means that there was a gap from position 32,025 up to position 42,548 (about 11 kb), which was not analysed after Bgl II digestion. Most of this area was also not probed after either EcoR I or Hind III digestion (Figure 6-12). Although the DNA in this gap can be analysed by BamH I digestion, some breakpoints are still missed after a single enzyme digest for the reasons discussed above. Therefore, three regions totalling 19,157 bp (about 62%) of the 30,960 bp PSR were actually covered with only one restriction enzyme. It is possible, therefore, that the number of patients with a rearrangement in the PSR as determined by Southern blotting is an underestimate.

The whole ABL breakpoint cluster region is approximately 192 kb in length. This is the distance between the most 5' and 3' breaks that have been unambiguously determined, and assumes that the sum of the gaps between ABL1 and ABL2, and between ABL2 and ABL3 is 70 kb (see section 6.2.4.1). The size of this region is likely to be an

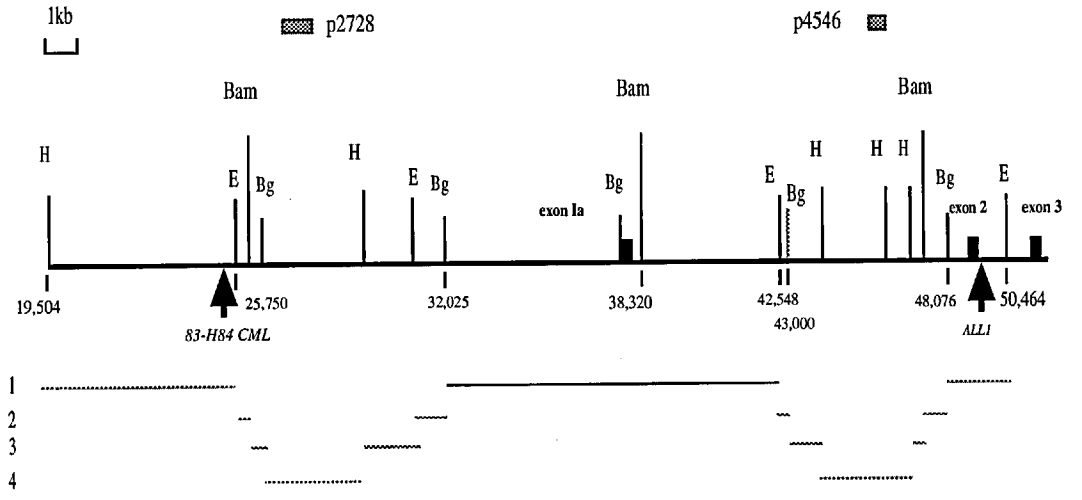


Figure 6-12. Actual coverage of the PSR by Southern analysis

An unexpected Bgl II site is represented by a dotted line. The position and length of the unexpected region covered with BamH I alone is indicated by a horizontal solid line. The other symbols indicated are the same as Figure 6-11.

underestimate, since it is unclear how far upstream of exon Ib that breaks may occur. Furthermore, if the missing sequence in the gaps between ABL1 and ABL2, and ABL2 and ABL3 was in fact 150 kb rather than the estimated 70 kb, then the finding of a rearrangement in 12/64 patients within the PSR by Southern blotting would actually indicate a statistically significant subcluster. Therefore, the accuracy of length of the missing portion in ABL gene is an important factor in deciding whether there really is a subcluster or not. The previous reports had estimated that the approximate length of the first intron region is about 175 kb between exon Ib and exon Ia by using PFGE analysis (Bernards *et al* 1987; Jiang *et al* 1990). The sequences available within this region now total about 110 kb in length (Chissoe *et al* 1995). According to the available data, it is unlikely that the missing length is as much as 150 kb, although it is possible that the PFGE analysis was misleading, for example, if there had been some bands that co-migrated on the gels, leading to an underestimate of the total size.

6.3.1.3. Possible biases in the groups of patients analysed

Considering the patients who were analysed by Bubble PCR in my study, patients were selected initially because they were due to have, or had already undergone, allogeneic BMT. Eligibility for BMT is made on the basis of age (< 55 years of the Hammersmith) and availability of a related or unrelated donor. It is unlikely that any of these parameters are related to the positions of the breakpoints unless perhaps the position is significantly related to the course of disease. If, for example, certain breakpoint position resulted in a highly aggressive disease, these individuals may not have been considered for BMT, or may have died before this option was considered. As mentioned above, however, there is no clear consensus whether the position of breakpoints within the M-BCR is or is not associated with the clinical course. Whether there is any association between the position of the breaks in ABL and any clinical parameter is unknown. The successful amplification of breakpoints by Bubble PCR is dependent on a Rsa I site in ABL within roughly 1 kb downstream of the breakpoint on the chromosome 22q. The distribution of Rsa I sites in the human genome was estimated from some genes randomly selected from Genbank database, and about 50% of Rsa I fragment were smaller than 1 kb (see Chapter 4). However, in some region of the genes the size of the fragments were quite large, for example, more than 2 kb.

The frequency of Rsa I site in non-coding regions depends largely on the composition of the DNA sequence. The frequency of the recognition site will become rarer if percentage of G-C pairs deviates from 50% (see Chapter 4). However, no grossly skewed G-C content distribution was found in the sequence of ABL1, 2 or 3 and therefore it is unlikely that the requirement for a Rsa I site relatively close to the breakpoint would significantly bias the overall distribution of breakpoints that were determined. In my Southern blotting analysis, DNA was extracted either from patients at presentation or after cryopreservation prior to BMT. As above, a significant bias

would be expected therefore only if positions of the breakpoints were very significantly related to the course of the disease.

Turning now to consider those patients whose breakpoints were characterised by other groups, it is possible that a bias was introduced since a particular probe was necessary to detect and isolate the rearranged DNA fragments from a genomic library. The probes used to identify and isolate the positive clones would highly restrict the patients that would be successfully analysed to those with a breakpoint within a region around the probe.

The breakpoint in patient 0319129 was identified with v-abl probe 1.7. The breaks in the other five patients (7701C, 83-H84, 0311068, ICB1 and 02120185) characterised by this group (Heisterkamp *et al* 1983; Groffen *et al* 1984; Heisterkamp *et al* 1985; Grosveld *et al* 1986; de Klein *et al* 1986) were determined using M-BCR probes. The break in cell line K562 was initially identified with a v-abl probe as well by Southern blotting (Heisterkamp *et al* 1983) and later was localised to a region that is not close to the PSR (Grosveld *et al* 1986; Chissoe *et al* 1995). The breakpoint in patient CML A was identified with BCR probes and its location in ABL was subsequently characterised by inverse PCR (Litz *et al* 1993). For the patients with AML or ALL, three out of six were characterised with an ABL probe (van der Feltz *et al* 1989; Papadopoulos *et al* 1990 and Soekarman *et al* 1990) and the others were initially identified with BCR probes (Chen *et al* 1989a; Chen *et al* 1989b). For patients NA and BON the position of the breaks in the BCR gene was first localised by Southern blotting and Northern blotting, and BCR probes were isolated to screen patient genomic libraries. The position of the break in ABL was then characterised after isolating, cloning and sequencing chromosome 9q+ and 22q- derived clones (Chen *et al* 1989c; Chen *et al* 1989a; Chen *et al* 1989b). The breakpoint characterised in cell line SUP-B13 was also randomly selected by a PFGE approach although ABL probes had been used (Papadopoulos *et al* 1990; Rubin *et al* 1988). The breakpoints in patient FY and ALL1

were selected on the basis of ABL probes (van der Feltz *et al* 1988; Soekarman *et al* 1990). However, AML SA breakpoint was identified without selection for ABL (Chen *et al* 1989b). In total, of the 14 BCR-ABL breakpoints identified by other groups, 5 were selected by use of ABL probes. Four of those five patients actually had a break in the PSR. If these five patients are excluded, then 9/19 (47%) unselected patients had a break in the PSR, which still indicates a significant subcluster (see 6.2.4.1).

6.3.2. Comparison of PFGE results with sequencing data

My study has suggested a clustering of breakpoints in the PSR, but not anywhere else in ABL. A previous report had described three regions of breakpoint clusters between exons Ib and Ia, which were localised by PFGE (Jiang *et al* 1990). In that study, the first cluster α was located 30 kb downstream of exon Ib and was about 10 kb in size. The second cluster β was situated approximately 90 kb downstream of exon Ib and spanned about 25 kb. The last cluster γ was about 130 kb downstream of exon Ib, or 30 kb upstream of exon Ia. This cluster spanned about 15 kb (Figure 6-13B). Of the 37 patients analysed by Jiang *et al*, 16 (43%) patients fell into cluster α, 9 (24%) into cluster β and 8 (22%) into cluster γ. For the remaining 4 (11%) patients no rearrangements were detected. No rearrangements were found in the 3' region of intron 1 near exon Ia. No rearrangements were seen between exons Ia and a2 in that study (Jiang *et al*, 1990).

It is possible to make an approximate comparison between the PFGE map and the position of ABL1, 2 and 3 (Chissoe *et al* 1995; Jiang *et al* 1990 and Bernard *et al* 1987). The position of cluster α defined by PFGE, where the breakpoints were rather condensed, should fall into ABL2 (Figure 6-13) because 33 out of the 37 patients all had a band that cohybridised with probes pHabl5', T39-1-2 and 3'-BCR (Figure 6-14; Figure 6-15). Probe T39-1-2 was known to be derived from a region immediately 3' of the breakpoint in the cell line K562 (Jiang *et al* 1990). Cluster α should be located

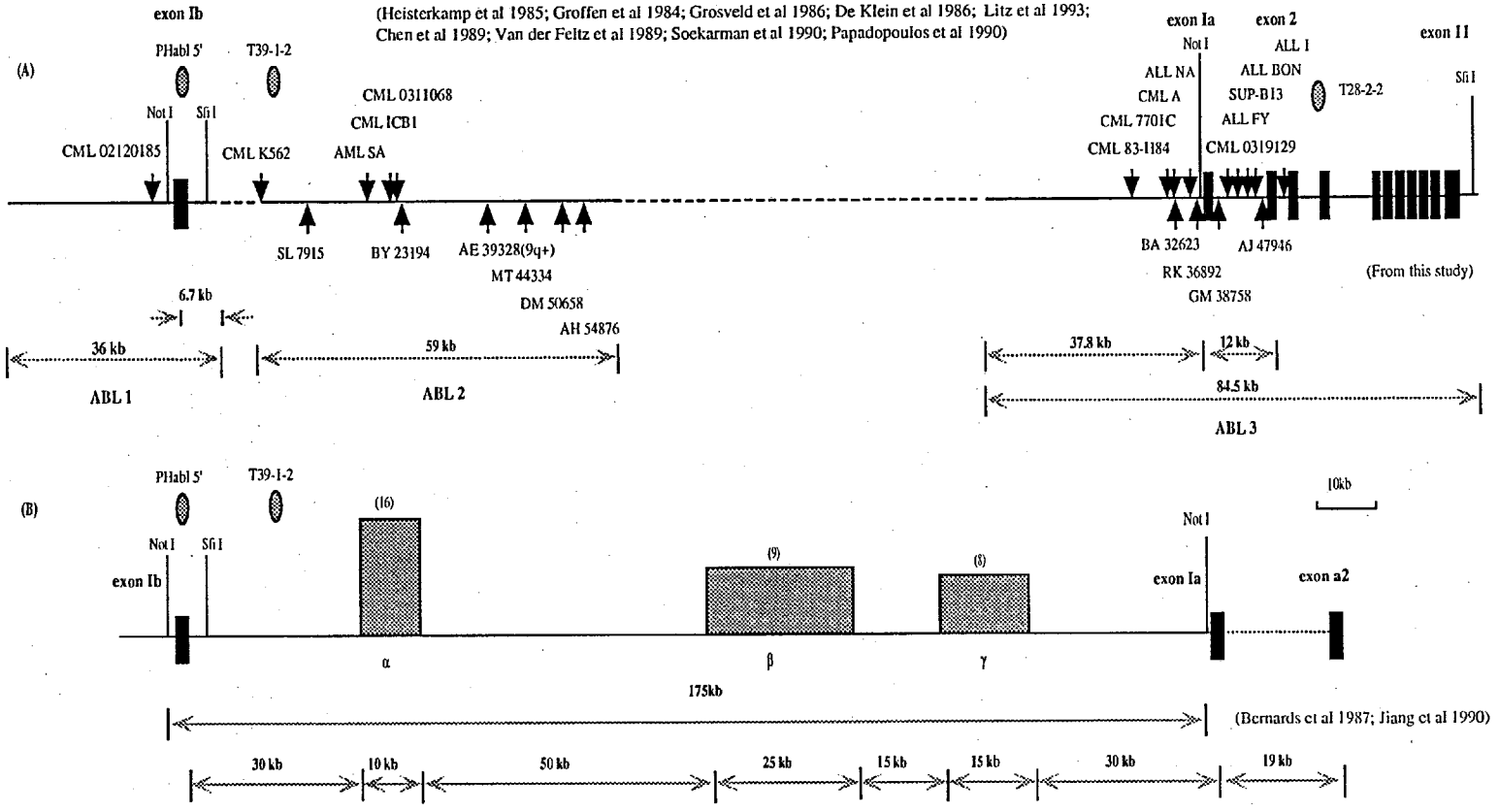


Figure 6-13. Comparison of the position of the sequencing breakpoints with that of PFGE breakpoints

(A) The breakpoints obtained by sequencing. (B) the breakpoints localised by pulsed field gel electrophoresis. Numbers in brackets indicate the number of patients whose breakpoints fall into the corresponding regions. The scale and size of DNA are indicated. The stippled ellipses indicate the positions of the probes used.

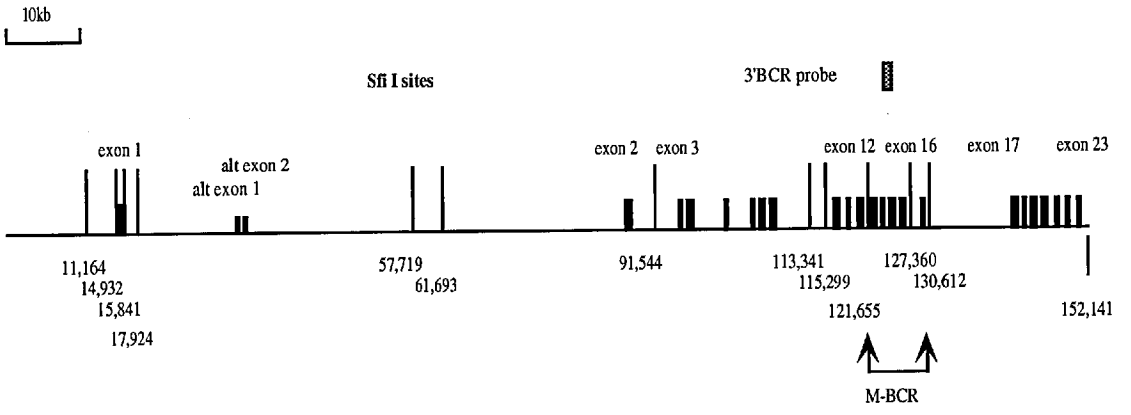


Figure 6-14. BCR with Sfi I restriction enzyme sites

The horizontal line indicates the sequence of the whole BCR gene. The vertical lines are Sfi I restriction enzyme sites. Solid rectangles represent exons. The alt exons are the alternative exons within the first intron of BCR. The position of the 3' BCR probe used in Jiang's study is illustrated by a stippled rectangle. The M-BCR is indicated.

downstream of probe T39-1-2. If probe T39-1-2 recognises a region downstream of cluster α, the size of the rearranged DNA fragments will be different from the one identified by probe pHabl5' with Not I digestion and the bands detected will be derived from chromosome 22q- rather than chromosome 9q+. The region where breakpoints AML SA, ICB1, 0311068 and BY lie may correspond to cluster α clarified by PFGE. However, the breakpoints in patients AE, MT, DM and AH would be situated within the 50 kb region that was cluster free, according to Jiang *et al* (Figure 6-13). The clusters β and γ should be mostly in the gap between ABL2 and ABL3 if the size of the PFGE fragments are correct. Cluster γ was situated about 30 kb upstream of exon Ia; the sequence of ABL3 includes 37.8 kb of sequence upstream of this exon.

This comparison suggests that the gap between ABL1 and ABL2 is much smaller than the gap between ABL2 and ABL3. From both PFGE and sequencing information, I estimate the gap between ABL1 and ABL2 to be only about 4 kb in size. The distance between exon Ib and cluster α, which includes the gap between ABL1 and ABL2, is approximately 30 kb (Jiang *et al* 1990). The distance between exon Ib and the end of ABL1 is 6.7 kb. Distance between the beginning of ABL2 and cluster α is unknown,

but I estimate that the four closely spanned breakpoints (patients AML SA, ICB1, 0311068 and BY; Figure 6-13) may correspond to cluster α. The distance between the beginning of ABL2 and the first of these breakpoints is 19 kb, suggesting that the gap between ABL1 and ABL2 is only 4 kb in size. If this was the case, then the gap between ABL 2 and ABL3 would be approximately 66 kb in length.

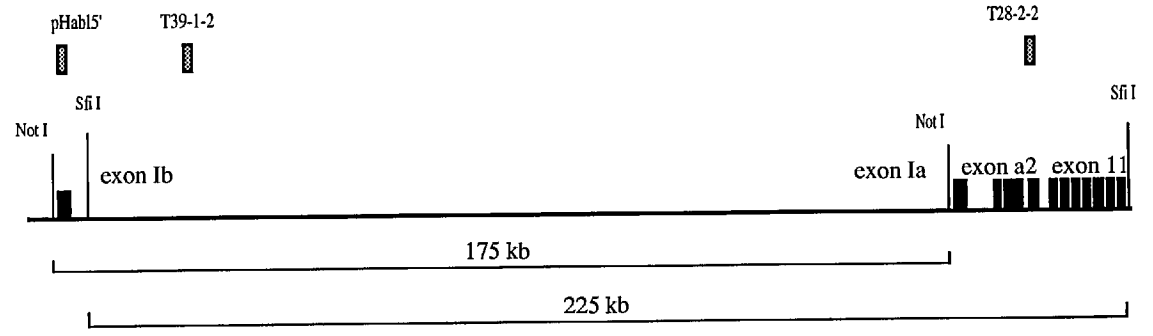


Figure 6-15. ABL restriction enzyme map with Not I and Sfi I sites

The stippled boxes indicate position of the probes used in Jiang's study. Other symbols are the same as Figure 6-6.

The results obtained by Jiang *et al* are quite different from the results obtained in my study. Only a small proportion of patients determined by sequencing (4/24) fell into cluster α, in contrast to the 43% of patients found by PFGE. Conversely the sequence data suggested that 54% of breakpoints fell into the PSR around ABL exon Ia whereas there were no breaks in this region as determined by PFGE. The possible reasons for these discrepancies are discussed below:

(i) PFGE results can not accurately localise position of the breakpoint in each patient. Errors of several kilobases are likely due to the inevitable mis-sizing of bands. For example, a previous PFGE report had defined the size between exons Ia and a2 as 19 kb (Bernards *et al* 1987), but recent sequencing data indicates the intron between these two exons is in fact 12 kb in length (Chissoe *et al* 1995), an overestimate of 58%. Other sources of errors would arise if two or more bands co-migrated on the gels, or if there were polymorphisms for the restriction enzymes employed. Either could result in mis-

interpretation of position of BCR-ABL breakpoints in ABL. Furthermore, if there was a significant deletion, insertion or duplication of sequence at the junction, the location of the precise break site could not be simply determined by only two probes.

(ii) According to the sequence of BCR gene (Genbank, U07000), there are no Not I sites in this gene. However, whether or not this sequence is the genuine representative of the human normal BCR gene is unknown. From my study for the PSR in ABL, Bgl II sites detected by Southern blotting were actually different from the ones defined in ABL3 (Genbank, U07563). Therefore, it may be possible that the actual Not I sites in BCR gene or around BCR gene would be different from the sites defined in U07000. If it is the case, then size of the fragments and position of clusters detected by PFGE will be mis-interpreted.

(iii) The results from both studies may also be skewed due to the relatively small sample sizes. Thirty seven patients were studied by PFGE (Jiang *et al* 1990). The breakpoints in 24 patients were identified by sequencing (Heisterkamp *et al* 1983; Grofffen *et al* 1984; Heisterkamp *et al* 1985; Grosveld *et al* 1986; de Klein *et al* 1986; Chen *et al* 1989a; Chen *et al* 1989b; van der Feltz *et al* 1989; Soekarman *et al* 1990; Litz *et al* 1993) or Bubble PCR (from this study).

(iv) No data about whether there was a DNA rearrangement in the M-BCR were available for the 37 CML patients studied by PFGE (Jiang *et al* 1990). If the breakpoint occurred outside the M-BCR, the estimated location of the breakpoint in ABL would be incorrect. This situation, however, is likely to be rare.

Ultimately, the conspicuous discrepancies between the PFGE and sequencing data will only be resolved by performing the same technique on the same group of patients so that the positions of any putative clusters could be compared directly.

6.3.3. The relationship between breakpoint positions and disease phenotype

6.3.3.1. Potential clustering of breakpoints in ABL in relation to BCR

Comparison of the position of the BCR-ABL breakpoints in both the BCR and ABL genes may help in understanding how the translocation occurs in CML. Of the 19 BCR-ABL breakpoints (18 CML; 1 ALL) currently characterised and localised in both the M-BCR and ABL, 13 patients had breaks in the 5' region of the M-BCR and 6 patients had breaks in the 3' region (i.e. 5' and 3' of the Hind III site; Figure 6-10). Of the 13 patients with 5' M-BCR breakpoints, 7 fell into ABL3; 5 into ABL2 and 1 into ABL1. In 6 patients with the 3' breakpoints, 2 fall into ABL3; 4 into ABL2. Although a greater proportion of patients with 5' M-BCR breakpoints fell into ABL3, the difference is not significant (7/13 versus 2/6; $p = 0.40$, Fisher's exact test). Of five patients BCR-ABL breakpoints localised into both ABL and m-BCR, four were identified within ABL3 and one was in ABL2 (Figure 6-10).

There is a breakage free region of 24 kb in size in ABL3 immediately the 5' of the PSR and a 16 kb gap without any breakpoints between patient BY and patient AE in ABL2 (Figure 6-3). Another gap between patient ICB1 and SL is about 13 kb in length. In ABL1 a single breakpoint was found upstream of exon Ib. A previous report (Morris *et al* 1991) demonstrated by Southern blotting that the breakpoints in 6 out of 31 (19%) patients who has a break in the M-BCR fell into the vicinity of ABL exon Ib, a region of about 16 kb in size (between the Bgl II site at position 18,135 and the EcoR I site at position 34,584 of ABL1; Chissoe *et al* 1995). Of these six patients, four had breakpoints upstream of exon Ib, with the most 5' breakpoint being about 11 kb from this exon. The remaining two patients had breaks about 5 kb downstream of exon Ib (Morris *et al* 1991). The break in patient 02120185 is also located within this region (at position 22,910) (Heisterkamp *et al* 1983; Groffen *et al* 1984; Heisterkamp *et al* 1985; Chissoe *et al* 1995).

There is a possibility that an association between breakpoints and disease status exist. As reviewed in Chapter 1, breakpoints in BCR are clustered in two regions, the m-BCR and M-BCR respectively. These two clusters are obviously related to leukaemic phenotypes. In CML, the breakpoints are mainly restricted to the M-BCR. Some groups have found that the position of the breakpoint within the M-BCR is related to the duration of chronic phase; other groups have found no such association (Kurzrock *et al* 1986; Mills *et al* 1988; Jaubert *et al* 1990; Mills *et al* 1991a; Inokuchi *et al* 1991; Opalka *et al* 1991; Aguiar *et al* 1995). In p190 BCR-ABL positive ALL, the breakpoints were found to partly fall into the m-BCR and partly into the M-BCR (Schaefer Rego *et al* 1988; Ohyashiki *et al* 1991). The reason why p190 BCR-ABL is of molecular heterogeneity is unknown but it is possible that this rearrangement is favoured in immature lymphoid cells (Craig *et al* 1990) perhaps as a result of aberrant recombinase activity. However, as discussed above, most rearrangements in Ph-positive ALL are not associated with obvious heptamer/nonamer signals. An alternative hypothesis is that the p190 BCR-ABL protein preferentially directs lymphoid development, and therefore ALL. Evidence in support of this theory was provided by analysis of p190 and p210 BCR-ABL transgenic mice. The p210 transgenic animals developed a variety of haemopoietic malignancies of both lymphoid (B or T cells) and myeloid phenotype. In contrast the leukaemias found in p190 BCR-ABL transgenic mice were predominantly of B-cell phenotype, similar to Ph-positive ALL (Heisterkamp *et al* 1990; Voncken *et al* 1995).

6.3.3.2. Breakpoint clusters in acute leukaemias

Group F (Figure 6-10) contains 4 breakpoints found in ALL patients (Chen *et al* 1989a; Chen *et al* 1989b; van der Feltz *et al* 1988; Papadopoulos *et al* 1990). These patients form quite a tight cluster with breaks at the 3' end of the m-BCR and within the ABL-PSR. This cluster suggests that there might be an association between the positions of

the M-BCR and ABL breakpoints. As described above, the ABL breakpoints for just one (FY) of these four patients was selected by use of a specific ABL probe. In addition to these four patients, two further ALL breakpoints had been described on the basis of Southern blot data alone: one at position F and the other at position D (Chen *et al* 1989c). In other words, all of the ABL breakpoints in p190 ALL characterised to date have been localised to the PSR. As discussed above, one possible explanation for this finding is that the M-BCR and PSR are relatively rich in signals that may misdirect the activity of immunoglobulin recombinase.

Southern blot analysis of different cell fractions in Ph positive ALL has shown that in some cases both myeloid and lymphoid cells are involved, whereas in other cases the BCR-ABL rearrangement is restricted to lymphoid cells (Chen *et al* 1988; Hirsch Ginsberg *et al* 1988; Bloomfield *et al* 1977). A striking difference in prognosis between these two groups of patients have been revealed (Secker Walker & Craig, 1993; Cuneo *et al* 1994). Event-free survival is poorer in the lineage-restricted cases then multilineage (Secker Walker & Craig, 1993; Anastasi *et al* 1996). Patients with a minor myeloid component involvement may be associated with a distinct cytogenetic pattern and even poorer responses to antilymphoid therapy than typical ALL (Cuneo *et al* 1994). It would be interesting to compare the positions of the breakpoints between these two groups of patients to see if there was any difference between them. It might be expected, for example, that lymphoid only rearrangements might have been mediated by aberrant recombinase activity whereas cases involving both lymphoid and myeloid cells could have arisen by a different mechanism.

The breakpoint in the single patient with Ph positive AML fell into the m-BCR and ABL2 (position E; Figure 6-10). Although this patient was not selected, it clearly is not possible to make any statement about the relationship between the breakpoint position and clinical phenotype in this rare case.

6.3.3.3. Breakpoint clusters in CML

The breakpoints identified in the M-BCR could be classified into four positions (A-D) (Figure 6-10). Inspection of the relative positions of the breakpoints does not suggest that there is any clear association between the position of the breakpoint in the M-BCR and the breakpoint in ABL (Figure 6-10). A total of 7 out of 9 patients (6 out of 8 cases of CML) in position D had 5' M-BCR breakpoints compared to only 2/6 patients in position C, but this difference is not significant and may be due to random sampling.

One study found that breakage in 3 (50%) out of 6 patients who had clinical features of essential thrombocythemia (ET) was situated within the vicinity of ABL exon Ib (Morris *et al* 1991). In another group of 28 patients with typical CML, only 2 (7%) patients had a break in this region. All patients showed rearrangement of the M-BCR (Morris *et al* 1991). This data suggests that the position of the breakpoint in ABL is related to disease phenotype. Such a relationship could arise theoretically if the predisposed breakpoint directs disease development, for example, by affecting the level of BCR-ABL expression, or if the break positions were influenced by the phenotype of the cells in which the translocation occurred, and this in turn was related to disease phenotype. In my study, however, no patients had a phenotype consistent with ET.

6.3.4. Involvement of Alu elements in the chromosomal translocation

Gene rearrangements caused by Alu-Alu recombination have been reported in some human diseases, for example, ADA deficiency (Markert *et al* 1988), lipoprotein lipase deficiency (Devlin *et al* 1990) and Lesch-Nyan syndrome (Marcus *et al* 1993). When DNA breakage occurs, Alu homologous sequences may enhance gene conversion between non-homologous DNA during the repair process (Hess *et al* 1983; Schimenti & Duncan, 1984). This process may result in partial DNA duplication and deletion of flanking sequence (Haber, 1995), such as I found for some BCR-ABL translocations.

Alu elements can retrotranspose into genomic DNA after reversion to cDNA and possess the capacity to fix the rotational and translation positions of nucleosome (Chang & Maraia, 1993; Martignetti & Brosius, 1993; von Sternberg *et al* 1992; Englander *et al* 1993; Englander & Howard, 1995). Therefore, it is reasonable to speculate that Alu-nucleosome interactions *in vivo* might play a role in gene regulation and chromatin structure. Chromatin structure may in turn be related to the probability of illegitimate recombination and therefore the possibility of translocation.

Of the 10 breakpoints in my study that were localised to both BCR and ABL, two patients (DM and MT) were within the M-BCR Alu and three patients (DM, MT and GM) close to an ABL Alu. One (patient MT) of those patients had an Alu sequence on both sides of the breakpoint on the Philadelphia chromosome (Figure 6-5). Whether and how the Alu elements are associated with the translocation in these patients is still not clear. Previously there were several reports in CML and ALL that claimed Alu elements at the junction or close to the break (Heisterkamp *et al* 1985; van der Feltz *et al* 1989; Chen *et al* 1989a; Papadopoulos *et al* 1990; Mills *et al* 1992; Koduru *et al* 1993; Morris *et al* 1996). It has been suggested that the translocation between chromosomes 9 and 22 may result directly from a homologous recombination between Alu elements within the BCR and ABL genes, or possibly because some sort of attraction between Alu elements preferentially brings these genomic sites together, so that the chances of recombination between them would be increased (Chen *et al* 1989a; Morris *et al* 1996). However, in my study no breakpoints were found to be located in Alu elements of both BCR and ABL gene in the same patients. Therefore, homologous recombination between Alu sequences is clearly not a common mechanism of translocation in CML. Furthermore, in those patients for whom I found an Alu element close to the both sides of the breakpoint (i.e. in ABL and BCR), I found no consistent orientation of these elements.

It is still possible that the recombination between BCR and ABL is mediated, at least in part, by Alu elements. In interphase, DNA molecules may be attracted and locally associated with each other by Alu elements or other repetitive sequences. DNA breakage and exchange could result, for example, from protein complexes binding to the repetitive sequences around the region where the two or more DNA molecules are associated together. Subsequent exchange might or might not involve homologous regions.

Alus constitute about 5% of the human genome and can be expected to occur on average roughly every ~4kb (Hwu *et al* 1986). Surprisingly, Alu elements were found to constitute a very high proportion (about 39%) of the sequence of ABL intron 1 (ABL1, ABL2 and ABL3) and also of BCR (about 39%). It is possible that either the BCR and ABL genes are really unusually Alu rich, or that previous studies had considerably underestimated the frequency of Alu elements in the human genome. If the density of Alu elements really is significantly higher in BCR and ABL compared to other genes, this might partially explain their propensity for illegitimate fusion.

Recent molecular analysis of the breakpoint clustered region of the ALL-1 gene on chromosome 11q23, which is characteristically associated with infant acute leukaemias, suggests that there are eight Alu sequences located within a 8-kb BamH I region, occupying 28% of the sequence. However, only 50% breakpoints fall into Alu sequences in this region (Gu *et al* 1994). This situation is very similar to the ABL gene.

Although it is possible that Alu elements may play a role in formation of the Ph chromosome, it is clear that breakpoints are not consistently associated with Alu elements. This implies that other factors are probably more important in the translocation process.

CHAPTER SEVEN DETECTION OF RESIDUAL DISEASE AFTER BONE MARROW TRANSPLANTATION FOR CHRONIC MYELOID LEUKAEMIA

7.1. Introduction

It has recently been suggested that some Ph chromosome positive CML cells may not actively express the BCR-ABL fusion gene (Keating *et al* 1994; Bedi *et al* 1993). Such cells would escape detection by RT-PCR but could be detectable by methods that recognise the rearrangement in genomic DNA. It is conceivable that such transcriptionally 'silent' CML cells are present in patients after BMT and, after reactivation, could contribute to relapse. In order to test the hypothesis that CML patients after BMT possess a pool of leukaemia cells that do not express BCR-ABL mRNA, I have compared results of residual disease detected by RT-PCR with results obtained by amplification of BCR-ABL breakpoints from genomic DNA. In this study, Bubble PCR was performed to amplify the breakpoints from patients of interest, the ABL sequences flanking the junctions were defined and patient-specific primers were designed for DNA-PCR to detect residual disease in a series of samples for the patients post BMT.

7.2. Results

7.2.1. Qualitative PCR from genomic DNA

7.2.1.1. Specificity of amplification

In Chapter 4 I described the use of Bubble PCR to amplify BCR-ABL genomic breakpoints from 17 patients with CML. Ten of these patients subsequently underwent BMT. Specific ABL primers were designed for each patient and tested in combination with various M-BCR primers for specificity of amplification. Combinations of ABL

and M-BCR primers enabled the BCR-ABL breakpoints to be amplified from chronic phase DNA of each of the 10 patients studied (Table 7-1). The sequence of each primer is listed in Table 2-1 (see Chapter 2). As expected, primer pairs were patient specific, i.e. a particular combination of primers only resulted in an amplification product from a single patient (Figure 7-1). Primer pairs and amplification conditions were selected that gave no non-specific amplification from other CML patients or from normal individuals.

Table 7-1. Primer combinations for detection of minimal residual disease

Patients	BCR primers		ABL primers		Annealing temperatures (^{o}C)	
	1st step	2nd step	1st step	2nd step	1st step	2nd step
AE	Z4A	Z4B	1489ABL	1489ABL	54	56
AH	Z4A	Z4B	1620BA	1620BA	60	60
AJ	B3A	B3B	1535ABL	1535ABL	64	64
BM	B2A	B2B	1797BA	1797BA	60	64
BY	Z4A	Z4B	1818BA	1818BA	64	62
DM	B3A	B3B	1854ABL1	1854ABL2	64	54
ML	B2A	B2B	MaisABL	MaisABL	64	66
MT	B3A	B3B	1472ABL1	1472ABL2	56	58
RK	Z4A	Z4B	1732BA1	1732BA2	60	62
SL	B2A	B2B	1572BA	1572BA	62	62

7.2.1.2. Definition of DNA-PCR sensitivity

Chronic phase DNA from each patient was serially diluted with either normal human genomic DNA or DNA extracted from the BCR-ABL negative cell line HL60. Dilutions were subjected to two-step PCR with 30 cycles of amplification in each step using either nested or hemi-nested combinations of patient-specific primers (Table 7-1). Using 100 ng genomic DNA as a template, the 10^{-4} dilution usually resulted in a specific amplification product but at the 10^{-5} dilution BCR-ABL was not detectable. By increasing the amount of template DNA to either 2 or 5 μg, and the first step reaction volume to 100 μl, an amplification product was routinely obtained from the 10^{-}

^{5} dilution (Figure 7-2). A specific product was obtained using a 10^{-4} dilution for all patients when 500 ng of genomic DNA was amplified.

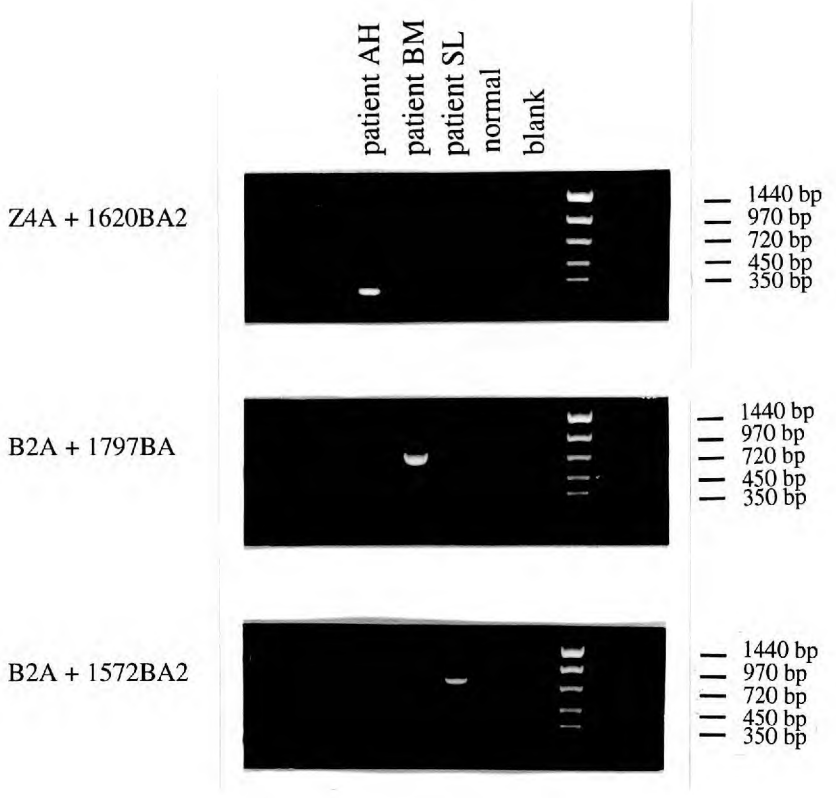


Figure 7-1. DNA-PCR specificity with patient specific primers

Specific primer combinations were tested for DNA-PCR specificity. Each specific combination of PCR primers amplified the genomic BCR-ABL breakpoint from one particular patient only. Z4A and 1620BA2 amplified the breakpoint from patient AH but not patients BM and SL. Similarly, primer combinations B2A and 1797BA, B2A and 1572BA2 specifically amplified the breakpoints from patients BM and SL respectively.

7.2.2. Integrity of sample DNA

To avoid false negative results, the concentration and integrity of all DNA samples were measured. DNA was quantified using a spectrophotometer and the concentration and integrity were confirmed by agarose gel electrophoresis. The spectrophotometer can not distinguish between intact and degraded DNA which is why an agarose gel was also used. The 'amplifiability' of the extracted DNA from each sample was confirmed

by amplification of a 318 bp BCR exon 1 fragment from 500 ng genomic DNA with primers BCR-B (5' CCCCCGGAGTTTTGAGGATTGC 3') and BCR-D (5' ATGGAA GGCGCCCTCGTCATC 3') (Figure 7-3) for 30 cycles of 96°C, 30 seconds; 64°C, 50 seconds; 72°C, 1 minute. All 77 DNA samples described above were tested and resulted in the amplification of a clear product of the expected size after a single step

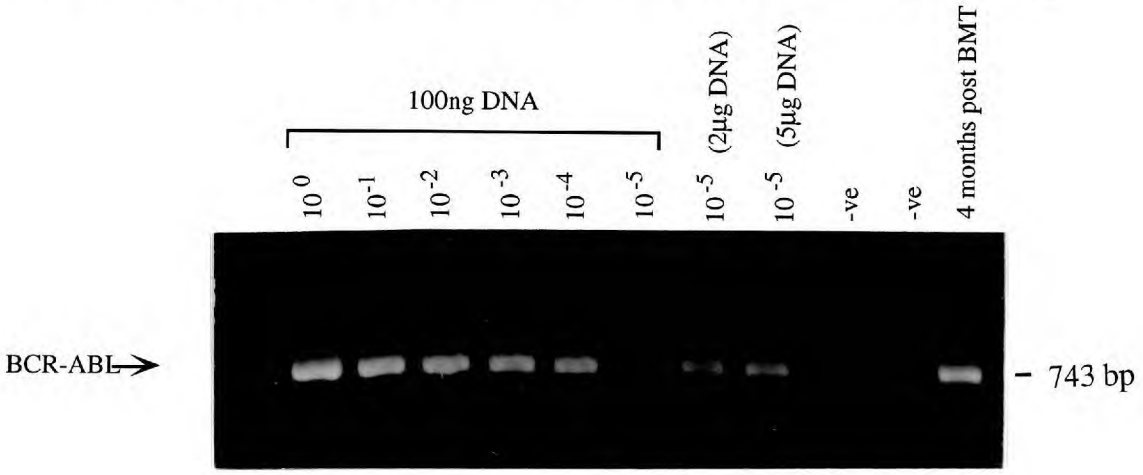


Figure 7-2. DNA-PCR sensitivity for patient DM

Patient chronic phase DNA was serially diluted into HL60 DNA and amplified by nested PCR with patient specific primers. The degree of dilution and amount of DNA per reaction is shown. Two blank reactions (-ve) were included, and also DNA extracted from the same patient whilst in remission 4 months post BMT.

PCR and were therefore considered to be of sufficient quality for analysis of residual disease. A BCR PCR product failed to amplify from additional four DNA samples extracted from bone marrow slides and these were therefore not analysed further. Despite these controls, I was still concerned that there may be some significant variability in the quality of the DNA samples, particularly those extracted from stained slides. For these samples I confirmed the DNA quality by competitive PCR. A competitor construct designed for quantification of p190 BCR-ABL cDNA was available in our laboratory. This plasmid had been constructed by cloning a 633 bp e1a2 junction fragment into the plasmid pGEM4Z, followed by insertion of a 134 bp bacteriophage λ fragment into the BCR exon 1 Bgl II site (van Rhee *et al* 1995). Since the insert is entirely within a single exon, this construct can also be used for quantification of genomic DNA by using BCR primers within exon 1 either side of the insert (BCR B and BCR D; Figure 7-3). Amplification of genomic DNA with these

primers gives a product of 185 bp whereas amplification of the competitor gave a product of 319 bp. In a titration assay, serial dilutions of linearised plasmid were added to fixed amounts of test genomic DNA and the mixture subject to standard PCR. The competitor BCR competed with the endogenous sample BCR gene for primers, enzymes and nucleotides in the reaction. If the initial number of the competitor molecules was much higher than the number of copies of the BCR gene, then only the competitor band was visible on the final gel. Conversely, if many fewer molecules of

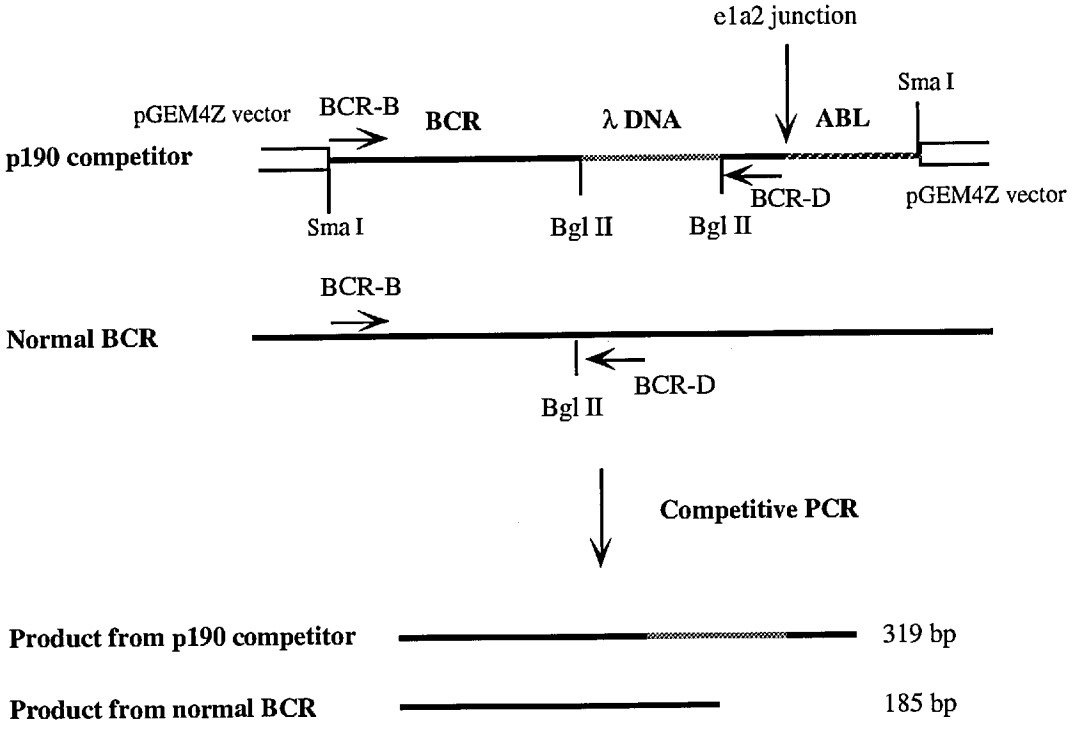


Figure 7-3. Diagram of the p190 competitor construct (van Rhee et al, 1995)

The vector pGEM4Z is not shown. The positions of primers BCR-B and BCR-D are indicated.

the construct are added, then only the sample BCR gene band was visible. If the starting reaction contained equal numbers of molecules, then the gel showed both bands with the ratio of the fluorescence intensity between the two, in proportion to the product sizes. Therefore, the number of the sample BCR molecules can be obtained after multiplying the number of competitor molecules added at the equivalent point by 1.7 (319 divided by 185). The accuracy and reproducibility of this assay have been reported

previously for quantification of BCR-ABL mRNA (Cross *et al* 1993a; van Rhee *et al* 1995).

Theoretically, 1 μg of genomic DNA is equivalent to 3.3 x 10^5 haploid genomes (Brisco *et al* 1991; Jeffreys *et al* 1988). Initially, I tested the competitive PCR system with HL60 genomic DNA that was considered to be good quality. One microgram of DNA was seeded into each PCR and different number of molecules of linearised

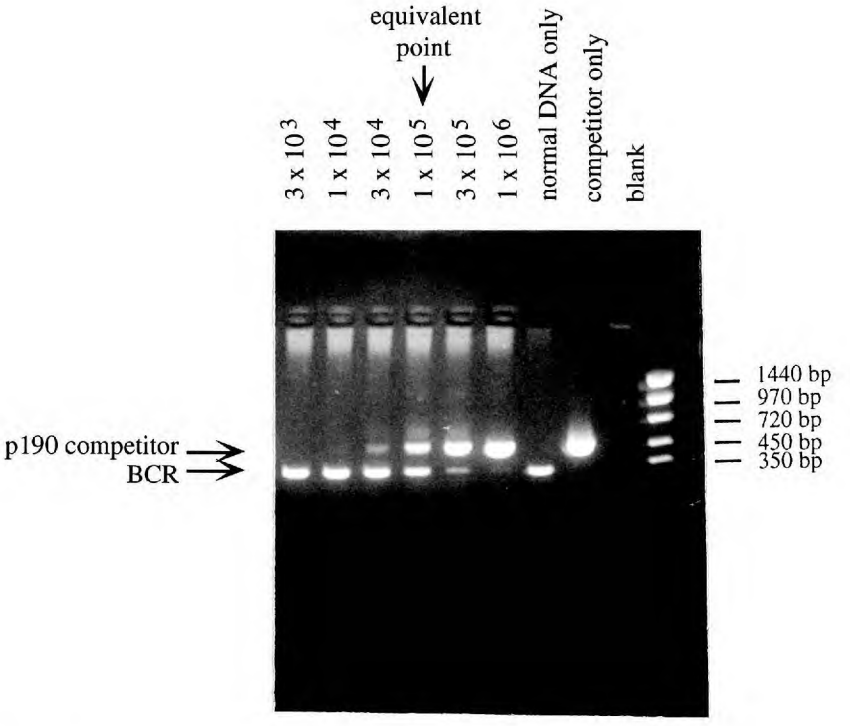


Figure 7-4. Quantification of the amount of DNA extracted from a bone marrow slide

Fixed amount of DNA were co-amplied with different number of molecules of competitor. Numbers of competitor molecules added are indicated alone each track. Controls for BCR (normal DNA) and competitor as indicated.

competitor were added. From 6 replicate analyses, the average number of molecules measured from the samples was 2.5 x 10^5 (range 1.8 x 10^5 to 3.5 x 10^5). This is very close to the expected number of 3.3 x 10^5. A typical result from DNA extracted from a bone marrow slide is shown on Figure 7-4. In this case 1 μl of sample DNA out of a total 20 μl was added to each reaction and co-amplified with standard known number of competitor molecules. The equivalence point was at approximately 1 x 10^5 competitor

molecules added, indicating 1.7 x 10^5 genomes after correction for the size difference of PCR products. For minimal residual disease (MRD) analysis in this case, 3 µl of DNA sample was loaded into each PCR reaction, roughly equivalent to 1.5 µg of amplifiable genomic DNA. For analysis of residual disease, at least 10^5 genomes were tested by nested PCR in each case. For quantification of DNA extracted from bone marrow slides, spectrophotometer analysis was not performed due to the limited amount of DNA available. Quantification was carried out by competitive PCR alone.

7.2.3. RT-PCR and cytogenetic analysis

All the MRD RT-PCR analysis was performed by Mr. F. Lin in our Department. RNA was extracted from peripheral blood leukocytes, reverse transcribed and tested for the presence or absence of BCR-ABL transcripts by nested PCR (Cross *et al* 1993b). Adequate cDNA quality was confirmed in the negative cases by amplification of part of the normal ABL gene. The number of BCR-ABL transcripts in positive specimens was quantified by competitive PCR (Cross *et al* 1993a, Lin *et al* 1996).

Cytogenetic analysis was performed by Mr. A. Chase. Bone marrow aspirates were cultured under standard conditions and, if possible, 30 consecutive metaphases were examined for the presence or absence of the Ph chromosome.

For the 10 patients post BMT that I studied, RT-PCR was totally performed for 36 timepoints. Of these 36 timepoints, sixteen were positive, eighteen were negative, and 2 single positive after repeated in triplicate (Figure 7-5, Figure 7-6, and Figure 7-7).

Cytogenetic analysis was performed on 18 of the 34 samples that were DNA-PCR negative and 15 of the 27 samples that were DNA-PCR positive. No Ph-positive metaphases were detected in any of the DNA-PCR negative samples nor in any of the 11 samples that were RT-PCR negative. Of the 15 samples that were DNA-PCR

positive, only 2 had detectable Ph-positive metaphases. Both samples were also RT-PCR positive (Figure 7-5, Figure 7-6, Figure 7-7).

7.2.4. DNA-PCR for patients post BMT

For the 10 patients after BMT, a total of 77 DNA specimens from 61 timepoints were analysed by two-step PCR for BCR-ABL. DNA was obtained from either from peripheral blood leukocytes (n = 30), fresh bone marrow specimens (n = 13) or stored, stained bone marrow smears (n = 34). For each DNA sample, at least 1 μg of DNA or 10^5 genomes (see below) was seeded into the first step amplification reaction. Twenty seven timepoints were DNA-PCR positive and 34 were DNA-PCR negative. At 48 timepoints a single sample was analysed whereas for 13 timepoints either 2 or 3 independent samples (total = 29) were analysed (Table 7-2). Complete concordance was found between results of contemporaneous DNAs extracted from fresh peripheral blood, fresh bone marrow and stained bone marrow slides (Table 7-2).

For most patients, the patterns of serial DNA-PCR results were similar to those that have been described for RT-PCR (Figure 7-5, Figure 7-6, Figure 7-7). Patients ML, BM and AJ were initially DNA-PCR positive after BMT but were subsequently persistently DNA-PCR negative. Patient BM was RT-PCR negative during the period of DNA-PCR positivity whilst patients ML and AJ were not studied by RT-PCR when DNA-PCR positive. Patients DM and AH were persistently DNA-PCR and RT-PCR positive. Patients BY, MT, AL and AE were persistently DNA-PCR negative; RT-PCR assays were also negative except at a single timepoint for three of these individuals. Patient RK was persistently DNA-PCR positive for 2 years after BMT. RT-PCR was initially negative but subsequently became positive. This patient was in cytogenetic remission for the first year, but at 21 months post BMT, 17% Ph-chromosome positive metaphases were detected in the marrow, rising to 40% Ph-chromosome positive metaphases at 24 months. Treatment for relapse by DLT was initiated at 21 months post

Table 7-2. DNA-PCR for detection of MRD post BMT

Patients	Time points	Tissue type	PCR results
AE	18 months	△	negative
	21 months	△	negative
	27 months	△	negative
	36 months	△	negative
	60 months	○	negative
	68 months	○ □	negative
	72 months	○	negative
AH	3 months	△	positive
	6 months	△	positive
	9 months	△	positive
	12 months	△ ○ □	positive
	19 months	△ ○	positive
	24 months	△	positive
	31 months	△ □	positive
AJ	6 months	△	positive
	12 months	△	negative
	20 months	△	negative
	24 months	△	negative
	36 months	△	negative
	48 months	□	negative
	60 months	○ □	negative
BM	2 months	○	positive
	3 months	○ □	positive
	6 months	○ □	positive
	10 months	○	positive
	12 months	○	negative
	14 months	○	negative
	18 months	○	negative
BY	3 months	○	negative
	24 months	○	negative
DM	2 months	□	positive
	3 months	△ □	positive
	4 months	○	positive
	5 months	○	positive
	6 months	△ ○ □	positive
	9 months	△ ○ □	positive
	12 months	△	positive
ML	5 weeks	○	positive
	6 weeks	○	positive
	7 weeks	○	positive
	11 weeks	○	negative
	12 weeks	○	negative
MT	21 months	△	negative
	24 months	△ □	negative
	36 months	△	negative
	44 months	○	negative
	56 months	○ □	negative
RK	3 months	○	positive
	6 months	△ ○	positive
	12 months	△	positive
	22 months	△	positive
	24 months	△	positive
	26 months	△	negative
	29 months	△	negative
	36 months	△	negative
	43 months	○	negative
SL	12 months	△	negative
	24 months	△	negative
	36 months	△	negative
	48 months	△	negative
	69 months	○	negative

Note: ○ peripheral blood; □ fresh bone marrow; △ bone marrow smear slide.

BMT. Four months later Ph positive metaphases were no longer detectable and no residual disease was detected by either DNA-PCR and RT-PCR. Subsequent PCR assays were also negative (Figure 7-5, Figure 7-6, Figure 7-7).

Twenty four paired samples were taken on the same day for analysis by RT-PCR and DNA-PCR and were therefore available for direct comparison. Nineteen samples were concordant, of which 9 were RT-PCR and DNA-PCR negative and 10 were RT-PCR and DNA-PCR positive. Of the 10 that were positive, 6 had high levels of BCR-ABL mRNA (arbitrarily defined as ≥ 50 transcripts / μg RNA) and 4 had low levels (< 50 transcripts / μg RNA). Five samples were discordant. Of these, 3 were DNA-PCR positive and RT-PCR negative. For these samples, RT-PCR was repeated in triplicate: one was persistently negative and two yielded a single positive result. Two samples were DNA-PCR negative and RT-PCR positive, of which one had a low level of BCR-ABL mRNA and one was not quantified (Figure 7-5, Figure 7-6, Figure 7-7).

7.3. Discussion

7.3.1. Potential applications of DNA-PCR for analysis of residual disease

Theoretically, DNA based PCR has some advantages over RT-PCR for analysis of residual disease. Extraction of DNA from patient samples is technically easier than extraction of RNA followed by synthesis of cDNA. Genomic DNA-PCR would result in amplification of BCR-ABL whether the fusion gene was actually expressed or not. Moreover, the use of patient-specific primers rather than a common set of primers for all patients may help to minimise the problem of PCR contamination. In CML, however, the t(9;22) breakpoints are dispersed over a very wide region of genomic DNA, particularly within the ABL gene (Bernards *et al* 1987). Thus, at least when I started this study, to amplify BCR-ABL from genomic DNA it is necessary to clone and sequence the breakpoint from each patient and to design patient-specific

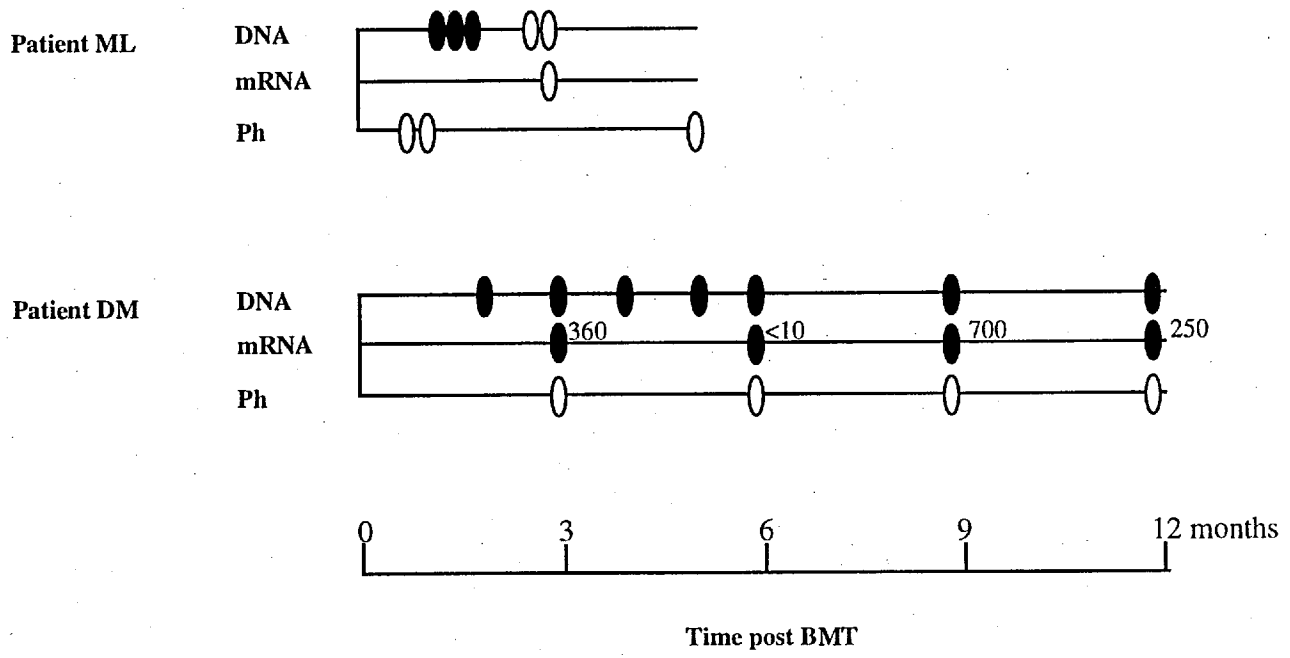


Figure 7-5. Minimal residual disease in patients ML and DM post BMT

Sequential DNA-PCR (DNA), RT-PCR (mRNA) and cytogenetic (Ph) results post BMT for patients ML and DM. Figures next to the RT-PCR results are the number of BCR-ABL transcripts/μg RNA determined by competitive PCR. Open ellipses represent negative results and solid ellipses are the positive results.

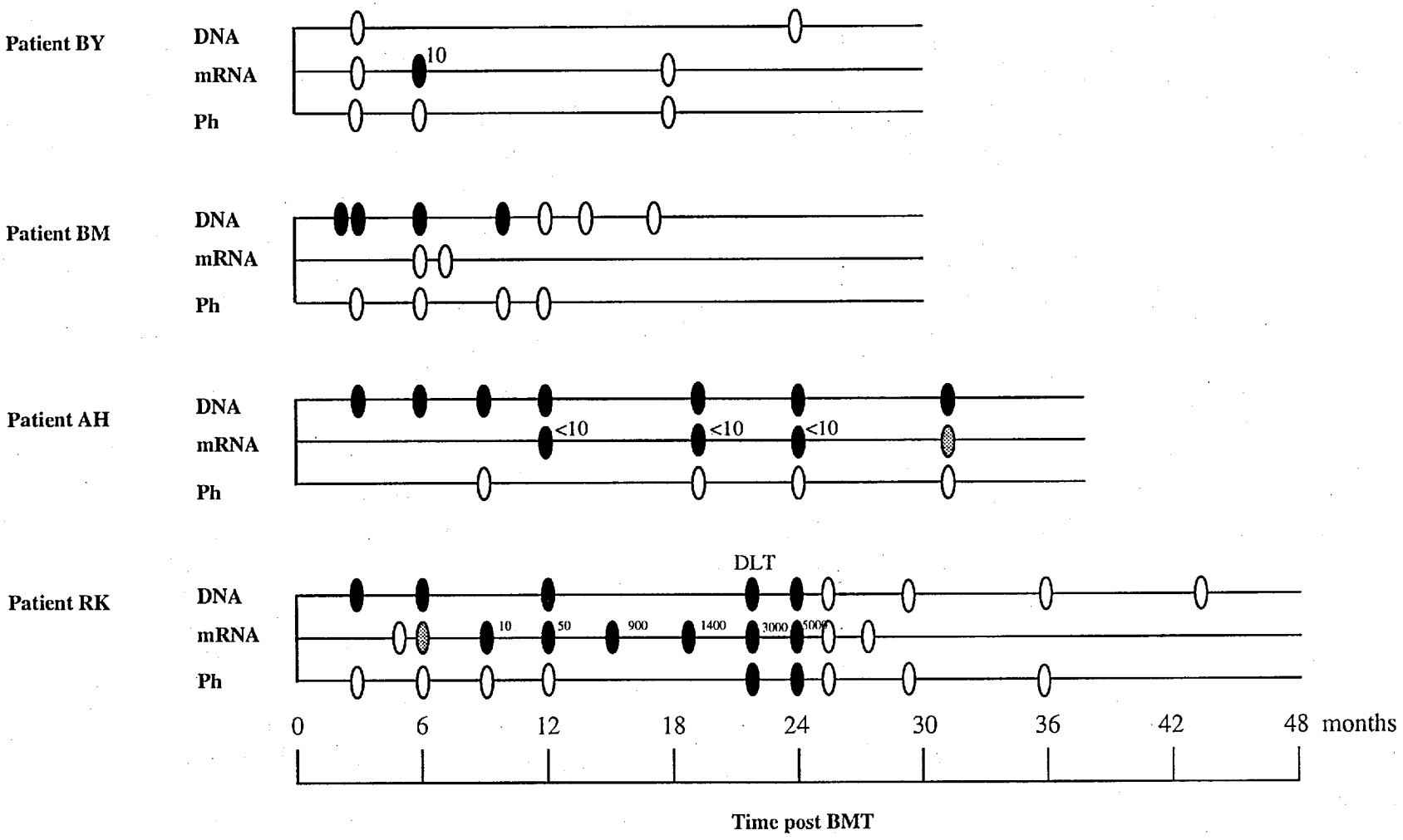


Figure 7-6. Detection of minimal residual disease for patients BY, BM, AH and RK

DLT indicates donor leukocyte transfusion for patient RK at 21 months after BMT. The stippled ellipse indicates the two cDNA samples that were repeated in triplicate and yielded a single positive result. Other symbols are the same as Figure 7-5.

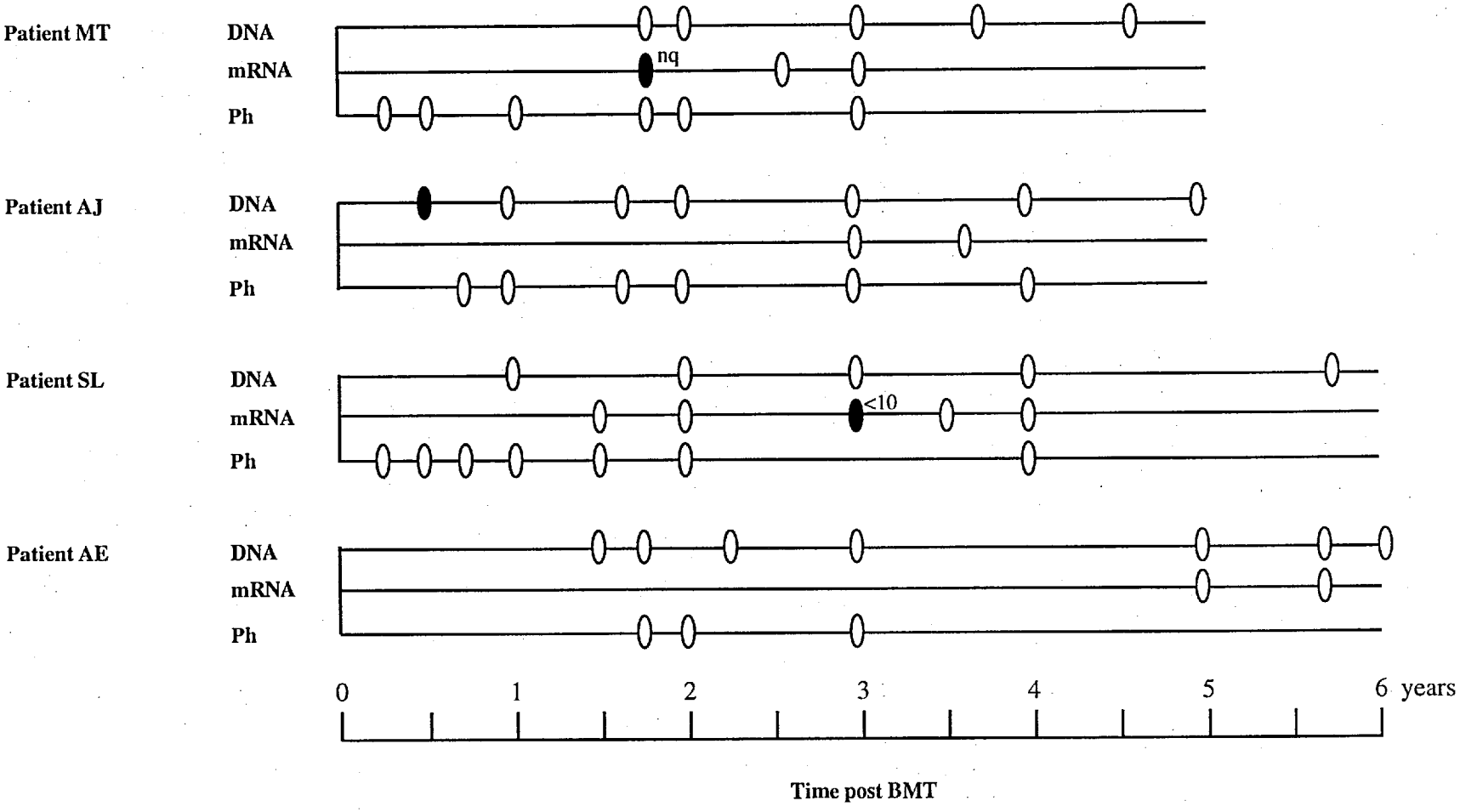


Figure 7-7. Detection of minimal residual disease in patients MT, AJ, SL and AE post BMT

nq means that this RT-PCR result was not quantified by competitive PCR. Other symbols are the same as Figure 7-5.

oligonucleotide primers. In Chapter 4 I described the use of Bubble PCR to facilitate cloning of BCR-ABL breakpoints. Whilst this technique is considerably easier than the traditional method of constructing a genomic DNA library from each patient, it does nevertheless involve an appreciable amount of time and effort. Furthermore, I was only able to amplify fragments harbouring genomic BCR-ABL breakpoints from 40% of patients. It is unlikely therefore that DNA-PCR could easily replace RT-PCR as a routine method for monitoring CML patients after BMT. Nevertheless, it is possible that DNA-PCR could be more informative than RT-PCR for detection of residual disease.

When a new treatment for CML is developed, such as interferon-α or donor lymphocyte transfusions to treat relapse post BMT, one needs to ascertain whether the treatment can reduce or eradicate leukaemic progenitor cells in the patient. Potentially, DNA-PCR is better than RT-PCR to detect and quantify CML cells, since there is only one abnormal allele in each leukaemic cell, whereas the number of BCR-ABL mRNA molecules per cell is unknown and presumably variable. Theoretically at least, a quantitative DNA based PCR assay would provide a more reliable estimate of the number of leukaemic cells in a sample than would be possible by RT-PCR. Importantly, DNA-PCR would detect CML cells regardless of whether the BCR-ABL fusion gene was being actively expressed or not.

DNA-PCR could be used as an efficient tool to track a given clone of leukaemic cells when it proliferates, differentiates and dies by specific amplification of the breakpoint as a genetic marker. This PCR could theoretically easily applied to haemopoietic colonies to obtain a visual result on a agarose gel. In contrast, RT-PCR may not always give reliable PCR results from single colonies in semi-solid cell culture due to very limited copies of mRNA molecules available for conversion to cDNA (Diamond *et al* 1995).

7.3.2. Strategy considerations

Bubble PCR was used to amplify products spanning the BCR-ABL breakpoint from patients with CML. These products were sequenced, and PCR primers designed that were complementary to the ABL side of the translocation. As discussed in previous Chapters, I found this strategy to be the best out of a number of possibilities that were available at the time.

Recently, however, some other strategies for detection of minimal residual disease at the DNA level have been explored by other groups. Inverse PCR has been used to specifically amplify BCR-ABL breakpoints and therefore is a potential useful for analysis of residual disease at the genomic DNA level (Miller & Blechert, 1994). However, due to the limitations and requirements of this technique, it likely that it is applicable to only a small number of patients (see Chapter 3).

Since the sequence of most of the ABL gene has been published (Chissoe *et al* 1995), it is possible to use Long-PCR to amplify genomic BCR-ABL breakpoints with a single BCR primer in combination with a panel of ABL primers (Dennebaum *et al* 1996). However, in this study only minority (5/10; 50%) of patients were successfully amplified using this technique. Theoretically, the frequency with which BCR-ABL breakpoints can be amplified could probably be improved if the whole of the ABL sequence were known and a larger series of primers that span at least the whole of ABL intron 1 were used. Long-PCR enables amplification of DNA sequences up to 10 kb to 40 kb in size (Cheng *et al* 1994b), in contrast to normal PCR for which the typical maximum target size is 2 - 3 kb. Therefore, 6 - 10 primers would theoretically cover the 200 kb breakpoint region in ABL. Long PCR is achieved by non-conventional conditions which include use of a modified buffer, and a combination of rTth DNA polymerase and Vent DNA polymerase instead of Taq polymerase (Cheng *et al* 1994a; Cheng *et al* 1994b). In some patients analysed, the BCR-ABL specific PCR product

was reported to be more than 10 kb in length (Dennebaum *et al* 1996). Although the products amplified well from chronic phase DNA, it is unclear whether this amplification is sensitive enough to detect residual disease. However, it is possible that Long-PCR could be developed as a routine method to detect minimal residual disease at the DNA level for CML if either the length of fragment to be amplified is reduced, for example by using more ABL primers, or if the efficiency of the PCR were increased. Alternatively Long-PCR could be used as part of a strategy of initial localisation of genomic breakpoints, followed by use of additional primers to narrow down the length of the fragments.

7.3.3. PCR sensitivity and contamination

7.3.3.1. Factors affecting sensitivity of DNA-PCR compared to RT-PCR

The sensitivity of PCR analysis is determined by a number of factors. One is of course the amount of target sequence present. The sensitivity of DNA PCR is limited by the amount of DNA that can be added to a PCR without inhibiting the amplification efficiency. Practically this is approximately 1-5 µg genomic DNA in a 100 µl reaction (Figure 7-2). 1 µg of genomic DNA is equivalent to 3.3×10^5 copies of human haploid genomes (Brisco *et al* 1991; Jeffreys *et al* 1988). The maximum sensitivity of DNA PCR is therefore one in 3.3×10^5 cells, if one µg of DNA is seeded into the first step reaction. Generally, therefore, DNA-PCR is less sensitive for detection of residual disease than RT-PCR which can achieve a sensitivity of 1 in 10^6 (Cross *et al* 1993a). A second reason that DNA-PCR may be less sensitive than RT-PCR is that genomic DNA is considerably more complex than cDNA, i.e. there are a much greater number of different sequences. This means that any particular target is effectively diluted and there is potentially greater competition due to non-specific amplification from the non-target sequences. Finally, most CML cells harbour only a single copy of the BCR-ABL fusion gene but each gene can give rise to multiple mRNA transcripts.

7.3.3.2. Prevention of false positive contamination and false negative results

Care has to be taken to prevent cross-contamination of sample, but by far the greater source of false positive results is due to contamination of reaction substrates with previously amplified PCR products (Kwok & Higuchi, 1989; Hughes *et al* 1990). This may happen when the same set of Gilson pipettes was used for manipulation of PCR products and substrates. Plugged tips were used routinely to prevent this happening and, more importantly, analysis and manipulation of PCR products was performed in a location physically separated from the area where DNA was extracted and the PCRs were set up using dedicated Gilson pipettes at each location. At least one negative control was included along with each PCR to detect any contamination. Theoretically, the problem of contamination is more problematic for BCR-ABL RT-PCR analysis since only two types of product (b2a2 and/or b3a2) are found in the great majority of patients. DNA-PCR results in patient-specific products and so the opportunity for contamination is considerably reduced. Nevertheless, the same extensive precautions need to be taken to ensure that false positive assessment of residual disease does not occur. In my study, three blanks were usually alongside each set of DNA-PCR reactions. Of a total of approximately 327 blanks performed, contamination was only observed on a single occasion. In this instance, the patient result was reanalysed using an independently extracted DNA sample.

In contrast, several reasons could result in a false negative results. Contaminants from the DNA extraction procedure can significantly inhibit the amplification efficiency. A positive DNA control with different PCR primers is necessary therefore to demonstrate amplifiable DNA: in this study I used amplification of BCR exon 1 as an indication of DNA quality. Second, an insufficient quantity of DNA or degraded DNA may also give rise to false negativity or reduced sensitivity, particularly when the frequency of BCR-ABL positive cells in a sample are at a low level. Quantification of the amount of amplifiable DNA for each sample is therefore a useful way of determining the real

amount of DNA present. Too much DNA in the PCR can also inhibit the amplification efficiency, because the DNA competes for Mg^{2+} with Taq polymerase, leading to inefficient amplification of the target DNA. In my experience, a maximum of 5 μg of DNA could be added to a first step PCR of 100 μl volume without inhibiting the efficiency of amplification.

7.3.4. Comparison of DNA-PCR with RT-PCR and cytogenetic results

7.3.4.1. RT-PCR versus DNA-PCR

I have found that DNA-PCR can detect BCR-ABL when chronic phase DNA from a particular individual is diluted into normal DNA up to a concentration of 10^{-5}. RT-PCR is generally considered to be slightly more sensitive, capable of detecting a single CML cell against a background of 10^5 to 10^6 normal cells. It might be expected therefore that, at low levels of residual disease, RT-PCR could give a positive result while DNA-PCR would be negative. If high levels of residual disease were found by RT-PCR then DNA-PCR would also be expected to be positive, unless the positivity was due to a very small number of CML cells expressing high levels of BCR-ABL mRNA. Conversely, if there were CML cells that did not express BCR-ABL mRNA, then DNA-PCR could be positive and RT-PCR negative.

7.3.4.2. DNA-PCR and RT-PCR results were concordant for the majority of samples

Several publications have demonstrated consistent RT-PCR patterns of minimal residual disease in CML patients post BMT (Cross *et al* 1993b; Miyamura *et al* 1993; reviewed in Miyamura *et al* 1994). i) persistently PCR negative for BCR-ABL chimaeric gene (PN); ii) persistently positive (PP); iii) initially positive and subsequently persistently negative (late negative; LN); iv) initially negative but subsequently positive (late positive; LP). The proportions of patients with these

different patterns vary from study to study, but at the Hammersmith roughly 60% of patients fall into pattern LN; about 5% into pattern PN; 10% PP and 5% LP (Dr. N.C.P. Cross, personal communication). Occasional patients are intermittently PCR positive and negative (IP). Most groups have found that patients with pattern PN have a very good prognosis, i.e., a low probability of subsequent relapse. Some studies have found that patients with patterns PP or LP have significantly elevated risk of relapse; other studies have found no significant relationship between quantitative RT-PCR results and subsequent relapse (reviewed in Miyamura *et al* 1994).

In the 10 CML patients post BMT that I studied, the pattern of DNA-PCR results were similar to the patterns found by RT-PCR (Cross *et al* 1993b; Miyamura *et al* 1993). One patient (AE) was PN by both techniques. Three patients (BY, SL and MT) were PN by both techniques, apart from single positive RT-PCR assays that were either at a low level (patients SL and BY) or was not quantified (patient MT). Two patients (DM and AH) were PP by both techniques. Three patients (ML, BM and AJ) were LN by both techniques. Patient RK was PP by DNA-PCR prior to treatment for relapse; RT-PCR was initially negative but then became positive. After successful donor leukocyte transfusion (DLT) for treatment of relapse, DNA-PCR and RT-PCR were persistently negative.

Direct comparison of DNA-PCR with RT-PCR was possible for 24 timepoints. Nineteen of those timepoints showed concordant results in 9 patients with both techniques. Only five timepoints were discordant (Figure 7-6, Figure 7-7). Therefore, it can be concluded that DNA-PCR and RT-PCR generate concordant results in the majority of time points.

7.3.4.3. Disparities between DNA-PCR and RT-PCR results

Of the discordant results, 2 were RT-PCR positive and DNA-PCR negative. One of these RT-PCR samples was quantified and found to contain low levels of BCR-ABL

mRNA; the other sample was not quantified. It is likely that these discrepancies result from the slightly superior detection level of RT-PCR over DNA-PCR.

Of the 3 samples that were DNA-PCR positive, and RT-PCR negative, only one was persistently RT-PCR negative when the analysis was repeated in triplicate. The cDNAs from all three samples appeared to be of good quality as judged by amplification of the normal ABL gene by single step PCR and, given the greater sensitivity of RT-PCR over DNA-PCR, it is possible that these specimens did harbour CML cells that were not actively expressing BCR-ABL mRNA.

Alternatively, since the maximum sensitivity of the PCR assay cannot be absolutely guaranteed for every sample, the discordance could have arisen due to random variation. It could also have occurred when number of tumour cells is extremely low around the limitation of PCR detection. Of the three patients who had a DNA positive, RT-PCR negative sample on initial analysis, one (patient RK) subsequently relapsed and two (patients BM and AH) are in remission 21 and 24 months after the discordant result. Thus a single DNA positive, RT-PCR negative result is not necessarily an indicator of subsequent relapse.

Interestingly, the two patients who did not relapse were transplanted whilst in accelerated phase (patient BM and AH). This suggests that accelerated phase CML cells, at least after BMT, might be more likely to harbour a transcriptionally silent BCR-ABL fusion gene than chronic phase cells. Since neither patient has relapsed, it is possible that the malignant cells were damaged by the transplant ablative regimen such that they did not express typical levels of BCR-ABL mRNA but were still able to survive for an unusually long time post transplant.

7.3.4.4. Quantification of the number of CML cells by DNA-PCR

It would be interesting to quantitate the level of residual disease determined by DNA-PCR in those patients who had at least two sequential DNA positive results. For example, if the number of CML cells was quantified in patient AH by DNA-PCR, it might clarify whether transcript numbers declined at 31 months post BMT because of a decrease of cell number or a decrease in the transcriptional activity of the BCR-ABL gene (Figure 7-6). Since concordance between DNA-PCR and RT-PCR was found at both high and low levels of BCR-ABL mRNA, it is unlikely that RT-PCR detectable residual disease is due to CML cells that express unusually high levels of fusion gene transcripts. However, quantification of CML cells at the genomic DNA level would enable a better comparison to be made and could potentially uncover changes in transcriptional activity of the BCR-ABL fusion gene.

Quantification could be achieved theoretically by either competitive PCR or a limiting dilution assay (Brisco *et al* 1994). Competitive PCR would require the construction of a competitor for each patient, which would entail a considerable amount of work. Limiting dilution analysis would be more appropriate since it simply involves performing PCR analysis on multiple amplification of serial dilutions of the target DNA, scoring the results as positive or negative and analysis by Poisson statistics. I did attempt to quantify some DNA samples by limiting dilution PCR, however, in many cases all the DNA had already been used, or there was very little left. It was clear that the data I could have obtained would not have enabled a meaningful comparison to have been made.

7.3.4.5. Do quiescent CML cells exist?

Published reports have suggested that not all CML cells actively express the BCR-ABL fusion gene. Purified CD34 positive CML cells were shown to be RT-PCR negative despite being positive for BCR rearrangement by Southern blot (Bedi *et al* 1993). Similarly, some Ph-chromosome positive myeloid colonies were found to be BCR-ABL mRNA negative (Keating *et al* 1994). Whilst results of the latter study have not been confirmed (Diamond *et al* 1995) and both studies can have been criticised on the grounds of inadequate controls for cDNA synthesis (Cross *et al* 1994a; Melo *et al* 1994a; Taylor & Heasman, 1994), it remains possible that some CML cells that do not express BCR-ABL mRNA do in fact exist. Such cells could lead to false negative assessment of residual disease in patients after treatment by RT-PCR.

Regarding to the controls and criteria for scoring positivity or negativity used in some studies (Bedi *et al* 1993; Keating *et al* 1994) in the literature, the results from RT-PCR can be easily misinterpreted. The reference gene chosen to confirm adequate quality of cDNA was β-actin gene in the report by Bedi *et al* and ABL in the study published by Keating *et al.* Those controls for mRNA expression may not have been adequate. First of all, a PCR positive control should be cDNA-specific for detection of expression of BCR-ABL chimaeric gene since genomic DNA always contaminates RNA preparations (Melo *et al* 1994a). In the report from Keating *et al*, the control PCR for ABL gene was not cDNA specific. The first step PCR employed primers in ABL exons 2 and 3. There would be expected to give rise to distinct cDNA and genomic DNA sized PCR products since the intron between exons 2 and 3 is only 562 bp. The second step primers, however, were both in exon 2 and would therefore give rise to an identical product from cDNA and genomic DNA. Specimens in which all the RNA was degraded, or where there was a failure of reverse transcription, could still give rise to a positive signal on a agarose gel (Cross *et al* 1994a) due to amplification from genomic DNA. Therefore,

CML colonies could have been wrongly assessed as quiescent with respect to BCR-ABL transcription.

β-actin is a poor control for two reasons: first, this gene has at least 19 processed pseudogenes (Leavitt *et al* 1984). Pseudogenes are derived from cellular mRNAs by retrotransposition and as such lack introns. In the presence of contaminating genomic DNA, it is easy to misinterpret amplification of genomic pseudogenes as that of mRNA (Taylor & Heasman, 1994) and so produce false negative results. Second, β-actin is expressed at a much higher level than BCR-ABL. Amplification of BCR-ABL and β-actin from 100 - 1000 fold dilution of genomic free cDNA prepared from leukocyte RNA from chronic phase CML patients demonstrated that BCR-ABL was no longer detectable, but the β-actin signal was still clear (Cross *et al* 1994a), i.e. false negative results can be obtained.

7.3.4.6. Quiescent CML cells are not detectable in most patients after allogeneic BMT

Whether or not there is a pool of quiescent CML cells in the patients post BMT is still an interesting question in spite of the controversial literature. In the majority of patients in my study DNA-PCR results one year or more after BMT were negative. Five of these patients (ML, BY, BM, AJ and SL) were clearly negative one year post transplant in the available samples. Two patients (MT and AE) had no samples available for DNA-PCR and RT-PCR analysis in the initial 1.5 years. However, the samples from these two patients more than 1.5 years post BMT showed that BCR-ABL was persistently negative in both bone marrow and peripheral blood. Patient RK was negative by DNA-PCR and RT-PCR after DLT, indicating that this treatment eliminates CML cells rather than simply suppress them, at least at the sensitivity of detection.

Overall, on initial analysis of contemporaneous samples, I found that 19/24 (79%) gave concordant RT-PCR and DNA-PCR results. This high degree of concordance suggests that patients in remission do not generally harbour a substantial pool of leukaemia cells that do not express BCR-ABL mRNA.

It is of course possible that there might be a small number of dormant or transcriptionally silent CML cells in patients but beyond the level of PCR detection. However, it is clear that, for most patients, there is no clear evidence for a substantial proportion of CML cells that do not express BCR-ABL mRNA.

7.3.4.7. DNA-PCR versus RT-PCR in other leukaemias

Only a small number of studies have addressed whether genomic DNA-PCR or RT-PCR is better for detection of residual disease. Potter and his colleagues in 1992 found that an identical picture was given by both DNA-PCR and RT-PCR for IgH CDR-III in the B-lineage ALL patients (Potter *et al* 1992). Concordance was found not only for the pattern of positivity or negativity but also the patterns of discrete bands of the fragments on a agarose gel in the patients.

However, a report in 1993 found disparities between DNA-PCR and RT-PCR in amplification of the breakpoint between mbr region of BCL2 and JH segments of IgH (Soubeyran *et al* 1993). Four patterns were identified in comparison of DNA-PCR with RT-PCR in the patients in complete remission: i) DNA+ / RNA- (36%); ii) DNA+ / RNA+ (4%); iii) DNA- / RNA+ (16%) and iv) DNA- / RNA- (44%). In the same study three PCR patterns were found in the patients before treatment: i) DNA+ / RNA- (25%); ii) DNA+ / RNA+ (50%); iii) DNA- / RNA+ (25%). This situation is clearly different from CML, and is difficult to explain fully. At diagnosis or before treatment the DNA- / RNA+ results may result from splicing, for example, if the primer site were relatively widely separated in genomic DNA but were spliced close together in the mRNA. The length of fragment to be amplified from genomic DNA could have been

beyond the limit of PCR. The DNA+ / RNA- results could either be explained by a lack of translocation of the fusion gene or an uncharacterised splicing event that remove one of the primer binding sites from the mature mRNA. Alternatively, the DNA+ / RNA- results could have arisen due to downregulation of expression of BCL2. It is interesting to note that the frequency of DNA+ /RNA - results was greater in patients who were in complete remission. This could indicate that at least one of the therapeutic reagent, did in fact downregulate BCL2.

CHAPTER EIGHT METHYLATION OF THE BCR-ABL GENE IN CML

8.1. Introduction

It is generally believed that progression of CML from chronic phase to blast crisis is the consequence of multiple genetic alterations that accumulate in malignant stem or progenitor cells. Much effort has been invested in identifying these genetic alterations and, indeed, certain specific mutations and chromosomal aberrations are found to be associated with the blastic transformation. For example, mutation or deletion of the p53 gene is associated with approximately 10 - 30% of cases in myeloid transformation (Soussi & Jonveaux, 1991; Nakai *et al* 1992; Guinn *et al* 1995) and in murine *in vivo* study it has been demonstrated that loss of p53 function play an important role in blastic transformation of CML (Skorski *et al* 1996). Homozygous loss of the p16 gene is found in approximately 50% of cases in lymphoid transformation (Sill *et al* 1995; Serra *et al* 1995). Nevertheless, there is thus far no distinct molecular finding commonly related to tumour progression in CML that precedes and can, therefore, predict blastic transformation. In the search for an such event, changes in the pattern of DNA methylation have been a consistent finding in cancer cells. Both hypo- and hypermethylation have been observed at various loci. *De novo* methylation of normally unmethylated CpG islands and increased expression of DNA (cytosine-5)-methyltransferase are common characteristics of immortalised cell lines and human tumours (Laird & Jaenisch, 1994). In CML, a part of the BCR-ABL gene was found to undergo progressive *de novo* methylation during the course of disease (Zion *et al* 1994). Whether *de novo* methylation in CpG island of the BCR-ABL gene is a marker of disease progression in CML is still unclear. Is methylation status a feature of primitive cell compartment of CD34+ cells differentiating into normal tissue or is it related to truly transformed cells? To address these questions, I performed a preliminary analysis

to determine whether there are any changes in methylation in CpG island in the BCR-ABL chimaeric gene during disease progression, especially at the onset of blast crisis.

8.2. Materials and methods

8.2.1. Patients

Eight CML patients in chronic phase and seven different CML patients in blast crisis studied. Samples from patients in transformation had more than 50% blasts.

8.2.2. DNA extraction and quantification

Genomic DNA was extracted as a standard protocol (see Chapter 2). DNA was purified by using a QIAGEN kit (Germany) according to the manufacturer's instructions. DNA integrity was determined by running an agarose gel.

8.2.3. Determination of methylation status

8.2.3.1. Principle of determination

The aim of this study was to identify site-specific methylation in the CpG island immediately upstream of ABL exon Ia in CML patients. In general, methylation of CpG islands is usually at the C5 position of the cytosine residue in the dinucleotide sequence 5'...CG...3'. This sequence can be recognised by the restriction enzyme Hpa II when it is part of a CCGG sequence. This restriction enzyme will cut, however, only when the recognition site is unmethylated. If the site is methylated, Hpa II will not cut. Therefore, a PCR and Southern blotting strategy can be designed to detect the methylation status of specific Hpa II sites. After digestion of genomic DNA with Hpa II, PCR is performed using a pair of primers flanking the target site or sites; if the site

or sites were methylated, then the PCR results in an amplification product. If the DNA was not methylated, then PCR can not amplify the fragment since the fragment was digested with Hpa II. Similarly, Southern blotting can detect the methylated fragment after Hpa II digestion when a probe is designed to hybridise to a region next to the Hpa II site. Methylated sites give rise to larger bands than unmethylated sites (Figure 8-1).

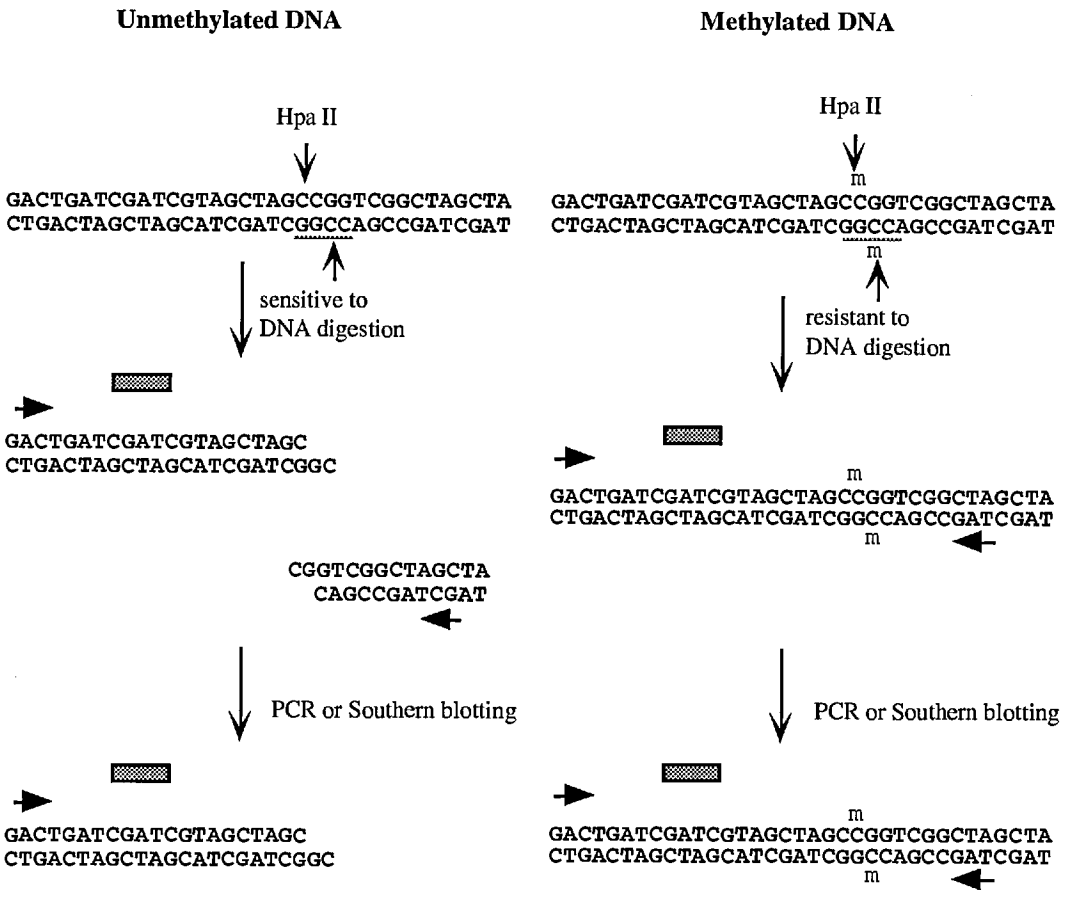


Figure 8-1. Diagram of detection of DNA methylation status

Stippled boxes indicate position of DNA probe for Southern blotting. Horizontal arrows represent the PCR primers. Hpa II restriction enzyme sites are underlined. 'm' indicates the methylated Hpa II site. After DNA digestion with Hpa II, PCR can not amplify the unmethylated fragment but Southern blotting of genomic DNA can detect the digested products. For the methylated DNA, PCR can amplify across the Hpa II site.

8.2.3.2. Procedures for defining methylation status

The method of analysis used was precisely the same as that described by Zion *et al* (1994). One μg of genomic DNA was digested with 20 units of Hpa II in 50 μl final volume for 6 hours. A further 10 units of enzyme was added and digestion continued overnight. 5 μl of the DNA was loaded on an agarose gel to check for digestion. 1 μl of the well-digested DNA containing 20 ng of DNA in each sample was pipetted into 25 μl of PCR reaction mixture, and amplified for 25 cycles of 100°C, 10 seconds; 97°C, 15 seconds; 58°C, 25 seconds; 60°C, 25 seconds; 78°C, 10 seconds; and 72°C, 50 seconds on a MJ Research Inc. programmable heating block with PCR primers, CG20 and Ia3 (Table 8-1). The region between the two primers contained three Hpa II sites immediately upstream of the exon Ia of the ABL gene (Figure 8-2). 20 μl of the PCR products were loaded into a 50 μl volume well on a 200 mm x 120 mm

Table 8-1. PCR primers used in methylation study

CG20	5'CGGGGGGGCGCGCGGGCCGAGC3'
Ia3	5'AGATAACAGCTGGAGGACGAGGAC3'
B2A	5'TTCAGAAGCTTCTCCCTGACAT
169BA-	5'AGCAGCAGCCACTCAGGGAGAAAC3'
GLMNABL	5'TTGAAAACAACAGAGAAGTCCAA3'
JGZ4	5'AGGaatTCCACCCAGGAAGGAC3'
Z3BW3	5'GCAACTCATGTGTTACCAGCCTT3'

agarose gel. The gel was blotted as described in Chapter 2 and hybridisation was carried out in aqueous solution with end-labelled oligonucleotide probe Ia 5 at 42°C for 2 hours or overnight. A brief wash was carried out with 6 x SSC at room temperature first and then again with 6 x SSC at room temperature for 5 minutes. The filter was exposed to film for 6 hours or overnight. Bands on the film indicated the three enzyme sensitive sites are all methylated at least in a proportion of cells. No bands indicated that at least one of the three sites is not methylated. The intensity of bands was graded visually as strong (+++), medium (++) or weak (+).

8.2.4. Controls in this study

Several important controls were employed in this study: DNA digestion control, DNA quality control and DNA amount control.

First, a control for complete DNA digestion is crucial for determining genuine methylation status. False positive results could arise due to incomplete digestion with Hpa II. Even if a small amount of DNA remain undigested, this could give rise to a

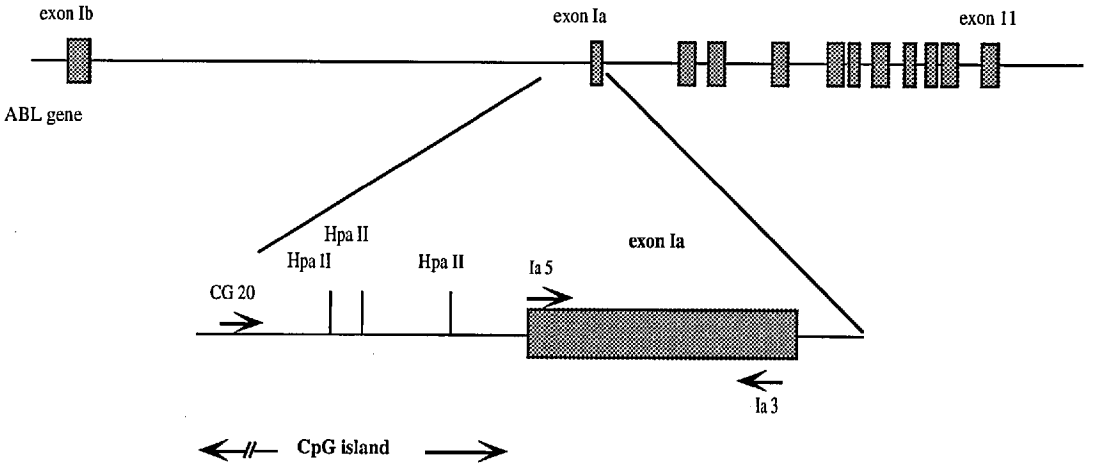


Figure 8-2. Schematic organisation of the ABL gene and restriction map of the CpG island containing exon Ia and its 5' flanking region

The short arrows indicate oligonucleotides. CG20 and Ia 3 are used as primers for PCR amplification; Ia 5 is used as a probe for Southern blot detection.

PCR product and therefore suggest that a sequence was methylated where it was in fact methylation free. In addition to running digestions on a minigel, two PCR amplifications were performed: the first, digested samples were amplified with primers CG20 and GLMNABL. The primer annealing site for GLMNABL, which was derived from Bubble PCR in the previous study (see Chapters 4 and 7), is 996 bp downstream of exon Ia. The amplification product with these two primers should be about 1,160 bp in size. This fragment contains 7 Hpa II sites in addition to the 3 sites in the CpG island. The second PCR was performed with primers JGZ4 and Z3BW3 in the M-BCR

flanking two Hpa II sites. Genomic DNA from patients in chronic phase was amplified for both before and after digestion with Hpa II. In all cases PCR products were generated from the undigested DNA, but not the digested DNA, indicating that the digestion was complete. Another second indirect control was also performed. DNA was digested with Msp I, a isoschizomer of Hpa II that is not inhibited by the methylation status. PCR with primers CG20 and Ia3 was performed after Msp I digestion and an amplification product could indicate incomplete digestion, suggesting that DNA sample was impure and that it might have been possible that the Hpa II digestion was also incomplete. However, all samples tested in this way yielded no band after Msp I digestion.

Second, a DNA internal positive control was used to ensure that a negative result was not caused by inhibition of the PCR reaction or degradation of DNA. PCR was performed with primers B2A and 169BA- (Table 8-1) in the M-BCR zone 2 for each Hpa II-digested DNA. This target region did not contain Hpa II enzyme sites and, as expected, all Hpa II-digested samples tested yielded an 310 bp band after amplification. In addition, false negative results could be caused by undescribed polymorphisms in the CG20 and Ia3 primer binding sites. To demonstrate that this was not the case, undigested DNAs were amplified with these primers and probed with the internal oligonucleotide Ia 5. Again, as expected, a 166 bp band was generated from all samples tested indicating the absence of polymorphisms, at least on one allele.

Third, the amount of DNA tested was carefully measured to ensure that intermediate density of positive bands after Southern blotting truly reflected partial methylation status and not simply different numbers of starting DNA molecules. The amount of DNA was measured both by a spectrophotometer and by competitive PCR (see Chapter 7).

Finally, negative controls were always included to detect false positives resulting from reagent contamination. At least one reaction without DNA sample was run alongside each set of PCRs and no contamination was detected.

8.3. Results

8.3.1. Chronic phase DNA

Of eight chronic phase patients tested, DNA was digested with both Hpa II and Msp I enzymes in two patients. The remaining patients were analysed only after digestion with Hpa II. In all samples, a positive band was detected when the oligonucleotide Ia 5 was hybridised to CG20-Ia3 PCR products, suggesting the presence of methylated ABL Ia promoter at least in a proportion of cells. In Msp I digested samples no positive bands were detected (Table 8-2).

The intensity of positive bands for chronic phase samples was variable from patient to patient. For five patients, the whole procedure was repeated; the relative intensities of each positive band remained the same. The strongest signal (+++) was consistently found for patient 1730. Medium positivity (++) was detected for patients 1633, 1472 and p132. Weak positivity (+) was found for patients 1573 (Figure 8-3) and 1854 (Table 8-2). In addition one sample from a normal individual was tested. A positive PCR product after Hpa II digestion was found in replicate experiments (not shown). In all cases, however, the signal intensity was less than that for undigested DNA suggesting that not all the DNA tested harboured methylated ABL Ia promoters.

8.3.2. Blast crisis DNA

Of seven blast crisis samples, two were methylation negative in two separate experiments, three were weakly methylation positive (+) in the first experiment and

negative in the second experiment. The remaining two samples were positive in the single assay that was performed: one was weakly positive (+) and the other medium positive (++). In two of those samples, Msp I digestion was also performed, and complete digestion was indicated by the absence of a PCR product (Table 8-2; Figure 8-3).

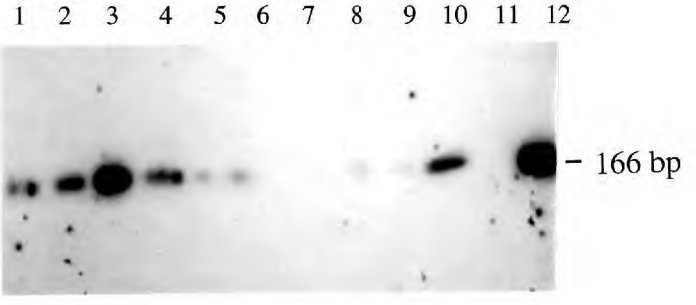


Figure 8-3. Southern blotting for detection of methylation in CpG island region of ABL

Lanes 1 - 5 shows results from chronic phase patients 1633, 1472, 1730, p132 and 1573. Lanes 6 - 10 shows the samples from blast crisis patients p15, ME, MA, RM and p171. Lane 11 is blank. Lane 12 is a sample of undigested DNA.

8.4. Discussion

8.4.1. Methylation status and disease progression in CML

A large number of studies have implicated the methylation of specific genes or genomic regions with the development of malignancy, including leukaemias (Ramsahoye *et al*, 1996). Global genome hypomethylation in most forms of cancer was first recognised in the early 1980s (Gama Sosa *et al* 1983). However, a growing number of specific genomic regions are recognised to undergo hypermethylation during disease progression. Changes in DNA methylation are relevant because methylation status is associated with transcriptional activity: in general expressed genes are hypomethylated whereas non-expressed genes are hypermethylated. Progressive hypermethylation may, for example, lead to a transcriptional block of tumour suppressor genes such as p16

Table 8-2. Results of methylation detection

patient	disease phase	DNA digestion	experiment 1		experiment 2	
			pos/neg	intensity	pos/neg	intensity
1854	CP	Hpa II Msp I	pos neg	+	pos	+
1546	CP	Hpa II Msp I	pos neg	+	pos	++
1633	CP	Hpa II	pos	++	pos	++
1472	CP	Hpa II	pos	++	pos	++
1730	CP	Hpa II	pos	+++	pos	+++
p132	CP	Hpa II	pos	++	pos	++
1573	CP	Hpa II	pos	+	pos	+
ML	CP	Hpa II	pos	+		
p11	BC	Hpa II	neg		neg	
p17	BC	Hpa II	neg		neg	
p15	BC	Hpa II	pos	+	neg	
ME	BC	Hpa II	pos	+	neg	
MA	BC	Hpa II Msp I	pos neg	+	neg	
RM	BC	Hpa II Msp I	pos neg	+		
p171	BC	Hpa II	pos	++		

(Merlo *et al*, 1995) or VHL (Prowse *et al* 1997). Alternatively, hypermethylation may be associated with genes such as calcitonin (Baylin *et al* 1986) that have no clear relevance to malignancy. Several lines of evidence have indicated that changes in DNA methylation may be relevant to CML.

In the majority of CML patients, the BCR-ABL breakpoints fall upstream of ABL exon Ia (see Figure 6-3; Chapter 6). For these individuals, it is possible, therefore, that the

BCR-ABL fusion gene would be transcribed in at least two ways: i) from the BCR promoter leading to the BCR-ABL fusion transcript and ii) from the ABL Ia promoter leading to normal ABL Ia transcripts. However, Zion *et al* (1994) found that the ABL Ia promoter within the BCR-ABL fusion gene was hypermethylated, in contrast to the normal ABL Ia promoter. RNase protection assays demonstrated that methylation of this region was associated with a lack of expression of ABL from the Ia promoter within the BCR-ABL fusion in CML cell lines without a normal chromosome 9. Expression of the untranslocated ABL gene, however, appeared to be unaltered in the cell lines with an intact chromosome 9. In an analysis of primary patient DNA, Zion *et al* found that methylation of the ABL Ia promoter was progressive during the course of the disease. Sample from CML patients at diagnosis were methylation free, but were invariably methylated in advanced phases.

Other studies have also implicated methylation in the progression of CML. For example, an analysis of the M-BCR showed different patterns of methylation between patients which related to both the stage of disease, the position of the breakpoint within the M-BCR and type of BCR-ABL transcript expressed (Mills *et al* 1993). Two patterns of methylation were observed: i) a decrease in Hpa II resistance of the rearranged fragment during disease progression was associated with b2a2 RNA type and a 5' genomic breakpoint; ii) no significant change in the level of methylation of the rearranged fragment during disease progression was related to b3a2 RNA splicing type and a 3' genomic breakpoint. A similar relationship between the position of the breakpoint in the M-BCR and the degree of methylation was also been found in another study (Litz *et al* 1996).

Hypermethylation of calcitonin gene at chromosome 11p15 was also found to increase during progression of CML (Malinen *et al* 1991). This data has been substantiated in a more recent analysis: Mills *et al* (1996) found that a gradual increase in the ratio of methylated : unmethylated calcitonin gene during chronic phase with a dramatic rise at

blast crisis. Their data also suggested that abnormal blast cell populations were expanding continuously, leading to subsequent blast crisis with an increased methylation status of the calcitonin gene (Mills *et al* 1996). The calcitonin gene was the first gene found to undergo tumour-associated hypermethylation in human lung cancer and lymphoma (Baylin *et al* 1986). The methylation status of the calcitonin gene was of particular interest as a marker of malignancy, partly because only one allele became methylated and also because this gene is located inside a hypermethylated hot spot on chromosome 11p15 (De Bustros *et al* 1988) in human neoplasms. The hyper-methylation of the genes on chromosome 11p has been shown to coincide with presence of 'inactive' chromatin patterns in the transcriptional regulatory region (De Bustros *et al* 1988). It is possible, therefore, that hypermethylation of the calcitonin gene serves as a marker for transcription silencing of an as yet uncharacterised tumour suppressor gene.

Patients with lymphoid and acute myeloid malignancies have been demonstrated to undergo hypermethylation of the calcitonin gene (Baylin *et al* 1987). However, not all studies have been consistent. Ritter *et al* (1995) found by a PCR based technique that only one of four CML patients revealed an indication of calcitonin gene hypermethylation during disease progression. The remaining three were apparently unmethylated on transformation (Ritter *et al* 1995).

8.4.2. Methylation results derived from my study

8.4.2.1. Possible explanations for discordant results

In complete contrast to the results obtained by Zion *et al* (1994), I found that the ABL Ia promoter was apparently at least partially methylated in chronic phase cells, but usually unmethylated at blast crisis. It is possible that the differences between the two studies may have come about due to technical problems. Complete digestion of the

target sites with Hpa II is a key problem in methylation analysis. This study was carried out by a PCR based technique; the sensitivity of this technique is so great that an incompletely digested DNA sample could lead to false positive results. In my residual disease analysis, I found that a single step PCR typically had a sensitivity of 10^{-3}, whereas with nested PCR the sensitivity was 10^{-5}. In this situation, therefore, a sample that was 99.9% digested could still be expected to give rise to a PCR product from the 0.1% of DNA that remained uncut.

I used a region of ABL as a control for adequate digestion, which covers the CpG target area. The region between primers CG20 and GLMNABL contains 10 Hpa II sites. Although this control always indicated complete digestion, it is well known that some apparently identical restriction enzyme recognition sites are cleaved less efficiently than others (Thomas & Davis, 1975; Oller *et al* 1991). Hpa II is one of those enzymes for which slow or inefficient cleavage sites have been recognised (Oller *et al* 1991). It is possible, for example, that the three target Hpa II sites are in fact relatively resistant to cleavage and may therefore be prone to giving rise false positive results.

In addition Hpa II is very sensitive to salts, which can partially or completely inhibit the enzymatic activity (Ritter *et al* 1995). However, Msp I is not as sensitive as Hpa II. In my experience, Msp I digestion was complete after a relatively short period of time. Msp I was used in my study as an additional control for DNA digestion, but it could not be excluded that the DNA was completely digested by Msp I but incompletely by Hpa II. This could explain why the sample from patient MA was Hpa II positive but Msp I negative in the first experiment (Table 8-2) but negative for both enzymes in the second experiment.

One way to minimise the problem of false positive due to incomplete digestion could have been to reduce the number of PCR cycles, for example, to 20, so that small

numbers of undigested molecules would not be detected. I was keen, however, to reproduce exactly the conditions that had been described by Zion *et al.*

One solution to the problem of determining whether digestion of a particular target sequence really is fully digested or not was provided by Ritter *et al* (1995) in a study of calcitonin gene methylation. A non-methylated DNA competitor was generated by PCR covering the same target region and amplifiable with the same primers, but giving rise to a smaller sized PCR product. This competitor was added to test samples DNA prior to digestion, incubated with the restriction enzyme. Since the competitor DNA is unmethylated, it should be completely digested by Hpa II. Presence of the competitive PCR product after digestion and amplification therefore was a sensitive indicator of incomplete digestion. A similar competitor would be of great value in determining the methylation status of the ABL promoter.

The target region around ABL exon Ia including the PCR primers is very GC rich. I found that amplification of this region invariably gave rise to several non-specific products and so each gel was blotted and probed with a internal oligonucleotide, as described by Zion *et al.* This second step would also increase the sensitivity, compounding the problems described above.

Secondly, it is possible that different and variable cell types studied may have influenced the results. Previous studies demonstrated that methylation patterns are strongly associated with cell lineage, for example, at the 3' CCGG sites flanking the X chromosomal hypervariable DXS255 locus (Hendriks *et al* 1991). In a study of murine cell lines, the SCL promoter (CpG rich) was hypomethylated in an erythroleukaemia cell line but hypermethylated in fibroblast and T-cell lines (Bockamp *et al* 1995). In addition, some reports have indicated that methylation patterns in cells at different developmental stages were different (Razin & Shemer, 1995). Similar studies for CML also indicated that the methylation status of the M-BCR varies with the cell type and

maturation in the marrow (Litz *et al* 1990; Litz *et al* 1992; Ohyashiki *et al* 1993). These data suggest that samples with different constitution of cell types can not be directly compared to each other. In my study, DNA was extracted from a mixture of cells. It is possible that the methylation positive chronic phase samples were in fact due to a small proportion of methylation positive cells.

Thirdly, the position of the breakpoint in ABL may influence the results. In my analysis (see Chapter 6), 17/24 (71%) patients had breakpoints upstream of ABL exon Ia and 7 (29%) had breakpoints downstream of this exon. Only those patients with breakpoints upstream of exon Ia have the capacity to transcribe ABL Ia transcripts from the normal ABL allele and from within the BCR-ABL fusion gene. If the breakpoint is downstream of ABL exon Ia, the Ia-ABL transcripts will be derived from the normal ABL allele only. Of 15 patients I analysed, I had determined the position of the breakpoint by Bubble PCR in only 3 cases (1854, 1546 and 1472) and so I am unable to draw any clear conclusion about the position of the breakpoint and methylation status. Similarly in the study of Zion *et al* (1994), no attempt was made to establish the relationship between the position of the breakpoint within the ABL gene and methylation status. Since this group found in CML cell lines that it was the ABL Ia promoter within the BCR-ABL fusion gene that was methylated, it will be important in future studies to relate the methylation status of the Ia promoter to the position of the breakpoint in the ABL gene.

8.4.2.2. Is methylation status a true marker of disease progression?

In chronic phase samples, there were marked differences in the apparent degree of methylation from patient to patient (Table 8-2). This may reflect a genuine heterogeneity, perhaps related to disease progression. Alternatively differences in methylation status may depend on the proportion of mature and immature myeloid elements.

If the proportion of immature myeloid cells is closely associated with disease development, and if immature myeloid cells do have a different methylation pattern compared to the mature cells, the methylation status could potentially be used as a marker to monitor the pathological process. However, the methylation pattern of immature myeloid cells, for example CD34 positive cells, is still unknown.

In one of my experiments, analysis of 10 samples (5 chronic phase samples and 5 blast crisis; Figure 8-3) was performed at the same time from purification to the final Southern blot. All chronic phase samples were the methylation positive and most of the blast crisis samples were negative. This experiment suggested that in blast crisis, the exon Ia CpG island is hypomethylated. Hypomethylation of the M-BCR has also been found during disease progression (Mills *et al* 1993). This study demonstrated a significantly low degree of methylation in the BCR-ABL gene at the onset of blast crisis. For patients who had lost exon b3 and expressed b2a2 mRNA, a decreased methylation of the BCR-ABL was observed prior to and during blast crisis. No change in the extent of methylation of the BCR-ABL was found in the patients who had retained exon b3 and expressed a b3a2 mRNA (Mills *et al* 1993).

Zion *et al* claimed that there was progressive methylation in the CML patients from chronic phase to blast crisis. Of 14 chronic phase samples in their PCR study, 10 exhibited an unmethylated pattern and the remainder displayed partial methylation. Of 11 blast crisis samples, 10 were classified as methylated and one was partially methylated. In their study there was no DNA quantification control mentioned. No controls were included to determine the extent of partial methylation, for example serial dilutions of methylated DNA into unmethylated DNA. No controls for complete digestion of DNA were used. Therefore, it cannot be excluded that methylation positivity could have arisen due to technical reasons.

Whether methylation is really a marker for disease progression from chronic phase to blast crisis in CML is still unclear. My experiments and those of Ritter *et al* (1993) suggest that there may be no direct association between the methylation of CpG islands in the BCR-ABL chimaeric gene and disease progression, although the number of patients in each analysis was very small. Zion *et al* (1994) found evidence of progressive methylation during disease progression of CML. It is clear that carefully controlled experiments on a large series of CML patients need to be performed before methylation can be accepted as a marker of disease progression. Ideally, various BCR-ABL CpG island should be examined and the methylation status of different cell types in normal and CML individuals need to be defined. Furthermore, methylation status needs to be related to the positions of the breakpoints within the BCR and ABL fusion genes.

CHAPTER NINE GENERAL DISCUSSION AND THOUGHTS FOR THE FUTURE

9.1. The mechanism of chromosomal translocations

9.1.1. Significance of the research

9.1.1.1. DNA recombination is a fundamental feature of genetics

Genomic DNA rearrangements are fundamental to the process of life. Genetic diversity is generated by DNA recombination during meiosis (Alberts *et al* 1994) and many other more specialised forms of DNA rearrangement exist, for example in mating and reproduction in the yeast *Saccharomyces cerevisiae* (Oshima & Takano, 1971), development of the nematode *Ascaris lumbricoides* (Muller *et al* 1991), rearrangement of the immunoglobulin and T cell receptor genes (Gellert, 1994), viral DNA or RNA infection (Popescu *et al* 1990; Yeh *et al* 1995) and evolution of LINE and SINE sequences (Soares *et al* 1985; Gillespie *et al* 1982; Childs & Ling, 1996; Brookfield, 1994).

Meiosis is the process in eukaryotes by which haploid germ cells are formed from somatic diploid cells. Meiosis is typically associated with by crossing over, the reciprocal exchange of segments of DNA between homologous chromosomes. The orderly distribution of chromosomes through meiosis is dependent upon this DNA recombination. Recombination certainly contributes to genetic variability as well and this may be an important factor for its maintenance in evolution. Many genes have been found to be associated with meiotic recombination, for example, RAD50 (Malkova *et al* 1996).

Haploid cells of *Saccharomyces cerevisiae* have two mating-type alleles, designated MATa and MATα, which govern the expression of many mating-type specific genes. MAT switching is accomplished by a highly programmed site-specific homologous recombination event in which mating-type-specific sequences at MAT are replaced by alternative DNA sequences copied from one of two unexpressed donors (Wu & Haber, 1995).

Ciliates have a very complex life cycle that includes programmed chromosome breakage and healing reactions occurring during the development of ciliate macronuclei. Development begins with multiple rounds of replication to form polytene chromosomes. Following this, much of the genome is eliminated. In Euplotes there is a massive excision and elimination of transposon like elements. All genes so far analysed in this species are interrupted by short internal sequences which are spliced out to make the functional, uninterrupted macronuclear genes. Finally, there is massive elimination of other repetitive non-genic sequences. Similarly in Ascaris, programmed chromosome breakage was first observed by Boveri in 1887 in the horse parasitic nematode *Parascaris equorum*. The single pair of germline chromosomes is broken into more than 40 pieces during development of the somatic tissue, and during this process all the heterochromatic regions of the genome are eliminated (Pimpinelli & Goday, 1989).

A central process in the development of a competent immune system is the series of genomic rearrangements that produce mature, functional immunoglobulin or T cell receptor genes in species that have relatively advanced immune systems. In this process, genomic recombination of VDJ segments allows the immune system to maximise the diversity of receptor binding specificity so that B and T lymphocytes can exert their functions in the organisms.

A number of repetitive sequences have been identified in higher organisms, at least some of which are capable of spreading by retrotransposition in a manner similar to

viral replication and integration. One such element is called LINE-1. Of the 100,000 copies of LINE-1 sequences per mammalian genome there are many shared sequence variants representing changes occurring within the propagating LINE-1 elements themselves. These shared sequence variants define families of LINE-1 elements which have spread within specific periods of time (Casavant & Hardies, 1994). Therefore, the variants can be used to mark genome evolution and identify relationship among the species of interests.

DNA recombination in the cases mentioned above is either programmed or is intrinsic to the biological behaviour of the sequences concerned. However, some instances of recombination are not programmed, and can result in pathological aberrations, for example, human diseases associated with cytogenetic abnormalities. The distinction between programmed and incidental DNA rearrangements is somewhat arbitrary, and depends on the point of view. For transposable elements and retroviruses, transposition or integration is a programmed part of their lifestyle, but as far as the host is concerned the event may appear random and unprogrammed. In another way aberrant non-pathological chromosomal abnormalities could also be seen as a part of the evolutionary process, at least if they occur in the germ line.

9.1.1.2. Chromosomal translocations and human malignancies

Chromosomal translocations are clearly documented to be associated with variety of malignancies in humans. A wealth of clinical and experimental evidence has demonstrated that chromosomal abnormalities are associated with establishment or progression of specific diseases. For example, translocations involving in chromosome 11q23 are found in 65% of cases of infant ALL (Taki *et al* 1996) and DNA rearrangements involving the MLL gene account for 58% of cases of infant AML (Sorensen *et al* 1994). In infant ALL, 11q23 abnormalities are known to indicate poor prognosis (Kalwinsky *et al* 1990). Many cases of B-cell malignancy have chromosomal

rearrangements involving 14q32 (Igκ light chain gene locus), 2p13 (Igλ light chain gene locus) (Sandberg, 1990; Haluska *et al* 1989; Rabbitts *et al* 1988). Similarly in T-cell malignancies, chromosome abnormalities frequently affect 14q11 (T-cell receptor-α and δ loci) (Dong *et al* 1995; Rabbitts, 1991), 7q35 (T-cell receptor-β locus) (Kaneko *et al* 1989; Robinson *et al* 1993; Hashim *et al* 1995), or 7p13 (T-cell receptor-γ locus) (Dewald *et al* 1986; Dyer *et al* 1993). In CML, the reciprocal translocation between chromosomes 9 and 22 are consistently found in over 90% of patients (Champlin & Golde, 1985).

Therefore, uncovering the mechanism by which translocations occur in humans would contribute to our understanding of leukaemogenesis and perhaps to development of novel therapeutic strategies.

9.1.1.3. The association between chromosomal translocations and DNA replication

Chromosomal translocation has been postulated to be associated with the process of DNA replication (Bierne & Michel, 1994; Moore & Lohman, 1995). DNA recombination in association with replication has been described previously in T4 bacteriophage and *Escherichia coli* (Alberts & Frey, 1970; Sedgwick & Bridges, 1974). Recombination is considered to be a solution for topological problems during DNA synthesis and replication (Wasserman & Cozzarelli, 1986).

Prokaryotes contain specific sites in their genomes where replication is specifically arrested or slowed down. It is possible that similar regions in humans could be particularly susceptible to genomic rearrangements (Moir *et al* 1992; Yap & Kreuzer, 1991). Illegitimate recombination occurs by a variety of mechanisms. The observation that some rearrangements might be facilitated by a pause in replication could provide a clue in elucidating these processes (Bierne *et al* 1991; Michel *et al* 1989; Painter &

Kapp, 1991). It is postulated that transient replication pauses or blocks create replication intermediates that may be substrates of either homologous or illegitimate recombination reactions.

9.1.1.4. Translocations in association with transcription

In prokaryotes, the biochemistry of DNA transcription has been well-documented (Liu & Wang, 1987; Wu *et al* 1988). DNA recombination in those species may be associated with the transcription (Dul & Drexler, 1988a; Dul & Drexler, 1988b), and it was postulated that transcription might stimulate DNA recombination. A similar association between transcription and recombination has also been found in the yeast system (Voelkel Meiman *et al* 1987; Thomas & Rothstein, 1989a, Thomas & Rothstein, 1989b). In higher eukaryotes, a correlation between translocation and recombination has been suggested on the basis of the fact that transcription of immunoglobulin genes occurs at the time when the cells are undergoing the B cell-specific VDJ recombination events (Blackwell *et al* 1986; Yancopoulos *et al* 1986).

However, it is not clear how transcription stimulates recombination. It is possible that transcriptionally active chromatin structures are more accessible to both RNA polymerase and recombinases. Alternatively, transcription could result in particular topological structures that induce or stimulate recombination.

9.1.1.5. DNA structures and recombination

DNA topological stress and local DNA structures have been suggested as important factors in eliciting recombinational events (Hou & Wei, 1996). Two principle views on DNA recombination may be discerned: 1) recombination is associated with specific DNA sequences, for example, DNA rearrangements in the VDJ segments of the TCR or

immunoglobulin genes, or 2) recombination is related to special DNA structures (Sugawara *et al* 1995; Canosa *et al* 1996).

These two views may not be mutually exclusive. The structure of DNA is of course related to its sequence. It is known for example that DNA triplet sequences can adopt unusual and stable spatial structures (Chastain *et al* 1995). It is possible therefore that particular sequences are prone to adopting particular conformations that are recognised by recombination enzyme complexes. However, what these structures are, and their dependence on particular sequences is not known. It is possible that recombinogenic DNA structures can be formed from quite different DNA sequences (Canosa *et al* 1996) and this might be the reason why the distribution of breakpoints in CML is not found to be closely associated with any obvious sequence features.

9.1.1.6. Illegitimate recombination might be an important pathway of DNA repair in mammalian cells

Investigation into the mechanism of homologous recombination has been intensively carried out in both prokaryotic and eukaryotic organisms (Hishida *et al* 1996; Eisen *et al* 1988; Park *et al* 1996; Roth & Wilson, 1985; Sargent *et al* 1997). In humans, homologous recombination sometimes was found to occur between Alu elements. In human males who have two X chromosomes and no Y chromosome, Y-specific DNA was found to be present at short arm of one of the X chromosomes, which resulted from exchange of genetic material (reciprocal translocation) between terminal parts of short arms of chromosomes X and Y. Sequence analysis revealed that the DNA recombination was in fact homologous, having occurred between two Alu elements in nonhomologous regions (Rouyer *et al* 1987). Similar reports of homologous recombination between Alu elements was found in human ADA deficiency (Markert *et al* 1988), the hereditary bleeding disorder Glanzmann thrombasthenia (Li & Bray,

1993), Lesch-Nyhan syndrome (Marcus *et al* 1993) and the type VI variant of the Ehlers-Danlos syndrome (EDS) (Heikkinen *et al* 1997).

However, non-homologous recombination is frequently found in a number of species, especially in mammalian cells (Green *et al* 1988; Phillips *et al* 1994; Hou & Wei, 1996; Sargent *et al* 1997). Many instances of non-homologous recombination in human genetic diseases have been reported. For example, in human lipoprotein lipase deficiency (Devlin *et al* 1990) recombination was non-homologous although there was an Alu element involved on one side of the junction. Furthermore, many cases of chromosomal translocation in myeloid leukaemias that have been examined in detail appear to result from illegitimate recombination (Tashiro *et al* 1994).

Recently the mechanism by which repair of DNA double-strand breaks (DSB) is made has become an area of intense research. Several model systems have been established. For the yeast *Saccharomyces cerevisiae*, illegitimate recombination is observed only in cells defective in the Rad52 pathway or in artificially constructed cells where there is no homology available for repair by homologous recombination (Kramer *et al* 1994; Moore & Haber, 1996; Sugawara & Haber, 1992). However, in mammalian cells, the repair of DSB by illegitimate recombination occurs with high efficiency (Roth & Wilson, 1988). The current data suggest that illegitimate recombination is an important competing pathway with homologous recombination for chromosomal DSB repair in mammalian cells.

These results are concordant with what I found in CML. In 10 CML patients, I was able to localise the 22q- breakpoint in both the BCR and ABL genes. The translocation in three of these cases was fully characterised. In all cases the sequence at the junctions was non-homologous, indicating that the Ph chromosome forms by illegitimate recombination. In all other cases studied, the recombination event between BCR and ABL was also found to be illegitimate (Groffen *et al* 1984; Heisterkamp *et al* 1985;

Grosveld *et al* 1986; de Klein *et al* 1986; van der Feltz *et al* 1989; Papadopoulos *et al* 1990; Soekarman *et al* 1990; Mills *et al*1992; Litz *et al* 1993; Kodurus *et al* 1993; Chissoe *et al* 1995; Morris *et al* 1996).

9.1.1.7. Cell division and DNA recombination

During meiosis, molecular and cytological analyses clearly indicate that crossover frequencies are not uniform over the length of mammalian chromosomes (reviewed by Lichten & Goldman, 1995). The human genetic map shows a general trend toward expansion near telomeres, and interstitial regions display considerable variation in terms of recombination frequencies per unit distance. In male meiosis of humans and mice, chiasmata show a strong telomeric/subtelomeric localisation. Interstitial chiasmata also show a nonrandom distribution (Laurie *et al* 1981).

DNA recombination has been observed in both mitotic and meiotic cells. There may be distinct molecular pathways for controlling those two types of cell cycling. In the budding yeast, the checkpoint genes RAD9 (G2 phase) (Weinert & Hartwell, 1989), RAD17 (G2 phase) (Weinert, 1992) and RAD24 (G2 phase) (Weinert, 1992) are generally accepted to play roles in mitotic process. These genes encode proteins that may interact directly with DNA during repair process (Lydall & Weinert, 1995). The checkpoint gene DMC1 (prophase arrest) (Bishop *et al* 1992), and RED1 and MEK1/MRE4 genes that encode chromosome structure component and protein kinase, are thought to be meiosis-specific (Bishop *et al* 1992; Xu *et al* 1997). Dmc1 mutants are defective in reciprocal recombination, accumulate double-strand break (DSB) recombination intermediates, fail to form the normal synaptonemal complex, and arrest late in meiotic prophase (Bishop *et al* 1992). The red1 and mek1/mre4 mutations define a unique, previously unrecognised aspect of recombination imposed very early in the process, during DSB formation. The red1 and mek1/mre4 mutations appear to alleviate prophase arrest directly (Xu *et al* 1997). Recently one analysis indicated that meiotic recombination and mitotic DNA damage were both dependent on RAD17, RAD24 and

MEC1 (Lydall *et al* 1996). They suggested that RAD17 and RAD24 were required to generate the primary signal for arrest in both meiosis and mitosis (Lydall *et al* 1996). Therefore, it has been generally accepted that some recombination genes play a role in checkpoint control of cell cycle (Lydall *et al* 1996; Lydall & Weinert, 1996; Xu *et al* 1997).

Checkpoint genes are also known to be important in maintenance of genomic DNA stability during cell cycling. For example, p53 functions as a checkpoint by arresting the cell cycle of G1 in response to DNA damage (Kuerbitz *et al*, 1992). DNA topoisomerase II is an essential nuclear enzyme involved in major cellular functions such as DNA replication, transcription, recombination, and mitosis (Roca & Mezquita, 1989; Sperry *et al* 1989; Heck *et al* 1989; Holm *et al* 1989). One experiment indicated that wild type p53 gene functioned as a transcriptional repressor of topoisomerase II α gene expression (Wang *et al* 1997). Inactivation of p53 may reduce normal regulatory suppression of topoisomerase II α and contribute to abortive cell cycle checkpoints, accelerated cell proliferation, and alterations in genomic stability associated with neoplasia.

The normal ABL gene has recently shown to be necessary for G1 phase arrest in response to DNA damage (Yuan *et al* 1996). On basis of the evidence in yeast that some checkpoint genes are involved in DNA recombination, it is possible that the activity of ABL gene may also be related to DNA recombination.

9.1.1.8. Methylation and chromosome translocation

Several lines of evidence suggest an association between DNA methylation with gene mutations and chromosome translocation (Laird & Jaenisch, 1994; Litz *et al* 1996; Jones & Gonzalgo, 1997; Lengauer *et al* 1997). Global hypomethylation or regional hypermethylation gives rise to changes in gene expression, chromatin structure and DNA replication (Laird & Jaenisch, 1994). Also, methylation has been found to be

associated with the DNA mismatch repair system and genetic instability (Lengauer *et al* 1997). Specifically, mismatch repair competent colorectal cell lines were found to be deficient in their ability to methylate reporter constructs, whereas mismatch repair deficient lines were methylation proficient. Taken together, it is possible that methylation facilitates chromosome translocations, and also the genomic instability frequently associated with cancer development and progression.

In support of this hypothesis, Litz *et al* (1996) found methylation abnormalities in the Ph-negative cells of three patients in remission after autografting or treatment with interferon-α, it was suggested that aberrant methylation patterns could have appeared prior to the DNA rearrangement of the M-BCR in CML. If this is true, aberrant methylation could be an event upstream of the chromosome translocation in CML. Studies of clonality in Ph-positive and Ph-negative cells in CML patients have also suggested that acquisition of the Ph-chromosome may not be the initial event in CML (Fialkow *et al* 1980).

9.1.2. Future directions of the research

Investigation of mechanism of chromosomal translocation in CML has been carried out for nearly 40 years, since Ph chromosome was found in 1960. The majority of studies have focused on one-dimensional genomic DNA features that may play a role in the translocation, for example, chromosomal telomeres, aberrant DNA methylation, mobile genetic elements, repetitive sequences, protein binding motifs, or other conserved sequences. It is clear from the current data however that no consistent sequence features are located at the BCR-ABL translocation breakpoints. My data has demonstrated that it is likely that staggered cuts or gene conversion are involved in the translocation process. In addition I have found that the translocation is frequently associated with very short regions (2 - 5 bp) of homology. However, this does not give a whole picture of the formation of the t(9;22).

It is likely that the Ph-chromosome will be understood better by consideration of the translocation within the three dimensional framework of chromatin structure. For example, one report in a study of MLL gene demonstrated an association between breakpoint position and the chromatin scaffold attachment region (Broeker *et al* 1996). In addition, studies using mini-chromosome VDJ recombination substrates have indicated that a change in methylation status may be associated with a change in chromatin configuration and chromatin accessibility to recombination machinery in the cells (Hsieh & Lieber, 1992). At the present time relatively little is known about the details and variation in local chromatin structure. In the future, such knowledge might help to explain the illegitimate recombination events that lead to chromosomal translocations. In CML, it will be necessary to complete the sequence of ABL intron 1 (Chissoe *et al* 1995) and determine the structural features associated with the BCR and ABL genes. Such features include scaffold attachment regions, relationship between methylation patterns or histone acetylation and chromatin spatial structure, and definition of any recombination hot spots. They are also likely to include three dimensional features that are not yet fully understood or elucidated.

Further investigation of the pathways and genes involved in both DNA recombination and the cell cycle will also be important. For example, checkpoint genes could regulate or modulate apoptosis, genomic structure, DNA replication and gene transcription via various pathways, maintaining integrity of the genome or leading to genomic instability. Clarifying the functions of the BCR, ABL and BCR-ABL gene products would help to link these pathways. A better understanding of these pathways may lead to a better understanding of chromosomal translocations.

9.2. Minimal residual disease

9.2.1. Significance of the study

Allogeneic BMT is generally considered to be the most effective therapeutic procedure for patients with CML (Goldman *et al* 1986; Thomas *et al* 1986). The probability of sustained remission varies according to the transplant procedure and stage of the disease when BMT is performed. For patients allografted in chronic phase, a low incidence of relapse is observed when unpurged donor marrow is transplanted (Goldman *et al* 1986). If the donor marrow is manipulated by T cell-depletion a very high incidence of disease recurrence is observed (Goldman *et al* 1988). A dramatic increase in the incidence of relapse is also seen if patients are grafted in a more advanced phase of the disease, particularly in blastic transformation (Goldman *et al* 1986; Bortin *et al* 1993).

Minimal residual disease is defined as those relatively few leukaemic cells which have survived initial remission-induction treatment. Monitoring minimal residual disease is considered to be an important procedure for therapeutic decision and medical care for the patients post BMT. A number of groups have developed effective approaches to detect the residual leukaemic cells in patients and several ways have been explored for this purpose in the past decade, for example, haematological and morphological examination, cytogenetics, Southern blot, FISH, immunological assays or PCR based methods. Each technique has particular advantages and disadvantages and no single technique is applicable or the best for all situations. However, there has been considerable interest in the use of PCR for detection of minimal residual disease due to its highly efficiency, sensitivity and specificity.

RT-PCR was considered to have great potential as a method to monitor expression of the BCR-ABL chimaeric gene in CML patients post BMT (Goldman & Hughes, 1991;

Hughes *et al* 1991a; Delage *et al* 1991; Morgan *et al* 1989). However the early literature is somewhat confused, partly due to unexpected findings, such as the persistence of BCR-ABL positive cells for many months in patients who remain in sustained remission, but also due to false positive and false negative assays. An RT-PCR strategy was established for CML (Hughes *et al* 1990; Hughes *et al* 1991b). In this study a series of precautions were taken to minimize contamination and several controls to detect both false-positive and false-negative results were used. The results obtained were re-evaluated by at least two separate RNA samples (Hughes *et al* 1991b). Once appropriate controls became established, several consistent RT-PCR patterns of minimal residual disease were identified (Cross *et al* 1993b; Miyamura *et al* 1993; Pichert *et al* 1995).

It became apparent that qualitative PCR did not provide sufficient information to enable accurate prediction of relapse after BMT for CML. Several groups found that patients who were persistently PCR positive, or PCR positive within certain time windows were more likely to relapse than those patients who were PCR negative, but some individuals remained persistently PCR positive without relapsing. Similarly qualitative PCR was of little use in determining patient response after treatment with interferon or other regimens. In response to this, several different quantitative PCR assays were established based on the principle of competitive RT-PCR (Lion *et al* 1992; Malinge *et al* 1992; Cross *et al* 1993a; Thompson *et al* 1992; Ferre, 1992). These techniques enabled the absolute or relative number of BCR-ABL mRNA molecules in samples to be estimated, and therefore, the actual amount of residual disease. In some patients there was a clear evidence of rising numbers of BCR-ABL transcripts before routine detection of cytogenetic relapse. Conversely patients without cytogenetic relapse generally had low or falling numbers of transcripts (Lion *et al* 1992; Cross *et al* 1993a). These techniques produced more information than conventional cytogenetics or nonquantitative PCR. They enabled identification of patients in need of therapeutic intervention before the

onset of overt relapse (Lion *et al* 1992; Cross *et al* 1993a), and enabled determination of patient response to interferon-α treatmeant (Hochhaus *et al* 1996).

RT-PCR relies on the detection of BCR-ABL RNA. Some experiments however have revealed that some Ph positive cells may not actively express the BCR-ABL fusion gene (Keating *et al* 1994; Bedi *et al* 1993). These transcriptionally quiescent CML cells could be present in large numbers but would not be detected by RT-PCR. After activation they could potentially lead to relapse. This could mean that remission status of patients who are RT-PCR negative could be misinterpreted. However, a PCR method that used for genomic DNA rather than mRNA would enable these transcriptionally quiescent CML cells to be detected. Since the BCR-ABL breakpoints in CML fall into a very large genomic region in ABL gene and the location of the breakage is extremely variable from one patient to another, PCR primers have to be designed for individual patients. This in itself is not prohibitive, for example, patient specific primers are routinely designed for specific VDJ junctions in order to monitor residual disease after treatment of ALL or other lymphoid malignancies. In CML, however, the problem is more difficult as the breakpoints between patients are so variable. In my study, I found that Bubble PCR is the best choice to establish DNA-PCR strategy for detection of minimal residual disease at the genomic DNA level (see Chapter 7). In my study, patient specific PCR primers were generated from Bubble PCR in 10 patients who had been transplanted. The results suggested that there was no substantial pool of quiescent CML cells in the patients investigated after transplantation. However, I did find some instances of samples that were apparently DNA positive, RT-PCR negative and therefore, it might be useful to make further comparisons between the two techniques in a larger group of patients undergoing a variety of treatment regimens.

9.2.2. Future directions

PCR techniques have redefined the concept of remission. Whilst most PCR assays to detect minimal residual disease are performed in highly specialised research laboratories, it is likely that in the future these assays will become increasingly routine and automated. For most diseases, including CML, a precisely standardised methodology has not yet been agreed upon. It is possible that DNA-PCR will play an important role in the detection of minimal residual disease in CML in the future. DNA-PCR has a number of advantages over RT-PCR: 1) The procedure for DNA extraction is simpler than that for RNA. 2) DNA is relatively stable, whereas RNA is extremely susceptible to degradation due to contaminating RNases. 3) The reverse transcription step in RT-PCR can lead to variation between assays and is a potential source of assay failure. 4) High quality DNA can be extracted from samples stored in a variety of ways, even slides, but good quality RNA can only be extracted from cells stored at -70°C or colder, or cells that have been lysed in appropriate buffers. 5) DNA-PCR can detect leukaemic cells whether they express BCR-ABL mRNA or not. 6) Quantification of the number of leukaemic cells is theoretically more accurate than RT-PCR since each CML cell contains one or two copies of the Ph-chromosome but may contain a variable number of BCR-ABL transcripts. 7) PCR contamination is less of a problem for DNA-PCR compared to RT-PCR partly because fewer steps are involved but mainly because of the different patient-specific primer combinations that are used.

These advantages suggest that DNA-PCR should not be overlooked as an alternative to RT-PCR, provided that the system can be considerably simplified. Whatever technique is eventually established as the standard, it is clear that PCR based detection of minimal residual disease will result in considerable and routine improvement of the clinical management of patients with CML.

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Characterization of genomic BCR-ABL breakpoints in chronic myeloid leukaemia by PCR

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Summary. In order to understand better the mechanism of translocation between the BCR and ABL genes in CML, we have exploited a 'bubble PCR' technique to clone genomic breakpoints. BCR-ABL junction fragments were successfully amplified and sequenced in 14/32 (43%) patients tested. Breakpoints were dispersed throughout the major breakpoint cluster region without any clustering or hot spots. In three cases Alu sequences were found at or near the breakpoint on the ABL side of the translocation but no other obvious sequence homologies were found either in BCR or ABL. The translocation event was characterized further in three other patients by amplifying the reciprocal ABL-BCR junction on the 9q+ chromosome and also normal ABL around breakpoints. In two of these patients a few nucleotides of BCR and ABL were either duplicated or deleted on translocation, suggesting that staggered cuts had been made in the DNA strands prior to recombination. In the third patient 50 bp of ABL was deleted and 159 bp of M-BCR including exon b3 was duplicated, indicating either that the single-stranded cuts may span a larger distance than previously thought or that another mechanism, perhaps involving gene conversion, may be involved in this instance.

Keywords: CML, BCR-ABL, breakpoint, translocation.

More than 90% of patients with chronic myeloid leukaemia (CML) have the Philadelphia chromosome (Ph or 22q−), which results from the acquired reciprocal translocation t(9;22)(q34;q11). At molecular level, this translocation is due to the aberrant joining of the ABL proto-oncogene on chromosome 9 with the BCR gene on chromosome 22, giving rise to a novel chimaeric gene (Groffen & Heisterkamp, 1987). BCR-ABL is transcribed and translated into a 210 kd (P210) fusion protein that is considered central to the pathogenesis of CML (Daley *et al.* 1990). The reciprocal product of the translocation, ABL-BCR on the 9q+ chromosome, is also transcribed in ~70% of cases of CML, but its significance in the pathophysiology of the disease is unknown (Melo *et al.* 1993).

Genomic DNA breakpoints are clustered in a region of 4–5 kb, termed the major breakpoint cluster region (M-BCR), in the middle of the BCR gene (Groffen & Heisterkamp, 1987). There are four exons in the M-BCR, conventionally referred to as exons b1–b4; these correspond to exons 12–15 of the complete gene. Most patients have intronic breakpoints in the ~3 kb region between exons b2 and b4 (Mills *et al.* 1991). In ABL, breakpoints are dispersed over a much larger region (~200 kb), although pulse field gel analysis has suggested that many breakpoints may cluster in three regions of the very large first intron of this gene (Jiang *et al.* 1990). Characterization of breakpoints in BCR and ABL has therefore traditionally required the construction of genomic libraries from each patient. In the great majority of patients, splicing of the primary fusion transcript results in an mRNA in which either BCR exon b2 or b3 is fused to ABL exon 2 (b2a2 or b3a2 transcripts). In Ph-positive acute lymphoblastic leukaemia (ALL) about 70% of patients have a break in the first intron of the BCR gene which leads to a p190 BCR-ABL protein. The remaining 30% of patients express a p210 BCR-ABL indistinguishable from that found in CML (Maurer *et al.* 1991).

The mechanism that underlies translocation t(9;22) in CML is unclear, partly because relatively few breakpoints have been fully characterized. Large regions of BCR downstream of the breakpoint on the 9q+ derivative are deleted in a minority of patients (Popenoe *et al.* 1986). The DNA sequence of the M-BCR has been determined (Sowerby *et al.* 1993) and found to contain a number of features that may be relevant to the mechanism of translocation, including an Alu element. However, detailed Southern blotting has shown that only a small minority of BCR-ABL breakpoints actually fall within this Alu sequence (Mills *et al.* 1991). Thus far, BCR-ABL breakpoints from nine CML

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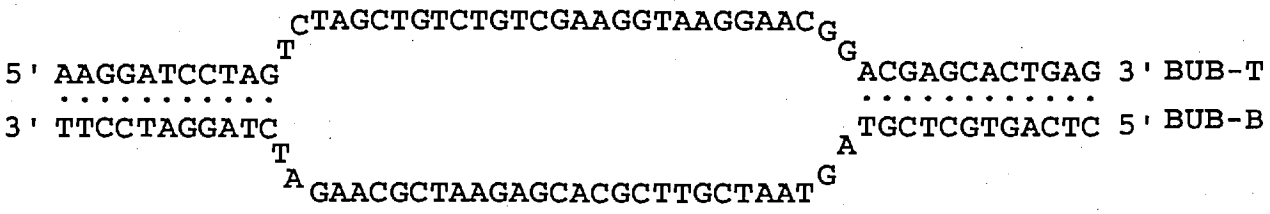


Fig 1. The bubble oligo formed by annealing the two oligos BUB-T and BUB-B.

patients have been characterized (Heisterkamp *et al*, 1985; de Klein *et al*, 1986; Mills *et al*, 1992; Sowerby *et al*, 1993; Litz *et al*, 1993), but no clear pattern has emerged. In order to gain a better understanding of the BCR-ABL translocation in CML, we have used a one-sided PCR technique – bubble PCR – to enable the relatively rapid amplification of breakpoints. Here we present the use of this technique to amplify and sequence BCR-ABL breaks in 14 CML cases. In three of these patients the translocation was completely characterized by deriving sequences from the 9q+ break and the corresponding regions of the normal chromosomes 9 and 22.

MATERIALS AND METHODS

Patient material. DNA was isolated from the cryopreserved blood or bone marrow cells of Ph-positive CML patients as described (Sykes, 1983). In some cases the approximate position of the BCR-ABL breakpoint was determined by Southern blotting (Jaubert *et al*, 1990) and the transcript type (b2a2 or b3a2) determined by RT-PCR (Cross *et al*, 1994).

Bubble PCR. The bubble oligo was a duplex that contained 12 bp of complementary region at each end and 29 bp of non-complementary sequence in the middle (Fig 1). The two unphosphorylated oligos BUB-T and BUB-B were annealed at a concentration of 136 μM at 4°C in 50 mM Tris pH 8·0, 10 mM $MgCl_2$, 50 mM NaCl for 16 h. 5 μg of genomic DNA was digested with the restriction enzyme RsaI and ligated to 1·5 μg double-stranded bubble oligo (Fig 2) in a volume of 20 μl (Sambrook *et al*, 1989). The reaction was denatured for 5 min at 100°C, cooled, and remaining unligated oligos removed by using a Geneclean kit (Bio 101). A BCR-specific primer was annealed to one-fifth of the ligated DNA and extended by Taq polymerase to the end of the bubble oligo. Usually three reactions were performed using different BCR primers (JGZ1, JGZ3 or Z4A). Amplification of this strand was achieved by PCR (96°C, 20 s; 66°C, 50 s; 72°C, 50 s; for 30 cycles) using the same BCR primer and a second primer, nvamp-1, which was complementary to the newly synthesized unique sequence of the bubble oligo. 1 μl of the reaction was reamplified with internal primers (either JGZ2, JGZ4 or Z4B and nvamp-2). The sequences of the primers used are listed in Table I and their positions in the BCR gene are shown in Fig 3. Assuming that a RsaI site occurs in the ABL sequence not too distant from the breakpoint and that there are no RsaI sites in BCR between the BCR primer used and the break, two amplification products are expected: one from the normal BCR gene and the other from the rearranged BCR-ABL. Rearranged bands were gel purified and directly sequenced or cloned into M13, and sequenced by the dideoxy-mediated chain-termination method (Sambrook *et al*, 1989). Sequences surrounding the breakpoints were submitted to the GenBank database under the accession numbers U19397–U19410 as annotations to the full-length BCR sequence (HSU7000).

RESULTS

Bubble PCR was performed on DNA from 34 Ph-positive CML patients. BCR-ABL junction fragments were successfully amplified and sequenced from 14 (43%). The remainder presumably failed to amplify due to lack of a convenient RsaI

Table I. Sequences of the primers used in this study. Lower case letters indicate bases that were changed in order to introduce restriction enzyme sites for cloning.

FORWARD PRIMERS	
1. JGZ1	CAGGGCACCTGCAGGGAGGGCA
2. JGZ2	CCTGAAttCTGATCCCCCCTTC
3. JGZ3	GTTTGGGGAGGAGGGTTGCAGC
4. JGZ4	AGGaatTCCACCCAGGAAGGAC
5. Z4A	GTGGAGACAGAAAGCTTACCA
6. Z4B	GGGAATtcGGTTGGGAGAGAGGA
BACKWARD PRIMERS	
1. NCB3-3	GAATGAACTTGAGACGAATTTAGG
2. NCB3-2	ACTGTAAGTCTTTGGGTATCTCG
3. Z3BW3	CGTTGAGTACACAATGGTCGGAA
4. Z3BW4	AAGTcttAAGACTGTCGAGGTCATC
5. Z4BW3	CCTTTGTGGTCGCAAATACTCCG
6. Z4BW4	GTCTCTtaagCCGGGTCCTCTTTCT
7. BALAL2	CCTGTATTCTCCTTGTTCAAAC
8. Z4BW-C	AAACttAAgCCCTACGTTCCAACTCAC
BUBBLE PCR PRIMERS	
1. NVAMP-1	TGCTCGTAGTAATCGTTCGCAC
2. NVAMP-2	GTTCGCACGAGAATCGCAAGAT

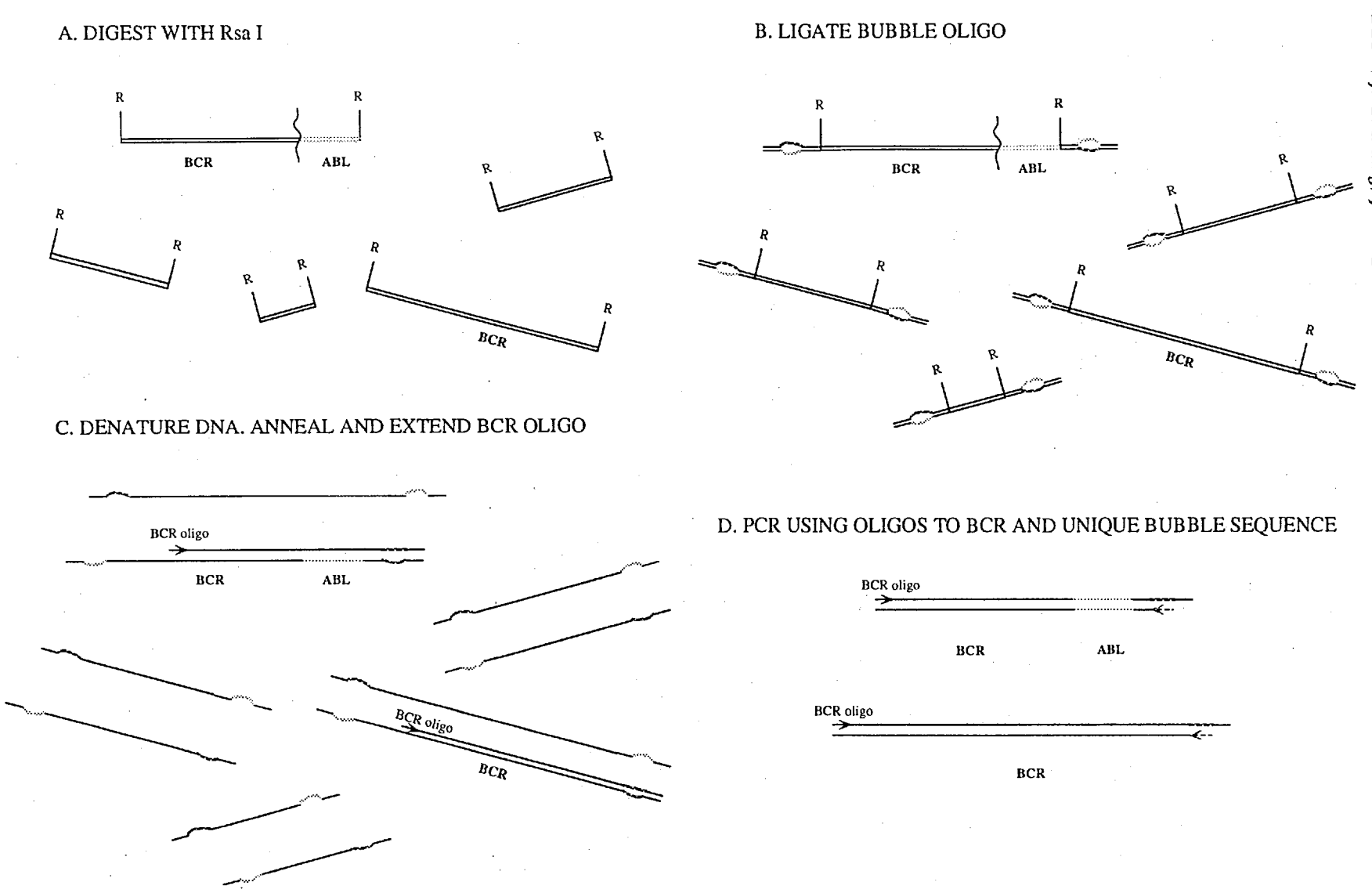


Fig 2. Outline of bubble PCR. Genomic DNA is digested with RsaI and ligated to the double-stranded bubble oligo. At this stage all fragments will have a bubble oligo at each end. Strand synthesis is then initiated by a specific BCR primer and proceeds to the end of the bubble oligo. The bottom strand of the bubble will only be copied for BCR and BCR-ABL fragments; in all other fragments the bubble remains uncopied. An oligo complementary to the copied single-stranded sequence of the bubble oligo bottom strand can therefore be used with a BCR primer to specifically amplify BCR and BCR-ABL by PCR.

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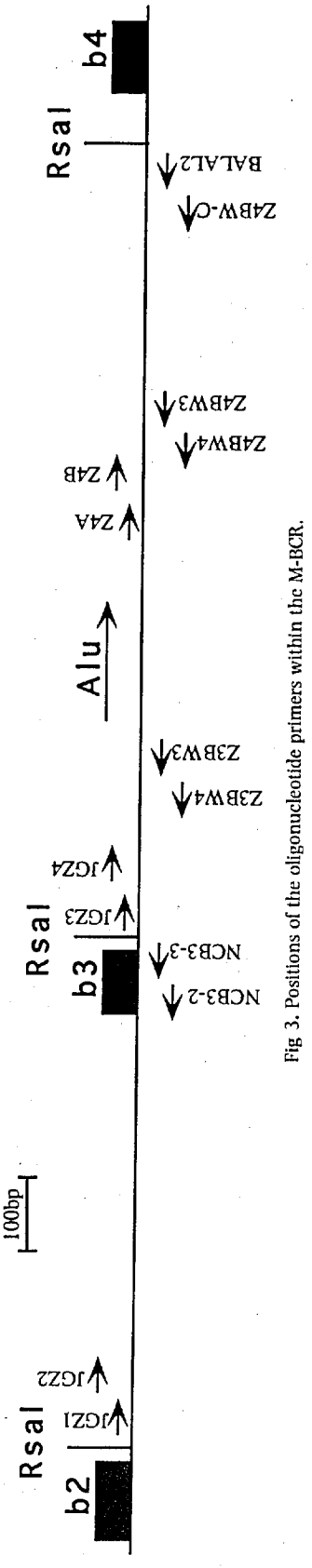


Fig 3. Positions of the oligonucleotide primers within the M-BCR.

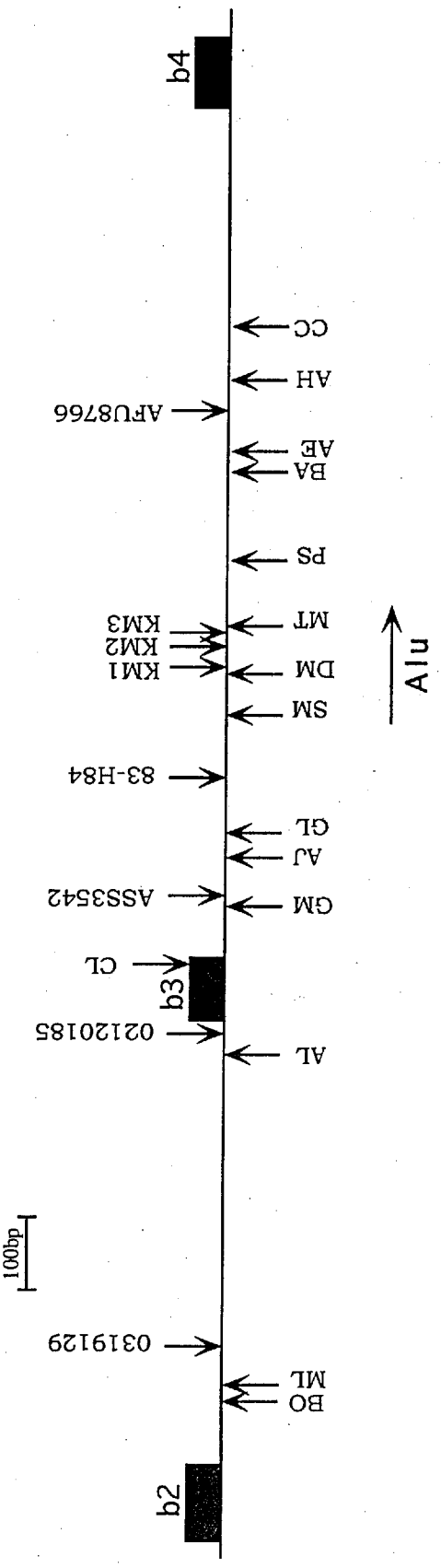


Fig 4. Distributions of BCR-ABL breakpoints within the M-BCR. The 14 patients in this study are shown below the line and nine from other studies above the line. Sources of the latter patients are: 0319129 and 02120185 (Heisterkamp *et al*, 1985), 83-H84 (de Klein *et al*, 1986), ASS3542 and AFU8766 (Sowerby *et al*, 1993), KM1, KM2 and KM3 (Mills *et al*, 1992) and CL (Litz *et al*, 1993).

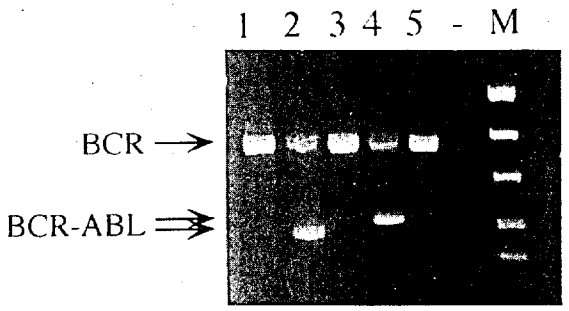


Fig 5. Amplification of BCR-ABL breakpoint fragments in two patients by bubble PCR. Forward primers used were Z4A and Z4B. All patients show a 900 bp BCR band from the primer to the RsaI site upstream of exon b4. Patients 2 and 4 each have an additional smaller band which contains the breakpoint junction. Markers are pEMBL/TaqI (1440, 970, 720, 470 and 360 bp).

site on the ABL side of the translocation. An example of successful bubble PCR amplification is shown in Fig 5 and the distribution of the 14 breakpoints in the M-BCR in Fig 4. Three breaks were in the BCR Alu element and the remaining 11 were distributed throughout the M-BCR. Table II shows the sequences 20 bp either side of the break for each case.

An average of 108 bp of ABL sequence (range 30–244 bp) was determined at each BCR-ABL junction and was used to search the GenBank database (release 81, February 1994). In two cases the break was found to occur within an ABL Alu sequence and in a third an Alu element was found 162 bp downstream of the breakpoint. These patients were different from the three above in whom the break was within the BCR Alu. All three ABL Alus were in the same orientation as the BCR Alu. No other significant sequence homologies were detected.

Table II. BCR-ABL breakpoint sequences for the 14 patients that were amplified by bubble PCR. BCR sequence is underlined. The full ABL sequences that were determined at the breakpoints were submitted to the GenBank database under the accession numbers U19397–U19410.

No.	Patient	BCR/ABL
1.	AE	ATGGATGAATTACATGACAT/TTGCAGAATCAGAAAAANTA
2.	CC	CCCGGGCTTGTCTCTCCTTG/GGTGCAGTGGCTCACACCTG
3.	GL	AATTGCAGGGGTTTGGCAAG/CACCTTTTCATATGTTTACT
4.	BA	CTAAAATTCTTTAAACCCTA/AAGCGGATTTACTCTAAGGC
5.	DM	GATTAGCCAGGCTAGGCAGT/TTTAAATCCTGGCTTTCCCC
6.	MT	GCATTCCAGCCTGGGCGACA/ATGCAAGAAAGAATAGTGAG
7.	ML	TTGATGGGACTAGTGGACTT/ATGACAAAATAACAATATGG
8.	GM	CTCATCGGGCAGGGTGTGGG/TCTTCACTTTCGTAGCTTCT
9.	AJ	TGGGAGCTGGTGAGCTGCCC/ATAATGACATGATTTGGGAT
10.	PS	GGGATTGTTGGGGAATGGGG/CTGCAGTGAGCCGAGATCGT
11.	AH	CCTCATAAACGCTGGTGTTT/GGCTTGTCATAGAAGGGCAT
12.	BO	GCACTTTTGATGGGACTAGT/CCTTATCCTTNGATTNGNTC
13.	SM	TCATGCCTGTAATCCCAGCA/AGGATCCTAGATCTTGCNAT
14.	AL	ACACAGTGTCCACCGGATGG/ATTTTATAAGTTGATCCAAC

PATIENT AH

ABL TGCGGCAAGCAGCTCTGCC**AGCT**TGTCATAGAAGGGCATT

BCR-ABL TCATAAACGCTGGTTGTTT**GGCT**TGTCATAGAAGGGCATT

ABL-BCR TGCGGCAAGCAGCTCTGCC**AGCT**CCCTCGTGGCCTCCCTGCA

BCR TCATAAACGCTGGTGTTTCCCTCGTGGGCCTCCCTGCA

PATIENT DM

ABL CCAAAGTTTGTCTACC**CAGT**TTTAAATCCTGGCTTTCCCC

BCR-ABL GCCAGGCTAGGCAGT.....TTTAAATCCTGGCTTTCCCC

ABL-BCR CCAAAGTTTGTCTACCCA.....ACTGCTTGGGAG

BCR GCCAGGCTAGG**CAGT**GGGCACCTGTAATCA**CA**ACTGCTTGGGAG

PATIENT GM

ABL TTTTATTTTTCTTTCCTGTG(50bp)TCTTCACTTTCGTAGCTTCT

BCR-ABL CTCATCGGGCAGGGTGTGGGTCTTCACTTTCGTAGCTTCT

ABL-BCR TTTTATTTTTCTTTCCTGTTAG***ATGATGAGTCTCCGGGGC***

BCR AGATGATG----159bp duplication-----GGTGTGGG

PATIENT AE

BCR-ABL ATGGATGAATTACAT**GACAT**TTGCAGAATCAGAAAAAGTA

ABL-BCR TACAGATCCCAATAG**GACAT**GCAGATTGCACCTTCATAAC

BCR ATGGATGAATTACAT**GACAT**GCAGATTGCACCTTCATAAC

Fig 6. Sequences surrounding the BCR-ABL and ABL-BCR breakpoints alongside normal BCR and ABL. BCR sequence is underlined and features that are referred to in the text are in bold type. Exon b3 in the ABL-BCR sequence of patient GM is shown in bold italics.

ABL sequences were compared pairwise with each other to see if in any case the breakpoint involved an identical or related region of the ABL gene, but no cross homologies were found. Moreover, the sequences did not contain any chi-like minisatellite recombination signals, GC[A/T]GG[A/T]GG (Krowczynska *et al*, 1990), lymphoid heptamer/nonamer sequences or consensus topoisomerase II cleavage sites, GTN(ATC)A(CT)A(CT)TN(GA)(GT)NN(AG) (Darby *et al*, 1986; Dong *et al*, 1993).

In four cases, BCR primers were synthesized downstream of the breakpoint in the 3′ → 5′ direction of the gene and used to amplify the reciprocal 9q+ (ABL-BCR) break. Two ABL primers were made, one each to the 22q− and 9q+ ABL sequences, and used to amplify the ABL sequence surrounding the breakpoints from a normal individual. In one case no normal ABL product was obtained, despite trying different primers, perhaps indicating that a deletion of ABL sequence had occurred on translocation and therefore the distance between the primers in unrearranged DNA was too large for PCR.

The sequences of the normal and translocated regions flanking the breaks are shown in Fig 6. Due to homologies or mutations at the breakpoints, different interpretations of the events are possible. In patient AH there appears to have been a clean break in the BCR sequence, whereas 3 bp (GCT) of ABL has been duplicated and an extra base (G) inserted at the 22q− break. Alternatively, there may have been a 4 bp ABL duplication (AGCT) with a concomitant mutation of A to G in BCR-ABL. In patient DM the exact positions of the breakpoints are ambiguous due to short stretches of homology (CAGT and CA) between BCR and ABL at the critical sites. However, it is clear that a total of 19 bp of sequence has been deleted by the translocation of which between 1 and 5 bp has been lost from ABL and between 14 and 18 bp from BCR. In patient GM, 159 bp of BCR including the whole of exon b3 is present on both the 22q− and 9q+ derivatives, and has therefore been duplicated during the translocation, whilst 50 bp of ABL has been deleted (Fig 7). Lastly, in patient AE, in whom normal ABL failed to amplify, 5 bp (GACAT) of BCR was duplicated.

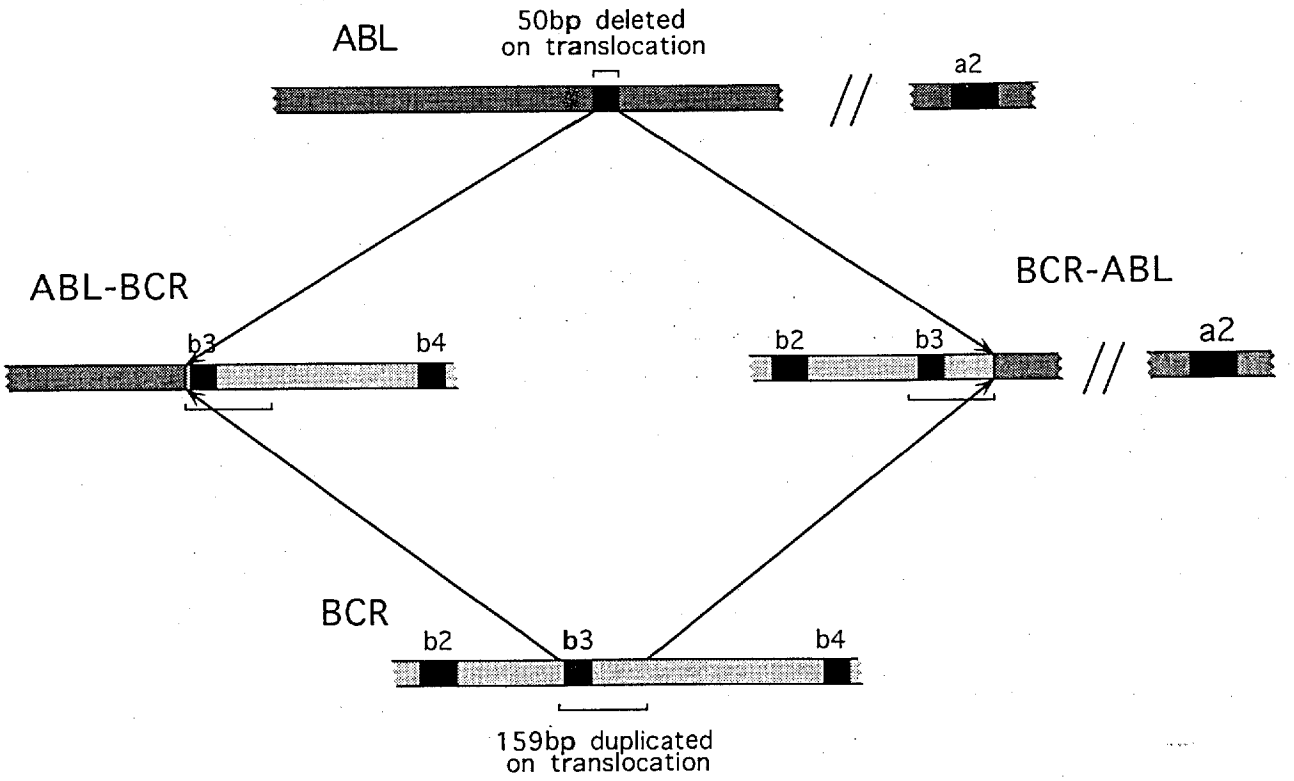


Fig 7. Diagrammatic representation of the translocation in patient GM. 159 bp of BCR including the whole of exon b3 is present on both the 22q− and 9q+ derivatives, and has therefore been duplicated during the translocation, whilst 50 bp of ABL has been deleted.

DISCUSSION

The mechanism by which the 9;22 translocation in CML is formed is unclear. Broadly, there are two possibilities: either there are specific sequence or topological features in BCR and ABL that lead to an increased aberrant recombination frequency, or the propensity for translocation of these two genes is no greater than background but there is selection for b3a2 and b2a2 products by virtue of the growth advantage they confer. These possibilities are not mutually exclusive. Evidence for the former includes the apparent subclustering of breakpoints within ABL intron 1 (Jiang *et al*, 1990) in CML and BCR intron 1 in Ph-positive ALL (Chen *et al*, 1989b), suggesting preferred sites of illegitimate recombination. On the other hand, irradiation of HL60 cells produced a variety of BCR-ABL fusion genes which are not found in CML or ALL (Ito *et al*, 1993), emphasizing the role of biological selection.

We have adapted a bubble PCR technique (Riley *et al*, 1990) to amplify BCR-ABL breakpoints in CML. Genomic DNA is digested with RsaI and ligated to the double-stranded bubble oligo. RsaI was chosen because this enzyme is a 4 bp blunt-ended cutter whose recognition site is under-represented in the M-BCR. Strand synthesis is then initiated by a specific BCR primer and proceeds to the end of the bubble oligo (Fig 2). This means that the bottom strand of the bubble will only be copied in BCR and BCR-ABL fragments; in all other fragments the bubble remains uncopied. An oligo complementary to the copied single-stranded sequence of the bubble oligo bottom strand can therefore be used with a BCR primer to specifically amplify BCR and BCR-ABL by PCR. The single-stranded bubble provides the specificity of the reaction: if a completely double-stranded oligo had been used then all genomic fragments would be amplified in the final step.

The deletion or duplication of a few base pairs of either ABL or BCR at the reciprocal breakpoints suggests that the nuclease involved in generating the translocation can make staggered cuts in the DNA strands. However, in the case of patient GM, 159 bp of BCR containing exon b3 was duplicated and 50 bp of ABL deleted. Previously we had found that the patient GM expressed both b3a2 BCR-ABL and 1b-b3 ABL-BCR transcripts (Melo *et al*, 1993), i.e. that exon b3 appears to be included in both reciprocal fusion mRNAs. Here we have confirmed at the genomic DNA level that this sequence is indeed present on both chromosome derivatives. Interestingly, the breakpoint on the 9q+ chromosome was only 2 bp upstream of exon b3. The polypyrimidine component of the splice recognition site (Green, 1986) has therefore been provided by the ABL sequence.

The relatively large region duplicated in patient GM suggests either that the single-stranded cuts may span a larger distance than previously thought or that another mechanism may be involved in this instance. For example, such an event could be explained by either two recombina-

tion events involving independent pairs of chromatids or by gene conversion. The latter explanation is probably more likely and has been proposed as a mechanism of sequence duplication of the thymidine kinase gene in a model mutation system (Brisebois & DuBow, 1993). A CML case similar to patient GM has been reported elsewhere (Litz *et al*, 1993); a 258 bp sequence containing most, but not all, of exon b3 was apparently duplicated. We have analysed the M-BCR sequence in this region in detail, but failed to find any direct or inverted repeats that could lead to structural aberrations.

Topoisomerase II may play a role in the formation of translocations because this enzyme catalyses both the strand breakage and ligation reactions that are necessary for their formation. A 15;17 translocation has been reported to have consensus topoisomerase II cleavage sites close to the breakpoints (Dong *et al*, 1993), but we have failed to find any similar homologies in our sequences. However, this does not rule out the involvement of topoisomerase II in BCR-ABL translocations, because a general cleavage site for this enzyme is poorly defined and differs considerably, depending on the drug used for induction of breaks (Cummings & Smyth, 1993). Comparison of the 14 BCR-ABL sequences described here with those of nine previously published cases reveals no apparent homologies at the site of breakage. Likewise, features of translocations in lymphoid cells, e.g. heptamer/nonamer sequences (Finger *et al*, 1986), poly-pyrimidine tracts (Boehm *et al*, 1989) or chi-like elements (Krowczynska *et al*, 1990), were not found at BCR-ABL breakpoints. Chi-like elements close to t(14;18) breakpoints in follicular lymphomas have been shown to bind a protein postulated to play a role in this translocation (Jaeger *et al*, 1993). The M-BCR has been shown to contain sequence-specific protein binding sites (Leibowitz *et al*, 1991), but whether these are relevant to the mechanism of translocation is unclear.

Alu elements have been found close to or at the breakpoint on the ABL side of the translocation in CML (de Klein *et al*, 1986) and in Ph-positive ALL (van der Feltz *et al*, 1989; Papadopoulos *et al*, 1990; Chen *et al*, 1989a). Breaks in the M-BCR Alu were found in 3/14 cases in this study and have also been reported elsewhere (Mills *et al*, 1992), no instances of homologous Alu-Alu recombination were observed. Alus have also been found at BCR intron 1 breakpoints in Ph-positive ALL and a model has been proposed whereby inverted Alu sequences lead to the formation of hairpin structures which facilitate the translocation (Chen *et al*, 1989b). However, only a single Alu exists within the M-BCR (Sowerby *et al*, 1993), suggesting that the model may have limited validity or that hairpin structures can form over larger distances, i.e. by pairing of the M-BCR Alu with an Alu or Alus outside the M-BCR. Although 3/14 (21%) breaks were in or near an ABL Alu in this study, only a relatively small ABL sequence was determined for each case, and so it is conceivable that this proportion is an under-representation of the true incidence of breakpoints near ABL Alu elements. Alus constitute about 3–6% of the human genome and can be expected to occur on average roughly every ~4 kb (Hwu *et al*, 1986). This element is therefore considerably over-represented in our breakpoint junction ABL sequences, suggesting either that Alus are causally involved in at least some BCR-ABL translocations or that ABL intron 1 is particularly rich in these elements. Further mapping of Alu elements and breakpoints in ABL intron 1 may help to distinguish these possibilities.

ACKNOWLEDGMENTS

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Comparison of Genomic DNA and cDNA for Detection of Residual Disease After Treatment of Chronic Myeloid Leukemia With Allogeneic Bone Marrow Transplantation

By Ji Guang Zhang, Feng Lin, Andrew Chase, John M. Goldman, and Nicholas C.P. Cross

To test whether patients in remission after allogeneic bone marrow transplantation (BMT) possess a pool of chronic myeloid leukemia (CML) cells that do not express BCR-ABL mRNA, we have compared the results and sensitivity of amplification of BCR-ABL from genomic DNA with conventional reverse transcription-polymerase chain reaction (RT-PCR). Bubble PCR was used to amplify the genomic BCR-ABL translocation breakpoints from chronic-phase DNA of 10 patients with CML who subsequently underwent BMT. After cloning and sequencing of the amplification products, patient-specific ABL primers were synthesized and tested for both specificity and sensitivity in nested or heminested combinations with a variety of primers derived from the major breakpoint cluster region of the BCR gene. In all cases, combinations of primers were selected that enabled the detection of chronic-phase DNA from a specific patient at up to a $10^5\times$ dilution into DNA from a normal individual. Patterns of residual disease obtained by serial RT-PCR and DNA-PCR analyses of blood and bone marrow samples obtained after BMT were similar for most patients, including one treated for relapse by infusion of donor leukocytes. Of the 24 samples for direct comparison of RT-PCR and DNA-PCR, results were concordant in 19 (79%) cases. Five results were discordant. In two instances, RT-PCR was positive, while PCR from genomic DNA was negative; this discrepancy might have arisen due to the slightly greater sensitivity of RT-PCR compared with DNA-PCR. In three samples from three patients, two of whom had been transplanted in the accelerated phase, PCR from genomic DNA was positive while RT-PCR was negative; this could mean that some CML cells in these samples had a reduced or absent capacity to express BCR-ABL mRNA post-transplant. Of these three patients, one subsequently relapsed; and two are in remission at 21 and 24 months after the discordant result. Thus, the finding of a single DNA-PCR–positive, RT-PCR–negative result does not necessarily predict relapse. Because the great majority of samples (79%) gave concordant results with the two assays, we believe that patients in remission do not generally harbor a substantial pool of CML cells that do not express BCR-ABL mRNA.
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CHRONIC MYELOID leukemia (CML) can be cured by allogeneic bone marrow transplantation (BMT) in the majority of cases, but some patients will relapse.[1,2] Cure is not achieved solely by high-dose chemoradiotherapy, but also involves elimination or suppression of the leukemic clone by an ill-defined graft-versus-leukemia (GVL) effect.[3] There is some evidence to suggest that the Philadelphia chromosome (Ph)-positive clone is suppressed rather than totally eradicated. First, evidence of residual disease has been found by reverse transcription-polymerase chain reaction (RT-PCR) in some long-term survivors after BMT.[4,5] Second, BCR-ABL–positive myeloid progenitor cells have been identified in some patients who remain in sustained remission.[6,7] Third, two female patients have been recently described who relapsed during or shortly after pregnancy despite having been in remission for 4.25 and 6.5 years after allogeneic BMT for CML.[8,9] It is possible that the immunosuppression associated with pregnancy[10] abrogated GVL, enabling a dormant or suppressed leukemia clone to re-emerge.

There has been considerable interest in the use of the PCR to detect residual disease in CML patients after BMT. Several groups have demonstrated that BCR-ABL mRNA may be specifically and efficiently detected by reverse transcription of leukocyte RNA followed by amplification (RT-PCR). While RT-PCR can identify groups of patients with an elevated risk of relapse, many patients are PCR-positive for several months or longer after BMT without subsequent relapse. Thus, this qualitative technique is of limited value in the clinical management of individual patients.[5,11-16] We and others have, therefore, developed a competitive PCR assay to quantitate BCR-ABL transcripts and have shown that rising levels of the fusion gene mRNA can be observed before detection of relapse at the cytogenetic level.[17,18]

It has recently been suggested that some Ph-positive CML cells may not actively express the BCR-ABL fusion gene.[19,20] Such cells would escape detection by RT-PCR but could be detectable by methods that recognize the rearrangement in genomic DNA. It is conceivable that transcriptionally silent CML cells are present in patients after BMT and, after reactivation, could contribute to relapse. To test the hypothesis that CML patients after BMT possess a pool of leukemia cells that do not express BCR-ABL mRNA, we have compared results of residual disease detected by RT-PCR with results obtained by amplification of BCR-ABL breakpoints from genomic DNA.

MATERIALS AND METHODS

Patients. Ten patients with CML who had undergone allogeneic BMT were studied. At the time of transplant, eight patients were in chronic phase, and two were in accelerated phase. Four patients received grafts from HLA-identical sibling donors and six from unrelated volunteer donors. The conditioning regimen in all groups included cyclophosphamide 120 mg/kg and fractionated total body irradiation to a total dose of 10, 12, or 13.2 Gy at a dose rate of 15 cGy/min. All patients received cyclosporin A (CSA) plus methotrexate (MTX) as graft-versus-host disease prophylaxis, and those with unrelated donors also underwent in vivo T-cell depletion using Campath 1G.[21] One patient relapsed and was treated by donor leukocyte transfusion (DLT).[22]

Cytogenetic analysis. Cytogenetic analysis of marrow cells was

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performed routinely on all patients at 3, 6, 9, and 12 months post-transplant and subsequently at 6-month intervals whenever possible. At least 30 consecutive metaphases were studied. All patients were Ph-positive before BMT.

RT-PCR for BCR-ABL. Peripheral blood or bone marrow cells were studied prospectively at various intervals posttransplant. Informed consent was obtained as required by the Declaration of Helsinki. After extraction of leukocyte RNA, samples were tested for the presence of BCR-ABL mRNA by reverse transcription followed by nested PCR.[14] BCR-ABL transcripts were quantified in 15 of the 16 RT-PCR–positive specimens by competitive PCR.[17] Results were standardized by deriving the number of BCR-ABL transcripts per microgram leukocyte RNA. PCR-positive samples were scored as having less than 10 BCR-ABL transcripts per microgram RNA if the competitor band predominated at 10 molecules of competitor added. High BCR-ABL levels were defined as 50 transcripts per microgram RNA or greater; detectable but low BCR-ABL levels had less than 50 transcripts per microgram RNA. Using competitive PCR, we have previously demonstrated concordance in levels of residual disease between blood and bone marrow.[23]

Cloning and characterization of BCR-ABL breakpoints. Characterization of genomic DNA breakpoints for patients M.L., D.M., A.H., M.T., A.J., A.L., and A.E. by bubble PCR has been described in detail elsewhere.[24] Breakpoints for patients B.Y., B.M., and R.K. were determined in exactly the same way. Briefly, DNA was extracted from peripheral blood samples collected before BMT. Fragments containing BCR-ABL breakpoints were amplified using a panel of oligonucleotide primers directed against the major breakpoint cluster region (M-BCR) in conjunction with primers complementary to the bubble sequence. Products were cloned into M13mp19 and sequenced. The position of the breakpoint was determined by comparison with the published M-BCR sequence,[25] and either one or two 22-bp to 25-bp primers were synthesized to match the ABL sequence for each patient.

DNA extraction. DNA was extracted from cryopreserved chronic-phase leukocytes as described[26] and from fresh peripheral blood (n = 29) or bone marrow specimens (n = 13) at various times after BMT. In some cases, DNA was extracted from stained marrow slides (n = 33). First, coverslips were removed after soaking the slide overnight in xylene. Using a razor blade, bone marrow material was scraped into 200 μL of 10 mmol/L Tris-HCl pH 8.0, 1 mmol/L EDTA, 0.5% sodium dodecyl sulfate (SDS). Proteinase K (40 μg) was added, and the sample was incubated at 65°C overnight. After extraction with phenol/chloroform, DNA was precipitated and resuspended in 10 μL to 50 μL water. DNA was quantified using a spectrophotometer, and the concentration and integrity were confirmed by agarose gel electrophoresis. Quality was confirmed by amplification of a 318-bp BCR exon 1 fragment from 500 ng genomic DNA with primers BCR-B (5′ CCCCCGGAGTTTTGAGGATTGC 3′) and BCR-D (5′ ATGGAAGGCGCCCTCGTCATC 3′) for 30 cycles of 96°C for 30 seconds, 64°C for 50 seconds, and 72°C for 1 minute. All DNA samples resulted in the amplification of a clear product of the expected size after a single-step PCR and were, therefore, considered to be of sufficient quality for analysis of residual disease.

Genomic DNA-PCR for BCR-ABL. Optimal PCR conditions and combinations of ABL and M-BCR primers were determined to yield specific amplification of BCR-ABL from chronic-phase DNA of each patient. For nested or heminested PCRs, an initial amplification reaction was performed with a pair of patient-specific primers. One microliter of product was reamplified in a second PCR using either a pair of patient-specific primers internal to the first set or the same ABL primer plus an internal M-BCR primer. Chronic-phase DNA was serially diluted with DNA of a normal individual to test the sensitivity of the assay for each individual. For analysis or residual disease, the first-step PCR was seeded with 1 to 2 μg genomic DNA. After reamplification with nested or heminested primers, products were fractionated on ethidium bromide-stained agarose gels, and results were scored as positive or negative. For both RT-PCR and DNA-PCR, rigorous precautions were taken to prevent contamination of samples and reaction components by PCR products.[14,17,27] At least two negative controls were performed along with each sample, none of which resulted in an amplification product.

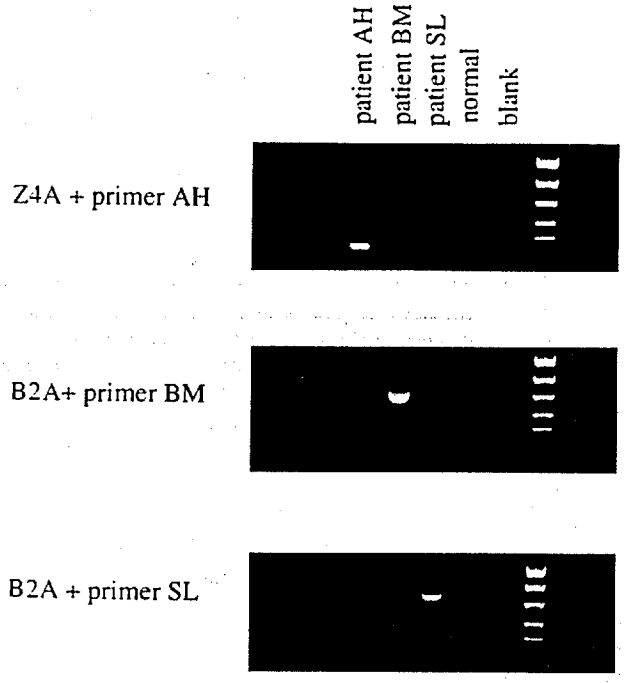


Fig 1. Specificity of DNA-PCR. Primer pair Z4A (M-BCR) and AH (ABL) amplify a fragment containing the BCR-ABL breakpoint from chronic-phase DNA extracted from patient A.H., but not chronic-phase DNAs from patients B.M. or S.L. Similarly, primer pairs B2A + BM and B2A + SL amplify specific products from patients B.M. and S.L., respectively. None of the primer pairs generates a product from normal genomic DNA.

RESULTS

Combinations of ABL and M-BCR primers enabled the BCR-ABL breakpoints to be detected in chronic-phase DNA from each of the 10 patients studied. As expected, primer pairs were patient-specific; ie, a particular combination only resulted in an amplified product from a single patient (Fig 1).

Chronic-phase DNA from each patient was diluted into normal DNA and subjected to two-step PCR using either nested or heminested combinations of patient-specific primers. Using 100 ng genomic DNA as a template, the 10^{-4} dilution usually resulted in a specific amplification product, but at the 10^{-5} dilution, BCR-ABL was not detectable. By increasing the amount of template DNA to either 2 or 5 μg, an amplification product was obtained from the 10^{-5} dilution (Fig 2). A specific product was obtained using a 10^{-4} dilution for all patients when 500 ng of genomic DNA was amplified.

For the 10 patients after BMT, a total of 75 DNA speci-

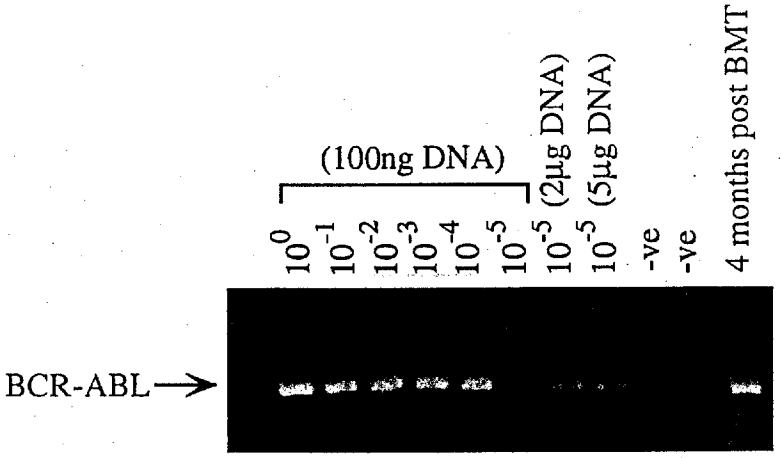


Fig 2. Sensitivity of DNA-PCR. Genomic DNA from patient D.M. was serially diluted up to 10^{-5} with DNA from a normal individual and subjected to two-step PCR. Using 100 ng genomic DNA as a template, the 10^{-4} dilution usually resulted in a specific amplification product, but at the 10^{-5} dilution, BCR-ABL was not detectable. By increasing the amount of template DNA to either 2 or 5 μg, an amplification product was obtained from the 10^{-5} dilution. No amplification product was obtained with normal DNA (−ve), and a sample at 4 months post-BMT was PCR-positive.

mens from 61 timepoints were analyzed by two-step PCR for BCR-ABL. Twenty seven timepoints were DNA-PCR–positive, and 34 were DNA-PCR–negative. At 49 timepoints, a single sample was analyzed, whereas for 12 timepoints, either two or three independent samples (total = 26) were analyzed. Complete concordance was found between results of contemporaneous DNAs extracted from fresh peripheral blood, fresh bone marrow, and stained bone marrow slides.

For most patients, the patterns of serial DNA-PCR results were similar to those described for RT-PCR (Fig 3). Patients M.L., B.M., and A.J. were initially DNA-PCR–positive after BMT but were subsequently persistently DNA-PCR–negative. Patient B.M. was RT-PCR–negative during the period of DNA-PCR positivity, while patients M.L. and A.J. were not studied by RT-PCR when they were DNA-PCR–positive. Patients D.M. and A.H. were persistently DNA-PCR– and RT-PCR–positive. Patients B.Y., M.T., A.L., and A.E. were persistently DNA-PCR–negative; RT-PCR assays were also negative except at a single timepoint for three of these individuals. Patient R.K. was persistently DNA-PCR–positive for 2 years after BMT. RT-PCR was initially negative but subsequently converted to positive. This patient was in cytogenetic remission for the first year, but at 21 months, 17% Ph-positive metaphases were detected in the marrow, rising to 40% Ph-positive metaphases at 24 months. Treatment for relapse with DLT was initiated at 21 months post-BMT. At 4 months later, Ph-positive metaphases were no longer detectable, and no residual disease was detected by either DNA-PCR or RT-PCR. Subsequent PCR assays were also negative.

Quantitative RT-PCR results have been described in detail elsewhere.[17,28] Results are shown in Fig 3 only for those RT-PCR–positive datapoints that had low levels of BCR-ABL mRNA (less than 50 transcripts/μg RNA). All other RT-PCR–positive datapoints had high levels of BCR-ABL (greater than 50 transcripts/μg RNA), except for one positive specimen that was not quantified. Twenty-four paired samples were taken on the same day for analysis by RT-PCR and DNA-PCR and were, therefore, available for direct comparison. Nineteen samples were concordant, of which nine were RT-PCR– and DNA-PCR–negative and 10 were RT-PCR– and DNA-PCR–positive. Of the 10 that were positive, six had high levels of BCR-ABL mRNA (greater than 50 transcripts/μg RNA), and four had low levels. Five samples were discordant. Of these, three were DNA-PCR–positive and RT-PCR–negative. For these samples, RT-PCR was repeated in triplicate: one was persistently negative, and two yielded a single positive result. Two samples were DNA-PCR–negative and RT-PCR–positive, of which one had a low level of BCR-ABL mRNA and one was not quantified (Fig 4).

Cytogenetic analysis was performed on 18 of the 34 samples that were DNA-PCR–negative and 15 of the 27 samples that were DNA-PCR–positive. No Ph-positive metaphases were detected in any of the DNA-PCR–negative samples or in any of the 11 samples that was RT-PCR–negative. Of the 15 samples that were DNA-PCR–positive, only two had detectable Ph-positive metaphases. Both samples were also RT-PCR–positive.

DISCUSSION

Theoretically, DNA-based PCR has some advantages over RT-PCR for analysis of residual disease. Extraction of DNA from patient samples is technically easier than extraction of RNA followed by synthesis of cDNA. Genomic DNA-PCR would result in amplification of BCR-ABL whether the fusion gene was actually expressed or not. Moreover, the use of patient-specific primers rather than a common set of primers for all patients may help to minimize the problem of PCR contamination. In CML, however, the t(9;22) breakpoints are dispersed over a very wide region of genomic DNA, particularly within the ABL gene.[29] Thus, to amplify BCR-ABL from genomic DNA, it is necessary to clone and sequence the breakpoint from each patient and to design patient-specific oligonucleotide primers. Recently, we have described the use of bubble PCR to facilitate cloning of BCR-ABL breakpoints.[24] While this technique is considerably easier than the traditional method of constructing a genomic DNA library from each patient, it does, nevertheless, involve an appreciable amount of time and effort. Furthermore, as shown elsewhere, we were only able to amplify

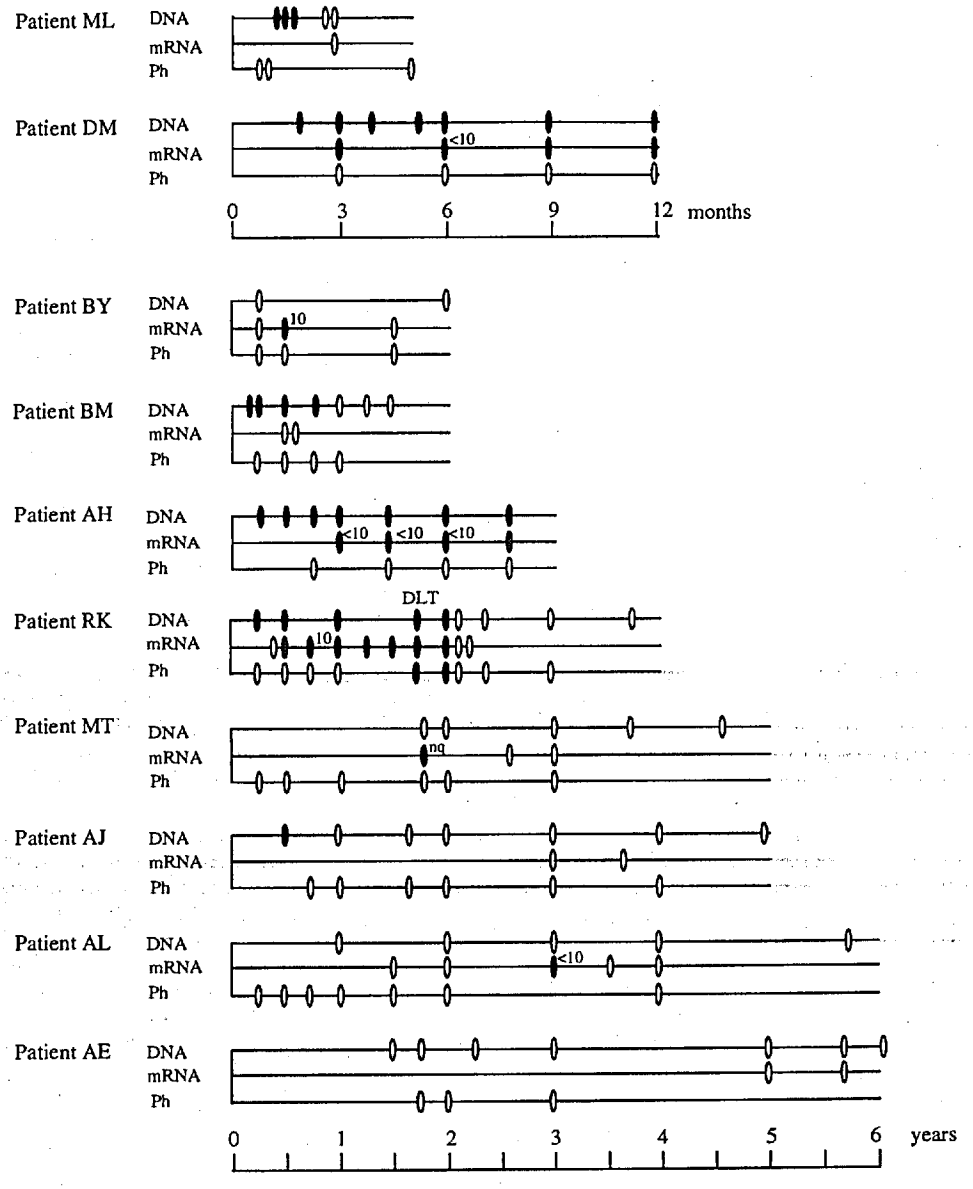


Fig 3. DNA-PCR, RT-PCR, and cytogenetic results of the 10 patients after BMT. Open ellipses indicate either PCR negativity or Ph-negativity. Solid ellipses indicate either PCR positivity or the presence of Ph-positive metaphases in the marrow. Stippled ellipses indicate the two cDNA samples that were repeated in triplicate and yielded a single positive result. Patient R.K. was treated for relapse with DLT at 21 months post-BMT. Competitive RT-PCR results are shown for those samples that were PCR-positive but had low levels of BCR-ABL mRNA (less than 50 transcripts/μg RNA). All other RT-PCR–positive datapoints had high levels of BCR-ABL mRNA (greater than 50 transcripts/μg RNA), except for one that was not quantified (nq).

fragments harboring genomic BCR-ABL breakpoints from 43% of patients.[24] It is unlikely, therefore, that DNA-PCR could easily replace RT-PCR as a routine method for monitoring patients with CML after BMT. Nevertheless, it is possible that DNA-PCR could be more informative for detection of residual disease.

Published reports have suggested that not all CML cells actively express the BCR-ABL fusion gene. Purified CD34-positive CML cells were shown to be RT-PCR–negative despite possessing the fusion gene.[20] Similarly, some Ph-positive myeloid colonies were found to be BCR-ABL mRNA-negative.[19] While results of the latter study have not been confirmed,[30] and both studies can been criticized on the grounds of inadequate controls for cDNA synthesis,[31-33] it is possible that CML cells that do not express BCR-ABL mRNA do, in fact, exist. Such cells could potentially lead to false-negative assessment of residual disease in patients after treatment.

We have found that DNA-PCR can detect BCR-ABL when chronic-phase DNA from a particular individual is diluted into normal DNA up to a concentration of 10^{-5}. RT-PCR is generally considered to be slightly more sensitive, capable of detecting a single CML cell in the background of 10^5 to 10^6 normal cells. The reason for the difference in maximum sensitivity is presumably that each CML cell carries a single copy of the fusion gene but, under steady-state conditions, may contain multiple copies of BCR-ABL mRNA. It might be expected, therefore, that at low levels of residual disease, RT-PCR could give a positive result, while DNA-PCR would be negative. If high levels of residual disease were found by RT-PCR, then DNA-PCR would also be expected to be positive, unless the positivity was due

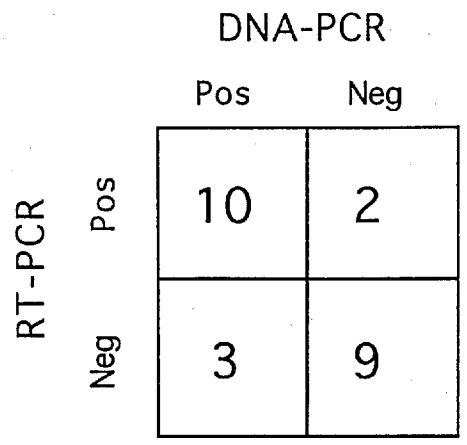


Fig 4. Concordance and discordance of DNA-PCR and RT-PCR on contemporaneous samples. Of the three samples that were DNA-PCR–positive, RT-PCR–negative on initial analysis, two yielded a single RT-PCR–positive result when repeated in triplicate, and one was persistently RT-PCR–negative. Pos, positive; Neg, negative.

to a very small number of CML cells expressing high levels of BCR-ABL mRNA. Conversely, if there were CML cells that did not express BCR-ABL mRNA, then DNA-PCR could be positive and RT-PCR negative.

Overall, we have found that for most patients the patterns of PCR results on serial analysis after BMT for CML were similar when tested with RT-PCR or DNA-PCR. On initial analysis of contemporaneous samples, we found that 19 of 24 (79%) gave concordant RT-PCR and DNA-PCR results. However, after repeating RT-PCR analysis for the three RT-PCR–negative, DNA-PCR–positive samples, 21 of 24 (88%) samples were concordant. This high degree of concordance suggests that patients in remission do not generally harbor a substantial pool of leukemia cells that do not express BCR-ABL mRNA. As concordance was found at both high and low levels of BCR-ABL mRNA, we believe it is unlikely that some CML cells express unusually high levels of fusion gene transcripts. Of the discordant results, two were RT-PCR–positive and DNA-PCR–negative. One of these RT-PCR samples was quantified and found to contain low levels of BCR-ABL mRNA; the other sample was not quantified. It is likely that these discrepancies result from the slightly superior detection level of RT-PCR over DNA-PCR.

Of the three samples that were DNA-PCR–positive, RT-PCR–negative, only one was persistently RT-PCR–negative when the analysis was repeated in triplicate. The cDNAs from all three samples appeared to be of good quality as judged by amplification of the normal ABL gene by single-step PCR, and, given the greater sensitivity of RT-PCR over DNA-PCR, it is possible that these specimens did harbor CML cells that were not actively expressing BCR-ABL mRNA. Alternatively, because the maximum sensitivity of the PCR assays cannot be absolutely guaranteed for every sample, the discordance could have arisen due to random variation. Of the three patients who had a DNA-positive, RT-PCR–negative sample on initial analysis, one subsequently relapsed and two are in remission at 21 and 24 months, respectively, after the discordant result. Thus, a single DNA-positive, RT-PCR–negative result is not necessarily an indicator of subsequent relapse. Interestingly, the two patients who did not relapse were transplanted while in the accelerated phase. This suggests that accelerated-phase CML cells, at least after BMT, might be more likely to harbor a transcriptionally silent BCR-ABL fusion gene than chronic-phase cells. As neither patient has relapsed, it is possible that the malignant cells were damaged by the transplant ablative regimen such that they did not express typical levels of BCR-ABL mRNA, but were still able to survive for an unusually long time posttransplant.

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