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HUMAN LEUKAEMIA AND THE BCR GENE : CYTOGENETIC AND HYBRIDISATION STUDIES IN CML

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

in the Faculty of Science

to the University of London

by

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PREFACE

The experimental work in this dissertation was carried out in the Medical Research Council (MRC) Leukaemia Unit, Royal Postgraduate Medical School, London between 1985 and 1988. The research was supported by the MRC and the Leukaemia Research Fund (LRF).

The nature of my study obliged me to collaborate with a number of research workers and their specific contributions have been acknowledged in the text. Therefore unless stated otherwise, the work presented in this thesis is believed to be original and performed by the author alone.

ACKNOWLEDGEMENTS

I am deeply indebted to Professor J M Goldman for his constant guidance and encouragement throughout this study. I would also like to thank Professor L Luzzatto, Chairman of the department of Haematology, who provided valuable support at many stages during the evolution of this work.

Because of the different techniques employed in the studies described in this dissertation, I was obliged to collaborate more or less closely with many other research workers in the Royal Postgraduate Medical School and elsewhere. I wish particularly to thank Dr S legon for his assistance with molecular biological techniques which helped overcome major obstacles in setting up the technique of in situ hybridisation, Mrs Guo Ai Pu and Dr C Price for assistance with interpretation of cytogenetic data, Drs P Martiat and Gao Ling Min for some of the Southern blotting analyses, Dr I Klisak for the clarification of initial in situ hybridisation experiments, Dr O Dreazen for the RNA protection studies, R Morilla working in Professor D Catovsky's laboratory, for marker studies on the blast cells of some patients, Dr L Wiedemann for the Southern and protein tyrosine kinase analysis and Dr L Foroni for the immunoglobulin and T-cell receptor gene studies in the patient with acute lymphoblastic leukaemia, Dr G Morgan for the polymerase chain reaction studies on the patient with acute myeloid leukaemia and Mr T'ng for his tireless assistance with cell culture techniques and the cryopreservation and storage of some of the cells used in my experiments.

ABSTRACT

I used a variety of techniques including conventional cytogenetic analysis, in situ hybridisation of gene probes to metaphase chromosomes and Southern analysis of DNA to study cells from patients with chronic myeloid leukaemia (CML) and Philadelphia chromosome positive acute leukaemia. I focused on five separate but related questions.

I studied a group of patients whose leukaemic cells lacked a classical Ph chromosome, so-called Ph-negative CML. I confirmed the presence in some of these patients of the same abnormal gene and mRNA that characterise Ph-positive CML but in situ hybridisation studies suggested that the BCR/ABL gene was created not by a mechanism of reciprocal translocation but by interstitial insertion. Studies designed to establish more precisely how much ABL-containing genetic material was inserted into chromosome 22 suggested that the amount of chromosome 9q34-derived material varied between different patients.

I studied a group of CML patients with complex translocations involving up to four chromosomes, in whom the sequence of translocation events was not clear, to confirm this impression that chromosomes 9q34 and 22q11 were always involved.

I studied the cytogenetic and molecular features of 4 patients with Ph-positive CML in blastic transformation to identify possible additional changes specific to transformation.

I had the opportunity to study two Ph-positive acute leukaemia patients with unique features. One patient at presentation had a minority of normal metaphases which at relapse had acquired an extra chromosome 19. Another patient had AML (FAB M6) arising de novo with a 'masked' Ph chromosome associated with extensive karyotypic changes.

My work provides support for the concept, now very widely accepted, that the acquisition of the BCR/ABL chimeric gene is a fundamental step in the pathway towards full leukaemic expression in CML and occasional cases of acute leukaemia. It highlights the important observation that genomic changes associated with specific chromosomal translocations may also occur in cytogenetically normal cells. It emphasises the point that other undefined genomic changes must also contribute, perhaps crucially, to the leukaemic phenotype in CML and Ph-positive acute leukaemia. Finally it addresses the question of what types of further change may underlie blast cell transformation.

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CHAPTER 1: CML AND THE PHILADELPHIA CHROMOSOME

1.1 CLINICAL AND HAEMATOLOGICAL FEATURES

1.1.1 Definitions of CML

The term chronic myeloid leukaemia (CML) describes a group of diseases characterised by autonomous proliferation of myeloid cells in the bone marrow and other sites. The most common type of CML, also called chronic granulocytic leukaemia (CGL), is characterised by progressive splenomegaly, leukocytosis, anaemia and marrow hypercellularity. The myeloid cells and some B cells in the majority of these patients have a specific cytogenetic abnormality, the Philadelphia (Ph) chromosome (see below). Between 3 and 10% of CML patients are atypical. Some of these patients have clinical and haematological features that resemble Ph-positive CML but their leukaemic cells lack the Ph chromosome; these patients have Ph-negative CML. A second subgroup of patients has a disease that superficially resembles CML but shows increased numbers of immature monocytes in the peripheral blood and also lacks the Ph chromosome. These patients are usually classified as chronic myelomonocytic leukaemia (a category included in the French-American-British classification of the myelodysplastic syndromes). A third group is those with juvenile CML. This disease occurs in young children and is characterised by leukocytosis, lymphadenopathy, rashes, a normal leukaemia cell karyotype and a poor response to therapy. Lastly, there are patients with splenomegaly, large numbers of mature neutrophils in the peripheral blood, a raised neutrophil alkaline phosphatase and a

normal karyotype. These patients probably have chronic neutrophilic leukaemia (reviewed in Shepherd et al, 1987).

1.1.2 **Clinical features**

Classically CML has two or three clinical stages or phases. The disease inevitably evolves sooner or later from a relatively benign 'chronic' phase to an 'acute' or blastic transformation; commonly there is an intermediate phase of 'acceleration' which then proceeds to full blastic transformation.

1.1.2.1 Chronic phase

The duration of the chronic phase may vary from one year to twenty or more years in different patients. The initial presenting symptoms are often nonspecific. There may be loss of energy, shortness of breath, or weight loss. Some patients may be completely asymptomatic and are diagnosed only after a routine blood count. Conventionally, however, uncontrolled expansion of the granulocyte mass leads to enlargement of the spleen and 90% of patients when diagnosed have a degree of splenomegaly that may range from being barely palpable to being so large that it occupies most of the anterior abdomen. The liver also is often enlarged in newly diagnosed patients. Lymphadenopathy is uncommon but bruising or purpura may be detected.

1.1.2.2 Accelerated phase

In perhaps 75% of cases the chronic phase is superseded by a phase of altered biological behaviour of the disease that still does not fulfil the criteria for blastic transformation. This accelerated phase is difficult to define precisely. The new features may for example be splenomegaly with resistance to cytotoxic drugs, unexplained fever or lymphadenopathy. However at this stage the peripheral blood and bone marrow may not show any concordant increase in immature cells.

1.1.2.3 Blastic phase

The onset of the blastic phase may be sudden or gradual. The disease becomes more aggressive and is said to have transformed. In 20% of cases transformation may be precipitous and is referred to as blast crisis. Initial symptoms may include fevers, sweating, bone pain and haemorrhage at various sites and are generally similar to those of the acute leukaemias. Some patients may show symptoms which are primarily due to anaemia and some may have a rapidly increasing blast cell count with infiltration of the lungs and central nervous system.

1.1.3 HAEMATOLOGY

1.1.3.1 Chronic phase

At presentation the white-cell count of a patient typically ranges from 20 to >500 x 10^9/l. The haemoglobin concentration is often slightly reduced with a mean of 9.7 g/dl (Spiers et al, 1976) and the platelet count is usually slightly elevated with a mean of 484 x10^9/l. The differential count shows a complete spectrum of granulocytic forms with prominent peaks in myelocytes and neutrophils and relatively fewer metamyelocytes. Blast cells may make up as many as 12% of the differential and their proportion increases in parallel with the increase in white-cell count. The level of neutrophil alkaline phosphatase is usually low or absent.

1.1.3.2 Accelerated phase

The haematological changes that characterise the accelerated phase are variable. There may be increasing leukocytosis, thrombocytosis (>1x10^{12}/l), thrombocytopenia, or basophilia. There may also be an increase in marrow fibrosis which may come to resemble primary myelofibrosis.

1.1.3.3 Blastic phase

The blastic phase is diagnosed with the appearance of more than 30% of blasts or blasts plus promyelocytes in the peripheral blood or bone marrow, accompanied or rapidly followed by a depletion of normal elements (Karanas and Silver, 1968). The blasts in approximately 20% of cases have membrane markers characteristic of lymphoblasts (mainly of the B-cell type); approximately 75% have membrane markers characteristic of myeloblasts. In about 5% of cases blast cells with both lymphoid and myeloid features are seen in the same patient.

1.2 THE PHILADELPHIA CHROMOSOME

1.2.1 Discovery

The story of the Philadelphia chromosome began in 1960 when Nowell and Hungerford described a small acrocentric chromosome in the peripheral blood of two male CML patients (Nowell and Hungerford, 1960). Due to technical limitations at the time the authors were unable to say whether this marker was a G-group chromosome or a small Y chromosome. Later that year the same authors (1960) described a similar abnormality in 5 more CML patients, some of whom were female. This result and the findings of Baikie et al (1960) excluded the possibility that this marker was a small Y chromosome and consigned it to the G group of autosomes. This small acrocentric G group chromosome was subsequently named the 'first' Philadelphia chromosome (Ph^1) after the city from

which it was first described. An international group that met to standardise chromosome nomenclature in 1971 decided that the Ph^1 chromosome should be defined as a deleted chromosome 22 (22q-) (Paris Conference, 1971). Later the superscript number 1 in the Ph^1 was dropped and the Philadelphia chromosome was abbreviated to Ph (Sandberg, 1985)

1.2.2 Further characterisation of the Ph chromosome

The methods for staining chromosomes available in the 1960s made it impossible to distinguish between the two pairs of G group autosomal chromosomes, 21 and 22. At that time uniform staining of chromosomes only allowed the human karyotype to be arranged in a sequence based on chromosome length and the position of the centromere. The introduction of techniques such as quinacrine (Q) and Giemsa (G) banding which produced unique transverse banding patterns on individual chromosomes made it possible for the Ph chromosome to be identified more precisely (Caspersson et al 1970a, Sumner et al 1971, Seabright et al 1971) .

Caspersson et al (1970b) and O'Riordan et al (1971) used the technique of Q-banding to define the Ph chromosome as a chromosome 22q-, resulting from a possible deletion or translocation. In 1973 Rowley, using both G and Q banding, demonstrated that the chromosomal material missing from 22 had been translocated onto the distal end of the long arm of chromosome 9 (Fig 1). This Ph translocation [t(9;22),(q34;q11)] was presumed to be reciprocal and therefore balanced. However, although the translocation of chromosomal material from chromosome 22 to

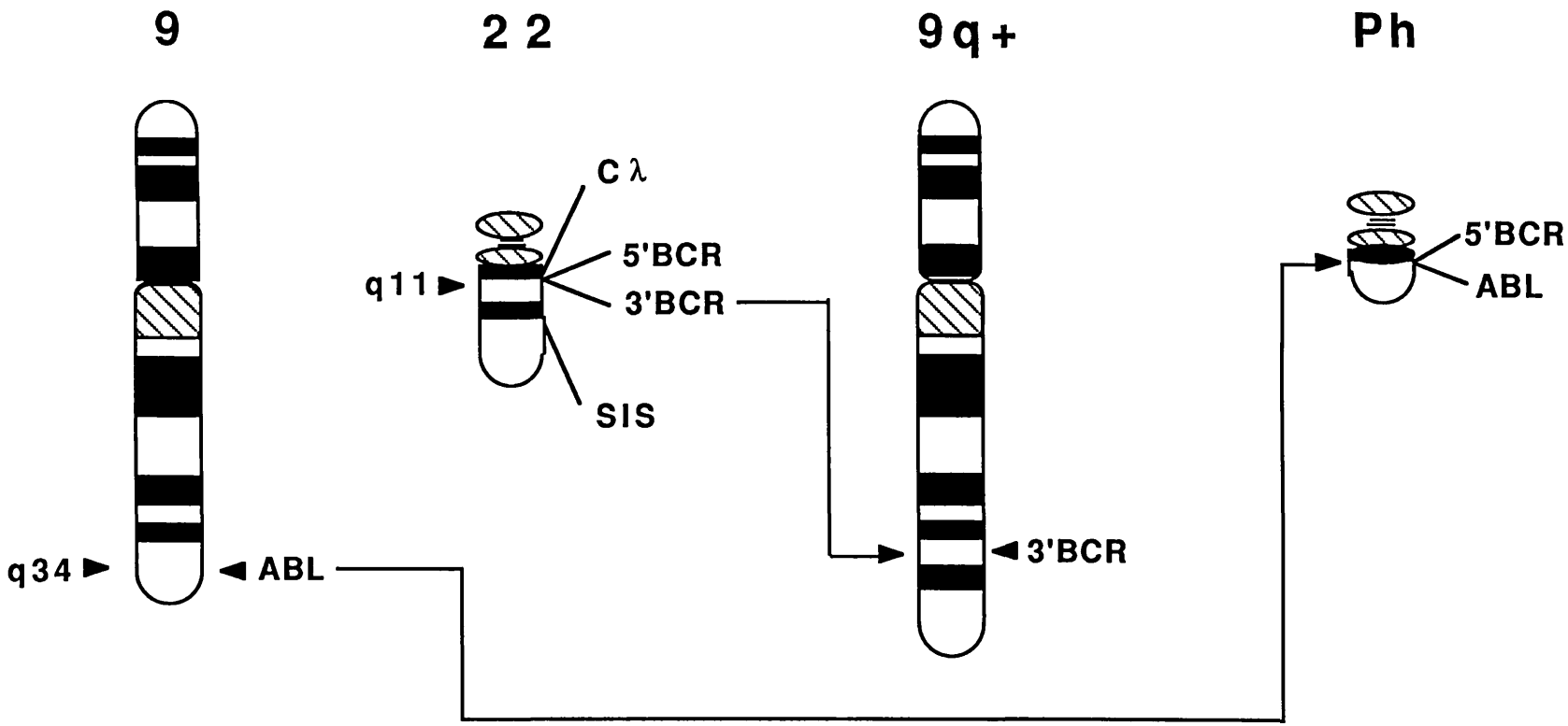


Fig 1 **A schematic representation of the Philadelphia chromosome**

The reciprocal translocation between chromosomes 9 and 22 results in the 9q+ and the 22q- (Ph). The black arrow heads indicate the positions of the chromosomal breakpoints 9q34 and 22q11, the ABL proto-oncogene and the BCR gene. The positions of the C λ gene and the SIS proto-oncogene relative to the BCR gene are also indicated.

chromosome 9 was microscopically visible by standard cytogenetic techniques, the translocation from 9 to 22 was not. Thus the reciprocal nature of the translocation was only confirmed a decade later with the use of molecular biological techniques (Bartram et al, 1983).

1.2.3 The molecular basis of the Ph chromosome

During the 1970s molecular biologists identified transforming genes in retroviral genomes which they called viral oncogenes or v-oncs (reviewed by Bishop, 1983). Initially it was thought that these retroviral genes may be responsible for some human cancers when introduced into normal cellular DNA. The picture became more complex when it was determined that these viral genes were derived from homologous genes present in normal eukaryotic DNA. These genes were called proto-oncogenes or cellular oncogenes (c-oncs), and although the precise function of these genes was not known, data suggested that they played a role in the regulation of growth and differentiation of the cell. The idea was thus proposed that these genes could cause cancers if expressed inappropriately.

In 1982 Heisterkamp et al using somatic cell hybrids mapped the human homologue of the transforming sequence of the Abelson murine leukaemia virus, ABL, to chromosome 9q34. In the same year Swan et al (1982) and Dalla-Favera et al (1982) mapped the human homologue of the transforming sequence of the simian sarcoma virus, SIS, to chromosome 22q12-3. Since both these oncogenes were localised in the region of break that produce the Ph translocation and since each chromosomal band may contain several

hundred genes, it was important to determine whether ABL or SIS (or neither) was specifically involved in the translocation.

de Klein et al (1982) showed that the cellular proto-oncogene, ABL, was mapped to the 22q- (Ph) chromosome. They used mouse-human fused cells containing selected human chromosomes, somatic cell hybrids. Some such cell lines contained the Ph chromosome and others contained the 9q+. They demonstrated by Southern blotting the presence of ABL in the Ph containing hybrid cells. A year later Bartram et al (1983) in the same laboratory used the in situ hybridisation technique to demonstrate hybridisation of ABL to the Ph chromosome in one CML patient and Groffen et al (1983) localised SIS to the 9q+ in CML. These results provided convincing molecular evidence that the Ph translocation was indeed reciprocal.

Although both SIS and ABL were reciprocally exchanged in the Ph translocation, it was still unclear if either of these proto-oncogenes played any role in CML. At first the cytogenetic evidence seemed to favour SIS as the more important gene because analysis of variant translocations often showed 9q34 not to be involved. Presumably therefore a gene normally located on chromosome 22 was likely to be involved in CML. However, conflicting evidence came from molecular analysis of complex variant Ph translocations. Bartram et al (1983) showed that in complex translocations involving chromosomes 9, 22 and another chromosome, ABL was always translocated to the Ph chromosome whereas the movement of SIS was variable. This finding verified the observation by Rowley et al (1982) that it was the translocation of ABL

and not of SIS that was important in the pathogenesis of CML. Thus it became necessary to define precisely the ABL gene and the molecular events leading to formation of the Ph chromosome.

The cloning of the ABL gene by several groups showed that it spanned approximately 230 kb and contained two alternative 5' exons (Ib and Ia) associated with a set of ten common exons (Fig 2), to yield two major RNA transcripts of 6 and 7 kb (Heisterkamp et al 1983, Shtivelman et al 1985, Shtivelman et al, 1986).

Heisterkamp et al (1983) and Bartram et al (1983) analysed events related to the translocation ABL onto chromosome 22 in considerable detail. They showed in one patient that the breakpoint on chromosome 9 occurred 14 kb upstream of the first common exon of the ABL gene. Subsequent data suggested that the breakpoints on chromosome 9 were variable and ranged from 4 kb (Leibowitz et al 1985) to > 100 kb upstream of the first known exon (1b) of the ABL gene (Grosveld et al 1985). However, the situation on chromosome 22 was quite different. Groffen and collegues showed that the breakpoints on chromosome 22 were clustered within a limited region of approximately 5.8 kb (Heisterkamp et al 1983, Groffen et al, 1984). This region, called the breakpoint cluster region (BCR or M-BCR), includes 5 exons and forms part of a larger gene which spans at least 70 kb (Hariharan et al, 1987, Heisterkamp et al, 1985, Lifshitz et al, 1987a, Lifshitz et al, 1987b, Mes-Masson et al, 1986) (Fig 3). Analysis of these breakpoints showed that they mainly occur within intronic sequences between exons 2 and 3 (b2 and b3) or 3 and 4 (b3 and b4) of M-BCR (Heisterkamp, 1985). (For a

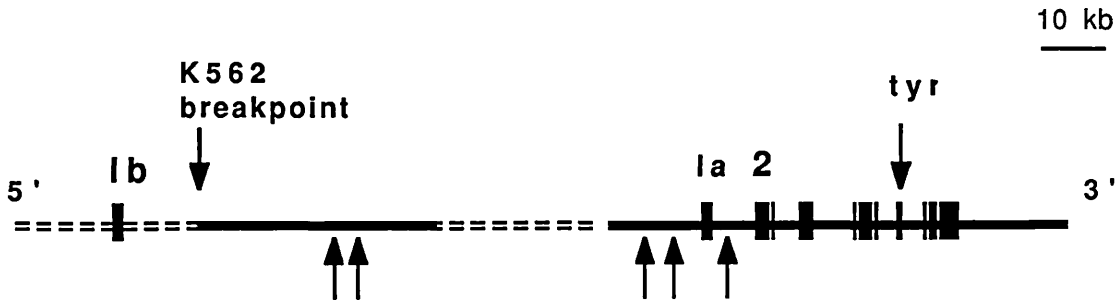


Fig 2 Schematic representation of the ABL proto-oncogene
The ABL locus is represented by the horizontal line, with broken and solid lines indicating the regions of the gene uncloned and cloned respectively. The positions of the variable exons 1b and 1a and exon 2 (usually known as the first common exon), as well as the location of the exon containing the phosphotyrosine acceptor site (tyr) are indicated. Arrows beneath the schematic diagram point to the positions of ABL breakpoints in 5 CML patients; the position of the breakpoint in the CML cell line, K562 is indicated above the schematic diagram by an arrow (modified from Groffen and Heisterkamp, 1987).

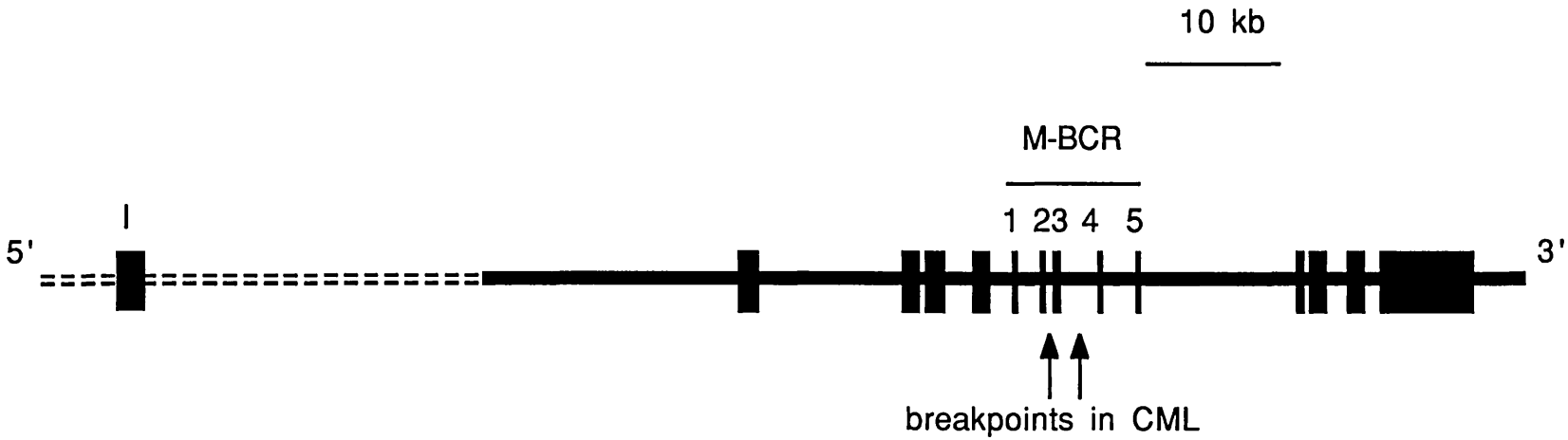


Fig 3 **Schematic representation of the BCR gene**

The solid and broken horizontal lines indicate cloned and uncloned parts of the gene respectively, which separate exon 1 from the main body of the gene. The two principle positions of the breakpoints in CML are indicated by arrows and are located between exons 2 & 3 (b2) and 3 & 4 (b3) of the major breakpoint cluster region (M-BCR). (modified from Groffen and Heisterkamp, 1987)

more detailed exposition of the BCR nomenclature used in this thesis, see section 1.71).

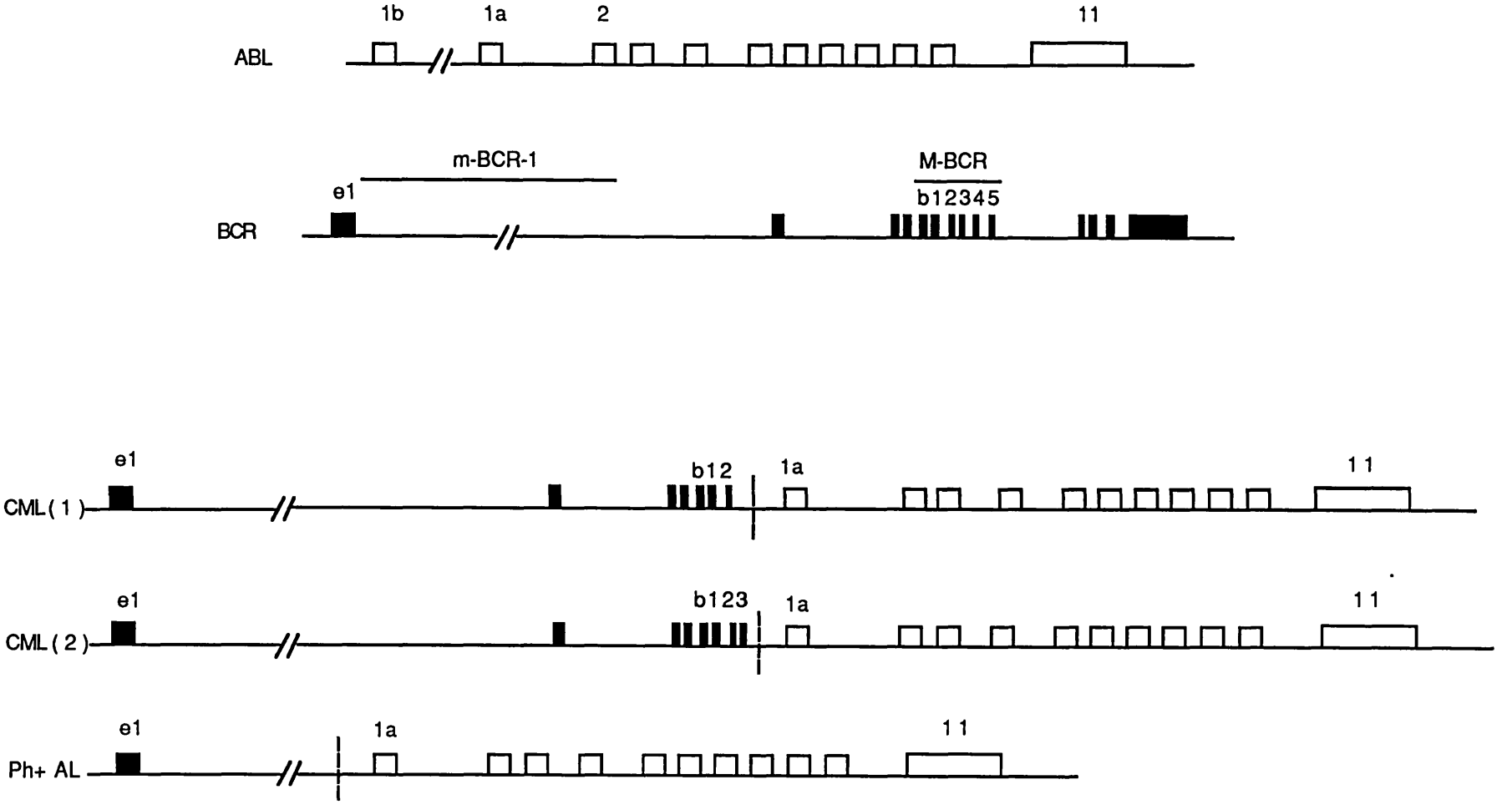
Studies focusing on transcription of ABL showed that the cells from Ph positive CML patients expressed a novel 8 kb mRNA and occasionally a 9 kb mRNA in addition to the normal 6 and 7 kb transcripts (Cannani at al, 1984, Gale and Cannani, 1984, Westin, 1982). The nature of this message was elucidated by Shtivelman et al (1985) who described a fused transcript of ABL and BCR in CML. This implied that the Ph translocation resulted in the creation of a new chimeric gene consisting of sequences from BCR upstream of the breakpoint on 22q11 linked to translocated ABL sequences (Fig 4). The striking uniformity seen in different patients at the mRNA level in spite of major size differences between the different BCR-ABL fusion genes is presumably achieved by post-transcriptional modification.

Protein studies have shown that both the BCR and ABL genes are expressed in normal cells although the precise function of the gene products is not known. BCR proteins of 190 kd and 160 kd and two ABL proteins (which differ at their amino-termini) were identified (Ben-neriah et al 1986, Grosveld et al, 1986, Konopka et al, 1984, 1985a, 1985b). In cells from patients with CML Konopka et al (1984) showed that the chimeric 8 kb BCR/ABL mRNA is expressed as a chimeric protein of Mwt 210 kd. This protein contains all the peptide sequences present in the normal 145 kd ABL protein and either 927 or 902 aminoacid residues of BCR (depending on the inclusion or exclusion of exon 3). In contrast to the normal ABL transcript, the 210 kd BCR/ABL protein has tyrosine

Fig 4 **Schematic representation of the Philadelphia translocation at the genomic level.**

The BCR and ABL genes are represented with the boxes indicating exons and the introns shown as lines. The M-BCR and m-BCR regions of the BCR gene are indicated. The representative positions for the breakpoints in CML and Ph-positive ALL are shown as vertical broken lines. Two breakpoints occur in CML between exon b2 and b3, and between exons b3 and b4. These two breakpoints give rise to two chimeric BCR-ABL genes, one containing an extra M-BCR exon (CML-2). The breakpoints within intron 1 (m-BCR) in Ph-positive ALL give rise to a chimeric BCR-ABL gene containing only exon 1 (e1) of the BCR gene.

Fig 4


1b
1a
2
11
ABL
m-BCR-1
M-BCR
e1
b12345
BCR
e1
b12
1a
11
CML(1)
e1
b123
1a
11
CML(2)
e1
1a
11
Ph+ AL

kinase activity readily detectable in vitro (Konopka et al, 1984, Kloetzer at al, 1985, Konopka et al, 1985).

1.3 DISTRIBUTION OF THE Ph CHROMOSOME IN DIFFERENT CELL LINEAGES

Nowell and Hungerford first observed the Ph chromosome in dividing myeloid cells obtained from the peripheral blood of a patient with CML (Nowell and Hungerford 1960a) (see above). In 1963 Wang-Peng et al demonstrated the presence of the Ph chromosome in 100% of the metaphases in the bone marrow of 3 patients. Evidence for the presence of the Ph chromosome in other cell lineages soon followed. Clein and Flemans (1966) demonstrated the presence of iron-containing granules and the Ph chromosome in the same cells of a patient in blast crisis, thus establishing the presence of the Ph chromosome in cells of the erythroid lineage. Subsequently Rastrick et al (1969), used radioisotopes of iron (^{59}Fe and ^{55}Fe) and demonstrated labelling of haemoglobin in Ph-positive red-cell precursors. The involvement of megakaryocytes was strongly suggested by the observation of 2, 4 and 8 Ph chromosomes in 4n, 8n and 16n mitotic cells, since such polyploid cells were assumed to represent the megakaryocytic series (Whang et al, 1963). By applying culture conditions favourable to macrophage growth, Golde et al (1977) demonstrated the presence of the Ph chromosomes in mononuclear cells, 80% of which were identifiable as macrophages.

The presence of the Ph chromosome in these cell lineages suggested that it arose in a pluripotential stem-cell. Fialkow et al (1967) studied the origin of granulocytes and red-cells using the sex-linked

polymorphic isoenzyme glucose 6 phosphate dehydrogenase (G6PD), one locus of which is subject to X inactivation in human females such that only one X chromosome remains genetically active. In three females with CML who were heterozygous at the G6PD locus, both isoenzymes A and B were found in their cultured fibroblasts. Their red cells and granulocytes however only expressed one isoenzyme. This finding was evidence compatible with a disease of a clonal nature with a common stem cell for red cells and granulocytes. The Ph status of the lymphoid lineage has also been of great interest. Rosenthal et al (1977) observed that the morphology of blasts in 21 out of 67 patients in blastic transformation was lymphoid in origin. About 33% of acute lymphoblastic leukaemias had the Ph chromosome (Peterson, Bloomfield and Brunning, 1976); they suggested lymphocytes could possibly have arisen from the same aberrant stem cell. Fialkow et al (1978) again studied the G6PD isoenzyme expression to determine whether lymphocytes originated from the same leukaemic progenitor as the leukaemic myeloid cells. They concluded that one population of T cells did not arise from the leukaemic clone (this population had both isoenzymes), whereas another population of lymphocytes with B cell characteristics, showed one isoenzyme type, suggesting that they arose from the CML stem cell. There have also been detailed analyses of T-cell involvement in chronic phase CML (Barr and Watt, 1978, Kearney et al, 1982, Fauser et al, 1982 Fauser et al, 1985, Bartram et al, 1987). Some of the patients studied had CML for ten or more years and direct analyses of T-cells showed no evidence of a Ph chromosome. However, in two studies, T-cell progenitors were reported to give rise to Ph-positive mature T-cells (Fauser et, 1982, Fauser et al, 1985).

Studies of in vitro skin cultures have demonstrated no Ph chromosome in this tissue, indicating that this disease process does not involve other somatic cells (Tough et al, 1961). It seems therefore that CML involves a stem cell common to the myeloid and the B and T lymphocyte series.

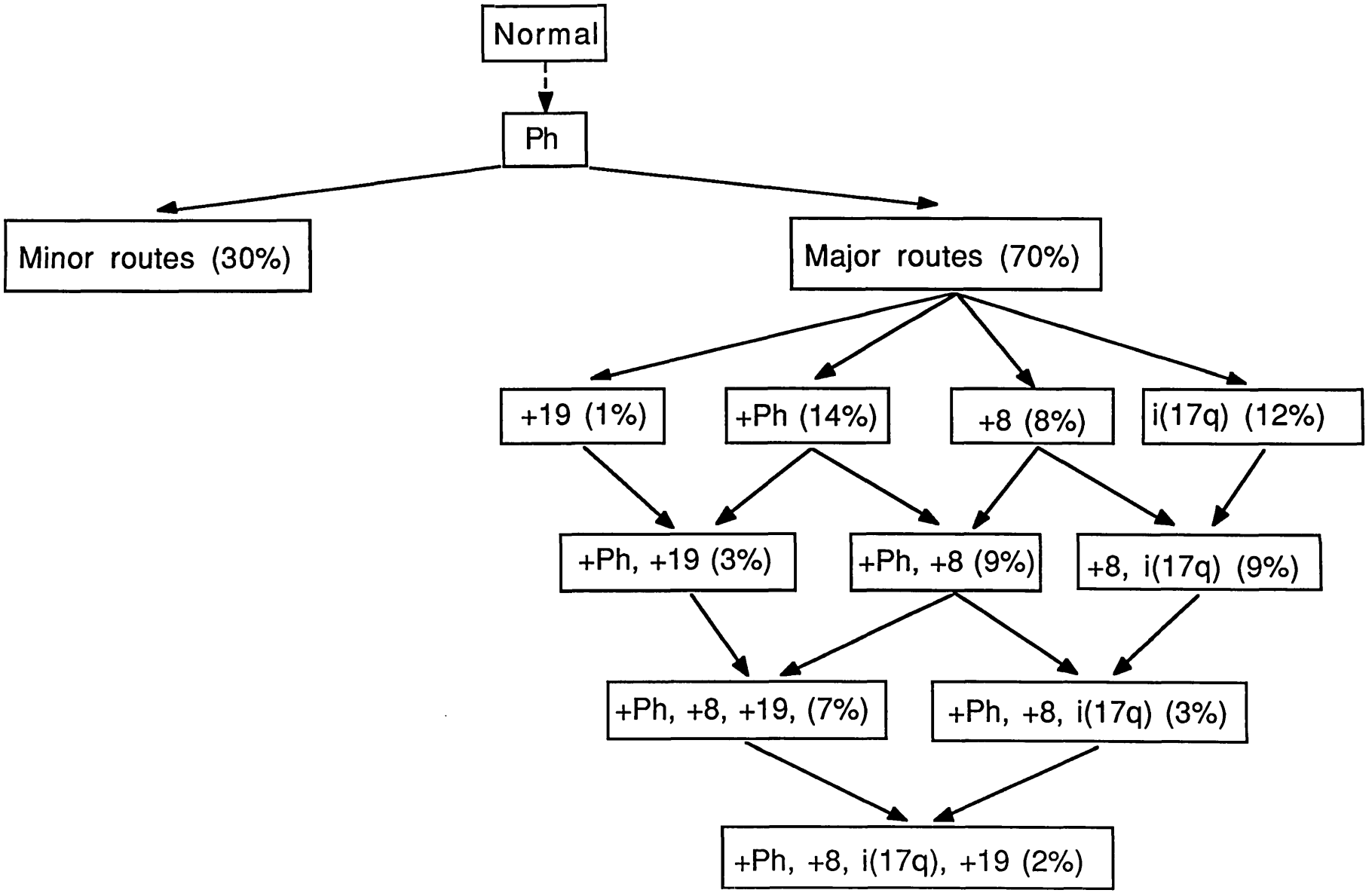
1.4 CYTOGENETIC EVOLUTION IN STANDARD Ph-POSITIVE CML

In the majority of CML patients the Ph translocation is the only cytogenetic abnormality during the chronic phase of the disease. However progression of the disease is heralded or accompanied by additional chromosome abnormalities in approximately 80% of patients. Early investigators, using unbanded chromosome preparations, believed that the chromosomal changes that occurred in addition to the Ph chromosome were non-random. These observations were later corroborated by studies using banding techniques. In 1976 Mitelman et al introduced the concept of the 'major' and 'minor' routes of cytogenetic evolution taken by CML in blastic transformation. The 'major' pathway referred to acquisition of an extra Ph chromosome, an extra chromosome 8, and (or) the occurrence of an isochromosome 17 [i(17q)]. These major additional chromosomal changes occur with similar frequencies. The gain of chromosome 19, on the other hand seems to occur later in karyotypic evolution and most often in combination with an extra chromosome 8 or a Ph. Furthermore whereas the the finding of i(17q) in combination with an extra chromosome 8 is quite common, the combinations i(17q) with +19, i(17q) and +8 with +19, and i(17q) and +Ph with +19 are very rare (Fig 5).

Fig 5 **Common pathways for cytogenetic evolution in CML**

This flow diagram indicates the 'major' and 'minor' pathways of cytogenetic evolution taken by CML in blast crisis. 'Major' routes are: the acquisition of an extra Ph chromosome, chromosome 8, chromosome 19 and an isochromosome of 17q. 'Minor' routes are are all other cytogenetic changes. This chart emphasises a dynamic cytogenetic picture where new clones emerge from a cytogenetically unstable neoplastic cell population. Those cells with proliferative advantage gradually outgrow the others as the disease becomes more malignant. (modified from Heim and Mitelman, 1987)

Fig 5


Normal
Ph
Minor routes (30%)
Major routes (70%)
+19 (1%)
+Ph (14%)
+8 (8%)
i(17q) (12%)
+Ph, +19 (3%)
+Ph, +8 (9%)
+8, i(17q) (9%)
+Ph, +8, +19, (7%)
+Ph, +8, i(17q) (3%)
+Ph, +8, i(17q), +19 (2%)

Secondary chromosomal aberrations have now been described in more than a thousand patients, and typical patterns of karyotypic evolution have been reviewed by several groups. The prognostic value of detecting additional chromosome changes in later stages of the disease is important when it comes to diagnosing accelerated phase CML or impending blast crisis. Thus the appearance of secondary chromosome aberrations is an ominous sign that usually precedes clinical blast crisis by 2-6 months. At times, the clonal evolution may be detectable in extramedullary tissues before it can be diagnosed in the bone marrow (Mitelman, 1975). However, the occurrence of additional cytogenetic aberrations is not an essential feature of blast crisis development and about 20% of all CML patients have the Ph chromosome as the sole cytogenetic abnormality throughout their disease (First International Workshop on Chromosomes in Leukaemia, 1978).

The clinical significance of these additional chromosomal changes in relation to blastic transformation is still controversial. Conflicting results have been reported with regard to the prognostic importance of additional aberrations. In some series, the survival times of the patients with and without additional aberrations have been similar; in other studies patients with the Ph chromosome as the only abnormality survived longer than those who developed secondary changes (Alimena et al,1982, Cervantes et al, 1986, Singh et al, 1986).

The precise nature of the secondary aberrations may also be of haematological and prognostic importance, although again opinions differ. A suggested association between i(17q) and thrombocytopenia

remains to be confirmed (Fleishman et al, 1981). Likewise the claim that patients with i(17q) have an especially poor prognosis has not been substantiated (Alimena et al, 1987). However, the association between i(17q) and basophilia, first suggested by Prigogina et al in 1978, has now been confirmed in other studies (Swolin et al, 1985, Alimena et al, 1982). Recently attempts have been made to correlate the chromosome abnormalities in blast crisis with the morphological, immunological and cytochemical features of the emerging blasts. The number of cases investigated thoroughly is still small, but the results of three recent studies indicate that the pattern of additional abnormalities correlates with the predominant lineage involved in the transformation (O'Malley and Garson, 1985, Yao, 1985, Parreira et al, 1986). It seems that in general the typical chromosome aberrations +8, i(17q), +19 and +Ph are found in blast crises with myeloid differentiation. The +8, i(17q), and +19 however are less common in the lymphoid blast crises.

1.5 Ph-NEGATIVE CML

Approximately 5% of CML patients have a form of leukaemia that resembles Ph-positive CML more or less closely but have a normal karyotype in their leukaemic cells. These patients are classified as having Ph-negative CML. The precise haematological classification of patients with Ph-negative disease is still controversial. Some investigators believe that these patients should be classified more correctly as having myelodysplastic syndrome. Pugh et al (1985), for example, studied 25 patients and classified all but one as myeloproliferative conditions other than CML or as myelodysplastic syndromes. In the same

vein, Travis et al (1986) suggested that Ph-negative CML was a mixture of other disease types, thereby making it a 'non-entity' (Travis et al, 1985). However other studies have contradicted these findings. Results of clinical and haematological study of Ph-negative CML, carried out as part of the Medical Research Council's second therapeutic trial involving 547 patients, showed that of 35 cases (8%) entered as Ph-negative CML, 18 (4%) cases had leucocyte differential counts indistinguishable from Ph-positive CML. The remaining cases were reclassified as atypical CML (10 cases) or chronic myelomonocytic leukaemia (7 cases). Therefore it seems clear that a proportion of patients with Ph-negative CML have a disease that is, by clinical and haematological criteria, indistinguishable from Ph-positive CML. Molecular studies in some of these patients show the same changes as in the Ph-positive disease - namely the translocation of ABL to a morphologically normal chromosome 22, formation of a chimeric BCR/ABL gene, expression of the 8 or 8.5 kb mRNA and translation of the 210 kd protein (Morris et al, 1986, Ganesan et al, 1986, Dreazen et al, 1987, Kurzrock et al, 1987, Wiedemann et al, 1988)

1.6 VARIANT Ph TRANSLOCATIONS

In the same year that the t(9;22) was identified, Hayata, Kakati and Sandberg (1973) reported the first case where material from the Ph chromosome had been translocated to a chromosome other than 9. They described a CML patient with a Ph chromosome and a normal chromosome 9 but with an additional terminal band on chromosome 2.

Since 1973 over three hundred such cases have been described. Based on the documentation of nearly 4000 Ph positive cases the overall frequency of variant translocations is 4.3% (De Braekeleer, 1986). These variant translocations were classified as 'simple' when the material lost from chromosome 22 was translocated to another chromosome other than 9, and as 'complex' if three or more chromosomes (including 22) were involved in the translocation. Sometimes during these complex chromosomal exchanges a piece of chromosomal material was translocated to the deleted 22, thus obscuring the identification of the typical Ph chromosome. The resulting chromosome is sometimes called a 'masked' Ph chromosome (Tanzer et al, 1977) .

The advent of reverse banding or R-banding (a staining technique that gives a banding pattern that is the opposite or reverse of the G-banding pattern) in conjunction with high resolution chromosomes (more extended chromosomes revealing more bands) and in situ hybridisation techniques has made it possible to define the variant Ph translocations more precisely. Simple variants were shown always to involve G-band negative terminal chromosome regions which were often difficult to visualise (Heim et al,1985, Maserati et al, 1986). Investigation of simple variants has shown involvement of chromosome 9 in each case (Oshimura et al 1982, Pasquali et al, 1979), thereby reclassifying simple variants as complex translocations. In situ hybridisation analysis has demonstrated that all variant Ph translocations involve a translocation of ABL to the Ph chromosome as in the standard Ph translocation (Bartram et al 1983, 1985a, Hagemeijer et al 1984, 1985). De Klein et al (1984) showed that variant Ph translocations also have rearrangement of BCR.

These findings demonstrate that variant Ph translocations have the same molecular lesions as the standard Ph translocation.

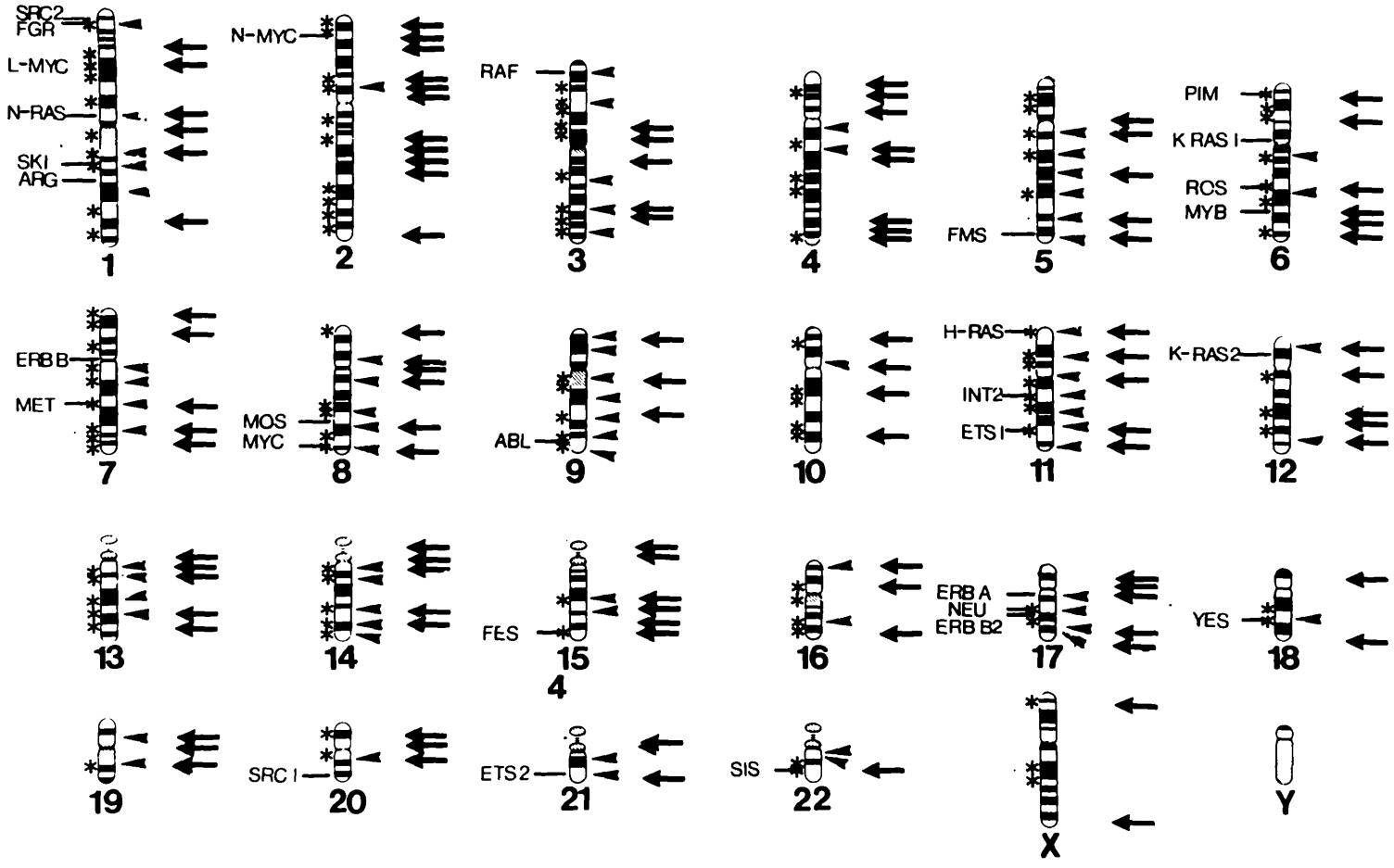
The significance of the involvement of chromosomes other than 9 and 22 in CML has been extensively reviewed. Although one report pointed to the possibility that the blastic phase may be somewhat shorter in patients with variant translocations, the general consensus is that the disease seems to have features that do not usually differ from cases with a standard Ph (Potter et al, 1981, Sandberg et al, 1980, De Braekeleer et al, 1987).

All chromosomes apart from the Y chromosome have been implicated in these variant translocations. The observation that practically every chromosome could be involved in these translocations seemed to suggest that their role in the pathogenesis of CML was not significant. Further, Heim et al (1985) found no obvious correlation between the breakpoints of all these chromosomes and fragile sites, oncogene locations or consistently occurring breakpoints in different malignancies. This conclusion was challenged in three separate studies. The authors of these studies showed that although most chromosomes were involved in variant translocations, particular chromosomal bands were involved more frequently than expected and many of these bands correlated with the positions of known oncogenes, fragile sites and non-random sites of rearrangement in other malignancies (De Braekeleer, 1987, Huret et al, 1986, Maserati et al, 1986) (Fig 6). However, although a correlation between breakpoints and oncogene sites has been proposed, additional studies allowing a more precise localisation of

Fig 6 Human chromosome map depicting the positions of oncogenes, fragile sites, breakpoints in human neoplasia and breakpoints in variant translocations of CML

On the righthand side of chromosomes: the breakpoints in human neoplasia are represented by an arrowhead ( ),
breakpoints in variant translocations are represented by an arrow ( )
On the lefthand side of chromosomes: fragile sites are represented by asterisks ( ), oncogenes are represented by lines.
The origin of the Human proto-oncogenes is listed below:
FGR Gardner-Rasheed feline sarcoma, SRC Rous chicken sarcoma,
L MYC Human lung carcinoma, N RAS Human neuroblastoma,
SKI SKV avian virus tdB77, ARG Abelson-related gene,
N MYC Human neuroblasoma, RAF 3611 Murine sarcoma,
FMS McDonough feline sarcoma, PIM Murine T-cell lymphoma,
K RAS Kirsten murine sarcoma, ROS Avian sarcoma,
MYB Avian myeloblastosis, ERB B Avian erythroblastosis,
MET Human osteosarcoma, MOS Moloney murine sarcoma,
MYC MC29 avian myelocytomatosis, ABL Abelson murine leukemia,
H RAS Harvey murine sarcoma, INT 2 Mouse mammary tumour,
ETS Avian E26 leukemia, K RAS 2 Kirsten murine sarcoma,
FES Snyder-Theilen feline sarcoma, ERB A Avian erythroblastosis,
NEU Rat neuroglioblastoma, YES Avian Yamaguchi (Y73),
SRC 1 Rous chicken sarcoma, SIS Simian sarcoma
(modified from Heim and Mitelman, 1987).

Fig 6


SRC2
FGR
L-MYC
N-RAS
SKI
ARG
1
N-MYC
2
RAF
3
4
FMS
5
PIM
K RAS I
ROS
MYB
6
ERBB
MET
7
MOS
MYC
8
ABL
9
10
H-RAS
INT2
ETS I
11
K-RAS2
12
13
14
FES
15
4
16
ERB A
NEU
ERB B2
17
YES
18
19
SRC I
20
ETS2
21
SIS
22
X
Y

breakpoints as well as molecular investigation of the relevant oncogenes, are necessary.

1.7 Ph-POSITIVE ACUTE LEUKAEMIA

1.71 Introduction

It has been known for some years that the Ph chromosome was not restricted to CML but was present in about 20% of cases with adult acute lymphoblastic leukaemia (ALL), 2-3% of cases with childhood acute lymphoblastic leukaemia and 1-2% of cases with acute myeloid leukaemia (AML) (Heim and Mitelman, 1987). The clinical similarities between the acute leukaemias and the blast crisis of CML led some investigators to propose that the Ph-positive acute leukaemias were nothing other than the blastic transformation of CML with a silent or undiagnosed chronic phase (Catovsky, 1979). However many features of the Ph-positive acute leukaemias argue against this interpretation. Firstly the onset of the acute leukaemia is often rapid. Secondly there is usually persistence of some Ph-negative metaphases throughout the course of the disease. Thirdly there is usually a true complete remission in response to treatment with the disappearance of all the Ph-positive metaphases (Sandberg et al, 1980). All these features contrast with the typical findings in CML in transformation. Furthermore, although the Ph chromosomes of ALL and CML are indistinguishable at the cytogenetic level several investigators have recently reported molecular differences between CML and Ph-positive AML and ALL (De Klein 1986, Hermans et al 1987, Clark et al, 1987, Chan et al, 1987, Kurzrock et al, 1987).

1.72 Ph-positive acute lymphoblastic leukaemia (ALL)

These leukaemias have been typed according to the French-American-British (FAB) classification as L1 or L2. The large majority of cases have antigenic markers corresponding to early precursor B-lymphocytes. These cells exhibit immunoglobulin heavy chain rearrangement, express terminal deoxynucleotidyl transferase (Tdt) and the membrane markers CD 19 (B4) and CD 10 (common ALL antigen (Rozendaal et al, 1981, Louwagie et al, 1985). Within the population of ALL patients the finding of a Ph chromosome constitutes a poor prognostic factor. Remission is usually obtained but relapse occurs early resulting in a short duration of life with median survival of only 10-12 months (Bloomfield et al, 1986).

50% of patients with Ph-positive ALL have additional chromosome abnormalities. These aberrations occur mainly as aneuploidy and to a lesser extent as structural chromosomal rearrangements. Monosomy of chromosome 7 is relatively common and translocations involving both chromosome 9s have been reported (Sandberg et al, 1985). 15% of Ph-positive ALLs have variant Ph translocations. These variants occur with greater frequency in Ph-positive ALL than in Ph-positive CML, the latter having an incidence of approximately 5% (Ribeiro et al 1987). Complex variants involving chromosomes 14q32 in particular have been reported (Takeuchi et al, 1981, De Klein et al, 1986).

Molecular analysis of the Ph translocation showed that about 50% of patients have a breakpoint within M-BCR (Erikson et al, 1986, De Klein et al, 1986). In these cases an 8 or 8.5 kb chimeric mRNA is

transcribed and a chimeric P210 protein is translated. The other 50% of cases did not have a break within M-BCR and failed to express an 8 kb mRNA. De Klein et al (1986) showed that ABL is consistently translocated to the Ph chromosome and 3' BCR sequences are sometimes deleted. Although no P210 was found in the leukaemic cells of these patients, a novel ABL related protein of 190 or 185 kd was detected (Chan et al, 1987, Clark et al 1987, Kurzrock et al, 1987). Hermans et al (1987) reported that, in these patients, chromosomal breaks occur 5' of M-BCR but within the first intron of the BCR gene (Fig 3). They suggested that in this way a new fusion gene was created which transcribed a 7 kb BCR/ABL mRNA (Fig 4). Most of the breakpoints within the first intron of the BCR gene have been shown to be clustered in a 10.8 kb region (Chen et al, 1988). However recently Chen et al (1989) reported two cases of Ph-positive ALL with breakpoints in a 5 kb segment of the first intron, 16 kb upstream of the previously described cluster.

1.7.3 **Ph-positive acute myeloid leukaemia (AML)**

Two-thirds of Ph-positive AML patients have cells with M1 features according to the FAB classification. The remaining one-third of cases are mostly M2, less commonly M4 and rarely M5. As in ALL, the presence of the Ph chromosome in an AML patient constitutes a poor prognostic factor (Bloomfield et al, 1978, Maddox et al, 1981). Although the number of cases studied is small compared with ALL, variant translocations have been reported (Kurzrock et al, 1987, Price et al, 1988).

Few molecular analyses have been reported so far in Ph-positive AML. Kurzrock et al (1987) have shown that in a patient with with no breakpoint in M-BCR the leukaemic cells expressed a p190 BCR-ABL protein. Erikson et al (1986) reported rearrangement of M-BCR in another patient.

1.8 NEW DEFINITIONS ARE REQUIRED

1.8.1 New nomenclature

As a result of molecular investigations of the Ph translocation carried out by different groups some confusion has resulted over nomenclature. When the breakpoints on chromosome 22q11 were first analysed in CML, the clustering of these breaks in a particular region of DNA led to the term Breakpoint Cluster Region (BCR) (Groffen et al, 1984). This region of DNA was then found to be part of a gene which contains at least 20 exons and which was then called the BCR gene by some investigators. Often the term BCR was used to refer to both the region, where the majority of breakpoints in CML occur, and also to the gene itself. One group suggested that the gene should be called PHL and the term BCR should refer just to the 5.8 kb region within the gene (Greaves et al, 1987). However after the observation of a second breakpoint cluster region 5' of BCR but within the first intron of the 'PHL' gene in some patients with acute leukaemia, it was recognised that a new nomenclature was needed to define both these areas clearly. Recently a provisional nomenclature was proposed by an informal group of investigators. They suggested that the 5.8 kb region previously designated BCR be termed M-

BCR (major breakpoint cluster region) and that the clustering of breakpoints within the first intron of the BCR gene be known as m-BCR (minor-breakpoint cluster region) (Gale and Goldman, 1988).

CHAPTER 2: MATERIALS AND METHODS

2.1 PATIENTS AND CONTROLS

Cytogenetic, in situ hybridisation and molecular studies were carried out on three groups of CML patients and two Ph-positive acute leukaemia patients. Each of these groups displayed features which posed interesting questions relevant to leukaemia and to the genesis of cancer in general.

2.1.1 Ph-negative CML

A selected group of eight Ph-negative CML patients was studied. The majority of these patients had been referred to us for investigation from haematology departments in the United Kingdom. These patients, initially studied locally, had clinical and haematological pictures resembling classical CML but their leukaemic cells lacked a Ph chromosome. Samples of peripheral blood and/or bone marrow were sent to us for confirmation of cytogenetic findings and further molecular investigation.

2.1.2 Variant Ph-positive CML

Four CML patients with variant translocations were identified, three 'complex' and one 'simple'. The 'simple' variant was studied by cytogenetics and in situ hybridisation to find out whether chromosome

9q34 and hence the ABL oncogene was involved in the translocation. The sequence of chromosome rearrangements in a patient with a 'complex' translocation involving 5 different chromosomes where the translocation pattern after R-banding ambiguous was investigated. The 'complex' translocations in the two other patients involved breakpoints in the region of the MYB and FMS oncogenes. A possible translocation of these oncogenes was looked for by in situ hybridisation.

2.1.3 **Blast crisis CML**

Four CML patients in blast crisis of myeloid lineage were studied by cytogenetic and molecular means in order to identify specific changes unique to blastic transformation.

2.1.4 **Acute leukaemia**

Two Ph-positive acute leukaemia patients with unique features were investigated by cytogenetic, in situ hybridisation and molecular means. One patient had acute lymphoblastic leukemia; at presentation he showed both myeloid and lymphoid cell surface markers and both Ph-positive and negative metaphases in the bone marrow. The other patient had acute myeloid leukaemia with a complex karyotype including a 'masked' Ph chromosome.

2.1.5 **Controls**

Control samples of peripheral blood and bone marrow were obtained from normal individuals (members of staff and/ bone marrow transplant donors) and patients routinely treated at our clinic with chronic phase CML and a standard Ph chromosome. Positive (Ph-positive CML) or negative (normal) controls were used in each experiment.

2.2 CELLS

2.2.1 **Handling of peripheral blood and bone marrow**

Samples of bone marrow (1-2 mls) were collected into sterile 'universal' bottles containing 4 mls of culture medium, consisting of 1 ml fetal calf serum in RPMI 1640 medium (Gibco), and 200 units of preservative-free heparin (PFH) (Paines & Byrne). Peripheral blood was usually collected in sterile universals containing 200 units of heparin without medium. These samples were delivered to the laboratory as soon as possible after collection and preferably processed on the same day. However if necessary and especially when the specimens were collected at some distance from the laboratory, the peripheral blood (PB) and bone marrow (BM) cells was stored overnight at 4°C and processed the following day. Sterile technique was employed throughout the procedures of handling and culturing cells.

2.2.2 **Cryopreservation and reconstitution of cells**

Peripheral blood from newly diagnosed CML patients were routinely cryopreserved in our laboratory. Peripheral blood and bone marrow was also cryopreserved from a number of CML patients in blastic transformation. The techniques of cell collection, storage and reconstitution of frozen cells were first described by Hill et al (1973). Leukaemic blood cells from newly diagnosed patients were collected by means of a continuous flow blood-cell separator (Cobe 2997) and bone marrow cells were collected by aspiration from the iliac crests with the patient under general anaesthesia. 10% dimethylsulphoxide (DMSO) was used as a cryoprotective agent and nucleated cells were routinely frozen together with the contaminating erythrocytes in 2 ml polypropylene ampoules (Sterilin) at a controlled rate of 1-1.5°C/min. Cells were stored at -196°C in liquid nitrogen containers (Planer Ltd). Frozen cells were reconstituted by diluting one ampoule of cells in 20 mls Hanks' balanced salt solution (Gibco) to prevent agglutination of leucocytes, before the separation of mononuclear cells.

2.2.3 **Separation of mononuclear cells**

Mononuclear cells from these BM and PB samples were usually separated on a density gradient using Lymphoprep (Nyegaard). Samples with very high white-cell counts (> 200 $x10^9/l$) were diluted with sterile Hanks' balanced salt solution (Gibco) and one part of cell suspension was added to one part of Lymphoprep solution. The cells were carefully layered

(in dropwise fashion) onto an equal volume of Lymphoprep and centrifuged at 2000 rpm for 30 mins. After centrifugation immature myeloid and other mononuclear cells lay in a well defined, white band at the interface between the serum and the Lymphoprep. This mononuclear fraction was removed using a 10 ml syringe with an attached quill and the volume made up to 20 mls with Hanks' solution in a sterile universal. These cells were washed twice by centrifuging the cells at 2000 rpm for 5 mins and finally resuspended in up to 5 mls of Hanks' solution at a cell concentration of approximately $10x10^6$/ml.

2.2.4 **Cell-counting procedures**

After separation of the mononuclear fraction and before culturing, the cells were counted manually using an improved Neubauer counting chamber. 10 ul of cell-suspension was mixed with 90 1 of a 1% acetic solution and gently introduced into a counting chamber (under a coverslip). The counting was done under a microscope using a x40 objective lens. The number of cells were counted within the central grid which was the equivalent of 0.1 micron. Taking the dilution factor into account it was possible to work out the concentration of cells in the final cell suspension.

2.2.5 **Cell-culture procedures**

2.2.5.1 **Preparation of conditioned media**

For optimum culture conditions it was often necessary to supplement our culture medium with conditioned media derived from three main sources:

(1) Activation of T-lymphocytes by alloantigens
Mononuclear cells from at least 2 individuals were combined and suspended at a concentration $1x10^6$/ml in culture medium and cultured for 7 days at 37°C. The cell-suspension was centrifuged at 5000 rpm for 5 mins and the supernatant used as conditioned medium.

(2) Antigen activation of T-lymphocytes with -mercaptoethanol
This conditioned medium was obtained by incubating mixed mononuclear cells with -mercaptoethanol at a final concentration of 10^{-5}M for 7 days at 37°C. The cell suspension was centrifuged at 5000 rpm for 5 mins and the supernatant was used as conditioned medium.

(3) Conditioned medium derived from bladder-carcinoma cell-line 5637
The supernatant from cell-line 5637 (Foch et al, 1978, Gabrilov et al, 1986), which appears to optimally support the proliferation of myeloid cell progenitors (CFU-GM) (Myers et al , 1984), was used as a conditioned medium in our cultures.

Conditioned medium was used at a concentration of 10% volume by volume (v/v).

2.2.5.2 **Setting up of cultures**

Our culture medium consisted of RPMI 1640 with added Hepes buffer (Gibco) suplemented with 20% fetal-calf serum, 10 ug/ml penicillin and 10 ug/ml streptomycin (Gibco), and 10 ug/ml units L-glutamine (Gibco). The mononuclear cells were cultured at a concentration of $1x10^6$/ml in 25 cm^2 tissue-culture flasks (Falcon) containing 7 mls of culture-medium and 10% conditioned medium. The cultures were incubated (with the flask-caps slightly loosened) for a period of 24-96 hours in an incubator (Leec) with 5% CO_2 in air at 37°C.

2.3 CYTOGENETICS

2.3.1. **Standard cytogenetics**

2.3.1.1 **Cell harvesting procedures**

Cultures were harvested at 24 hour intervals over a period of 96 hours. However, occasionally low cell numbers from PB and BM samples meant that a less than optimal concentration of cells was used. The best time for harvesting these cultures was assessed by closely observing the rate and type of growth under an inverted microscope.

Colcemid (Gibco) was added to the cultures at a concentration of 10 ug/ml 45 to 60 mins before they were harvested. Thereafter the cell suspension was transferred to 10 ml conical centrifuge tubes (Sterilin) and centrifuged at 1000 rpm for 5 mins. Most of the supernatant was

removed leaving behind approximately 1 ml in which the the cell-pellet was resuspended. 5 mls of 0.075 M hypotonic potassium chloride (KCl) was added dropwise to this cell suspension which was then incubated in a 37°C waterbath for 20 mins. Cells were then centrifuged at 1000 rpm for 5 mins and most of the supernatant removed. The resuspended cells were then fixed in a freshly made 3:1 methanol to acetic acid solution. Optimal fixation of cells was achieved by adding fix solution very slowly whilst constantly shaking the tube. The fixed cells were usually left for 30 mins at room temperature (RT) and then washed several times (3-4) in fix solution. The cell pellet was finally resuspended in fix solution (approximately 1 ml) so that the cell suspension appeared cloudy.

2.3.1.2 **Preparation of slides**

Pre-cleaned 76x26 mm twin-frosted (Chance Propper) microscope slides which had been soaked overnight (or longer) in absolute ethanol were used to make metaphase preparations. The slides were removed from the ethanol and wiped dry using clean paper towels.

Metaphase spreads were made as follows: The cell suspension was taken up in a glass pasteur pipette (Bilbate) and held approximately 15 cm above a slide held horizontally. The cell suspension was dropped onto the slide (one drop at either end) and then left in a vertical position to dry in air. One or two slides were then immediately stained using a 10% Giemsa (Gurr) stain made up in phosphate buffer pH 6.8 (Sigma) for 8-10 mins. These test slides were scanned to assess the quality and

quantity of mitoses obtained using a X40 lens and a X100 oil immersion lens. If mitoses were present but the chromosomes were overlapping, the cells were washed in fixative until the best preparations were achieved. However if the mitotic index and spread of chromosomes was acceptable then between 10 and 15 slide preparations were made. These slides were then labelled and clearly left to dehydrate in a 37°C dry incubator (Gallenkamp) for approximately 7 days before banding.

2.3.1.3 **Chromosome banding**

All metaphases were routinely banded according to the Trypsin-Giemsa method first described by Seabright et al (1971) and modified as described below.

Slides of metaphase spreads were placed in a coplin jar containing a 2x standard saline citrate (SSC) solution (see appendix) at 60°C for 45 mins. The slides were then rinsed in distilled water. A test slide was then exposed to a 0.5% v/v concentration of trypsin (Difco) at 4°C for 15 secs. This slide was then quickly rinsed in phosphate buffer and stained in 10% Giemsa stain for 10 mins. After rinsing the slide in distilled water the slide was air dried. The quality of banding was assessed by looking at metaphases with a X100 lens on immersion oil and the trypsinisation time was adjusted accordingly. Slides from different patients made at different times varied quite extensively in the trypsinisation time required to give optimal chromosome banding. However slides from the same patient spread on the same day required the same time of trypsinisation.

2.3.2 Cytogenetics of synchronised cultures

2.3.2.1 Principles

A technique for synchronising our cultures was also employed. This technique, when successful, improved the quality of the mitotic chromosomes (many prometaphase mitoses were observed) and the clarity of chromosome banding. Analysis of leukaemic cells has often been limited by the low mitotic index and the fuzzy and contracted nature of chromosomes obtained using conventional techniques (Sandberg et al, 1961). The mitotic yield and chromosome morphology have been improved by exposing cells to cell cycle blocking agents such as methotrexate (MTX) (Hagemeijer et al, 1979, Yunis, 1981), bromodeoxyuridine (BrdU) and fluorodeoxyuridine (FdU) (analogues of thymidine) (Viegas-Pequignot and Dutrillaux,1978, Weber and Garson, 1983). These agents block cell division at the G_1/S boundary of the cell cycle. Optimal blocking required exposure times of approximately 20 hours. A cohort of cells can be released from the block by adding thymidine; they then progress beyond G_1 through the cell cycle and can be arrested in mitosis 5-7 hours later (Fig 7).

2.3.2.2 Culture procedures

After 24 hours (BM) and 48 hours (PB) of mononuclear cell culture bromodeoxyuridine (BrdU) (Sigma) was added at a final concentration of 200 g/ml for approximately 15-17 hours (overnight). The cells were

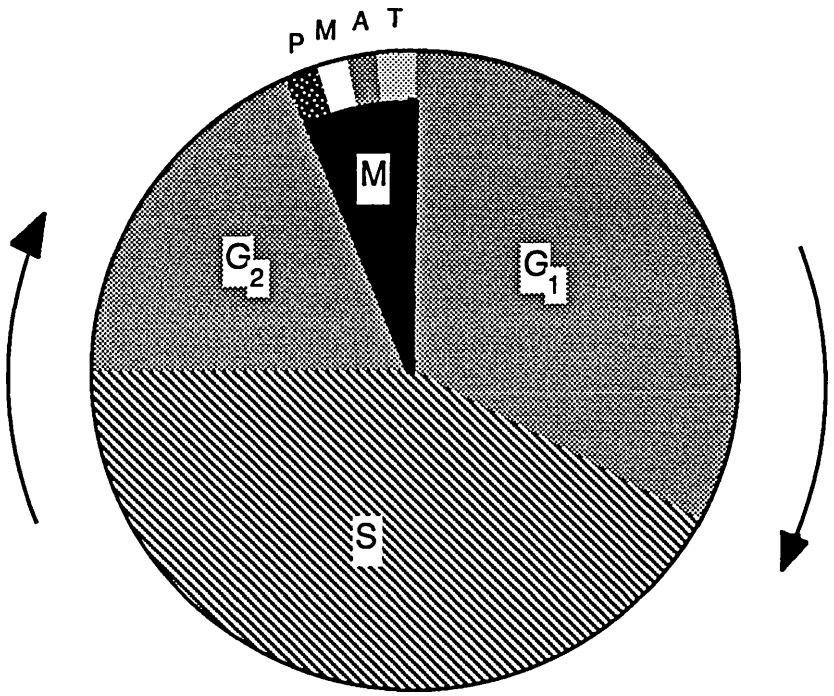


Fig 7 The mammalian cell cycle

The G1 and G2 (first and second gap) periods and the S (synthesis) period are the interphase period during which DNA and other macromolecules are synthesised. The whole cycle lasts approximately 15 hours.

The M (mitosis) phase lasts for about an hour and is the period in which the cell divides. Mitosis is conventionally divided into four substages: prophase (P), metaphase (M), anaphase (A), and telophase (T).

Synchronisation of cells is achieved by blocking cells at the G1/S border with BrdU. An accumulated cohort of cells is released from the block by addition of thymidine and they then progress through G2 into cell division.

then washed twice in Hanks' solution and reincubated with culture medium containing 0.3 g/ml of thymidine (Sigma) for a further 6-7 hours. 5 ug/ml of colcemid (Gibco) was added to the cultures for 30 mins and these were harvested according to standard techniques (see above).

2.3.2.3 **Chromosome banding**

Slides of metaphase preparations (see above) were stained with Giemsa after staining in Hoechst 33258 according to a modified method described by Perry and Wolf (1974). Slides were stained for 15 mins in Hoechst 33258 made up at a concentration 10 ug/ml with 2X (SSC). The slides were then rinsed in distilled water, mounted in 2X SSC and exposed for up to 2 hours under a HBO 200 mercury vapour UV lamp (Zeiss) at a distance of approximately 30 cm. After rinsing in distilled water once again, the slides were stained in 10% Giemsa for 10 mins. This method resulted in G (Giemsa), R (Reverse of Giemsa banding) and C (centromeric heterochromatin stained) banded cells. The percentage of different banded metaphases depended on the duration of culture after addition of BrdU.

2.3.3 **Chromosome analysis**

2.3.3.1 **Microscopy and photography procedures**

Slides of banded chromosome preparations were scanned on a Zeiss photomicroscope with a Hitachi CCTV camera and video monitor.

Metaphase spreads were localised using a X40 lens and were analysed using a X100 oil immersion lens with the aid of the video monitor. Detailed analysis was done to confirm and record the karyotype by photographing these metaphase spreads and cutting and sorting individual chromosomes according to ICSN (1985) criteria.

Metaphases were photographed using Pan F black and white film (Ilford) and developed in a Paterson System 4 Universal tank and reel apparatus using high contrast developing solution (Ilford). The exposed film was wound onto a reel by touch (in the darkroom) and placed in the light-proof tank. The developer was diluted 1:3 with water and used at 20°C. The film was developed for a period of 2.5 mins and the tank was inverted every 30 secs. The developing process was stopped in a 1% acetic acid solution for the period of 1 min and the film fixed by Amfix (May and Baker, diluted 1:3 with distilled water) for at least 3 mins. Once fixed, the film was washed under running water for 30 mins, with the final rinse containing a few drops of Ilfotol wetting agent (Ilford), and then hung up to dry. Black and white prints were made on Agfa-Gevaert Rapitone paper (P1-P4) by using a Durst enlarger with an E1 Nikkor lens and an automatic Agfa-Gevaert print processor.

2.3.3.2 **Evaluation of cytogenetic analysis**

All metaphases were analysed according to the guidelines laid down by the International Society of Cytogenetic Nomenclature (ISCN) in 1985. The necessary number of analysed metaphases required for a satisfactory evaluation of the cell karyotype depended largely on the findings of

each individual case; for routine purposes a detailed analysis of approximately 15 metaphases was generally adequate to exclude or confirm a simple abnormality of chromosome morphology or chromosome number, but the presence of a number of cells with more than one chromosomal constitution necessitated examination of further cells in order to confirm or exclude their clonal nature.

The First International Workshop in Leukaemia (1978) decided that the minimum criteria for identification of a clone of cells were 2 cells with the identical chromosome rearrangement or identical chromosome gain, or three cells with an identical chromosome loss. However, the technical limitations in procedures involved in the spreading of chromosomes (resulting in random chromosome loss and superimposition of one chromosome on another) often necessitated the analysis of more cells in order to reduce the risk of error.

2.3.3.3 **Cytogenetic nomenclature**

The symbols of cytogenetic nomenclature used in this thesis are illustrated in Table 1. Structural alterations of chromosomes were defined by the short system of nomenclature except in Chapter 6 where complex chromosomal rearrangments required a more detailed definition. As adopted by the Chicago conference (1966) single and three letter designations were used to specify chromosomes aberrations. For example a deletion of a chromosome is indicated by the symbol del and a translocation involving a chromosome was designated by the symbol t. Immediately following the symbol identifying the type of rearrangement,

Table 1 **Symbols for cytogenetic nomenclature**

A - G	chromosome groups
1 - 22	autosome numbers (Denver system)
X,Y	sex chromosomes
(+)	addition of chromosome
(-)	deletion of chromosome
i	isochromosome
mar	marker chromosome
p	short arm of chromosome
q	long arm of chromosome
t	translocation
der	derivative chromosome
ins	insertion
ter	terminal or end pter = end of short arm qter = end of long arm
:	break (no reunion as in terminal deletion)
::	break and join
⟶	from - to
parentheses ( )	used to surround structurally altered chromosomes
semicolon (;)	separates structural rearrangements involving two or more chromosomes.

the number of the chromosome involved in the change was specified within parentheses. If two chromosomes were altered then a semicolon (;) was used to separate their designations. If one of the chromosomes involved in the rearrangement was a sex-chromosome then it was listed first; otherwise the chromosome having the lowest chromosome number was always specified first. The breakpoints were indicated in parentheses immediately following the designation of the type of rearrangement and the chromosomes involved. In rearrangements affecting two or more chromosomes the breakpoints were specified in the same order as the chromosomes involved were specified, and a semicolon was used to separate the breakpoints. For example t(9;22) (q34;q11) defined a translocation between the long arms (q) of chromosomes 9 and 22 with breakpoints at bands 9q34 and 22q11 respectively. When we described a chromosomal translocation in detail we used the additional symbols: a double colon (::) to indicate a 'break and joining' event, an arrow ( ) meaning 'from-to' and the end of a chromosome arm was designated either by a band number or by the symbol 'ter' which was preceded by the chromosome number and the particular chromosome arm involved. The description of the chromosome translocation started with the end of the short arm and proceeded through to the end of the long arm, with the bands identified in the order in which they occurred in the rearranged chromosome. For example t(9;22) (9pter 9q34::22q11 22qter; 22pter 22q11::9q34 9qter) indicates that breakage and reunion occurred at band 9q34 and 22q11. The segments distal to these bands were exchanged between the chromosomes.

In recording the chromosome constitution of cells analysed from a patient, we recorded the number of cells analysed, followed by the number of chromosomes and sex chromosomes found in each cell and finally followed by the specific changes that occurred. For example 46,XY, t(9;22) (q34;q11) represented a translocation between chromosome 9 and 22 in a male metaphase with a total number of 46 chromosomes. 47,XY, +8 represents a male metaphase with a total of 47 chromosomes where the extra chromosome was chromosome 8. In complex karyotypes involving both gains and losses of chromosomes chromosome gains was always specified before chromosome deletion. For example 46,XX, +5, +8, -6, -9 indicated the gain of chromosome 5 and 8 and the loss of chromosomes 6 and 9. If cells of more than one chromosomal constitution were present then the number of karyotypes with each pattern out of the total number of metaphases analysed was indicated.

2.4 DNA PROBES ACQUIRED

The DNA probes used in our experiments were kindly provided by a number of collaborators or were acquired from commercial sources. These probes were sent in the form of DNA fragments, DNA inserted into plasmid vectors or bacterial stab cultures. In all instances (except probes inserted into Phage) the use of these probes entailed making preparations of supercoiled DNA from bacteria and eventually cutting out the probe insert from the plasmid vector. The probes I employed are described below:

2.4.1 **ABL**

The ABL probe was a 1.5 kb mouse cDNA PstI fragment (kindly provided by Drs. George Daley and David Baltimore). It corresponded to an internal fragment of ABL containing almost the entire second exon (the so-called first common exon) and virtually all of the kinase domain. In order to make more probe this DNA sequence was ligated into plasmid vector pAT 153.

2.4.2 **BCR probes** (Fig 8)

2.4.2.1 5' BCR

This 5' BCR probe was a human cDNA Bam HI fragment, 1.4 kb in length (kindly provided by Dr Owen Witte). This was an internal fragment of the BCR gene mapping 889 bp 5' (proximal) to the K562 breakpoint. This BCR gene fragment was ligated into plasmid vector pAT 153.

2.4.2.2 BCR 'full length'

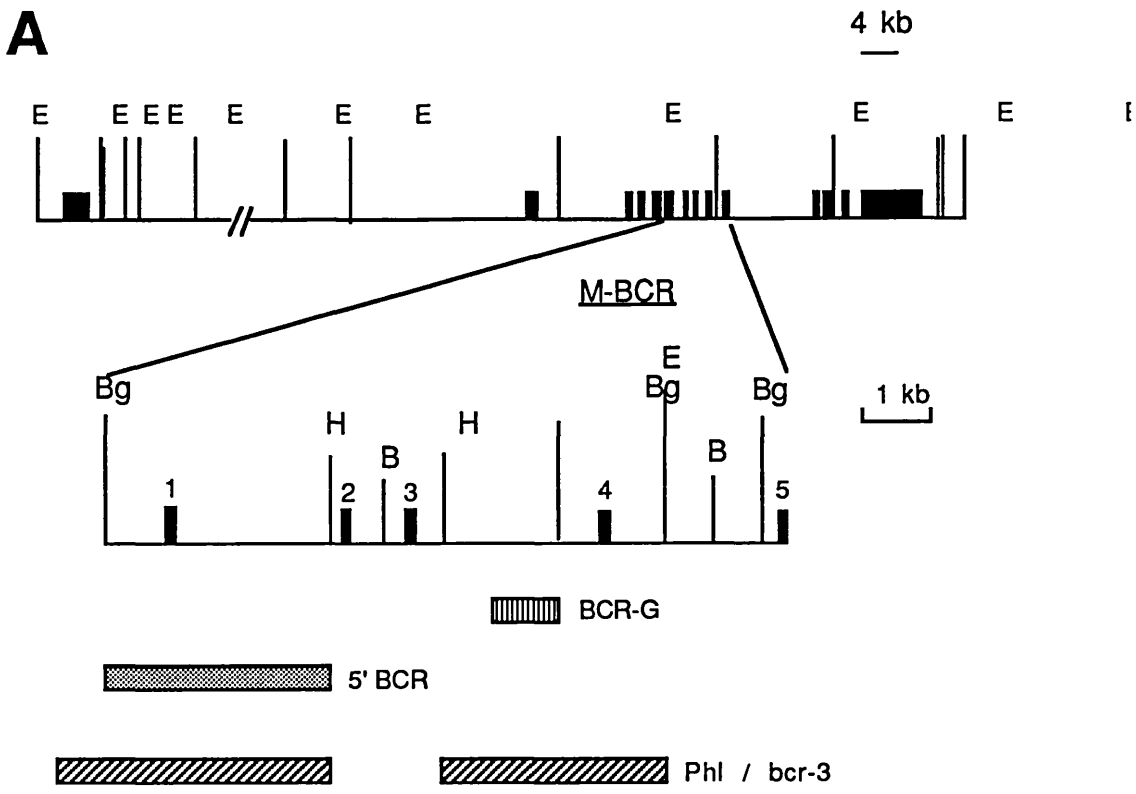
This was a 4 kb Eco RI cDNA probe (kindly provided by Dr. Eli Canaani) inserted into the pUC plasmid vector. This probe corresponded to approximately 90% of the BCR gene. This DNA fragment lacked about 300 base pairs (bp) at the 5' coding region and 5' untranslated region and also lacked exon 5 of M-BCR (according to the nomenclature of Heisterkamp et al, 1985)

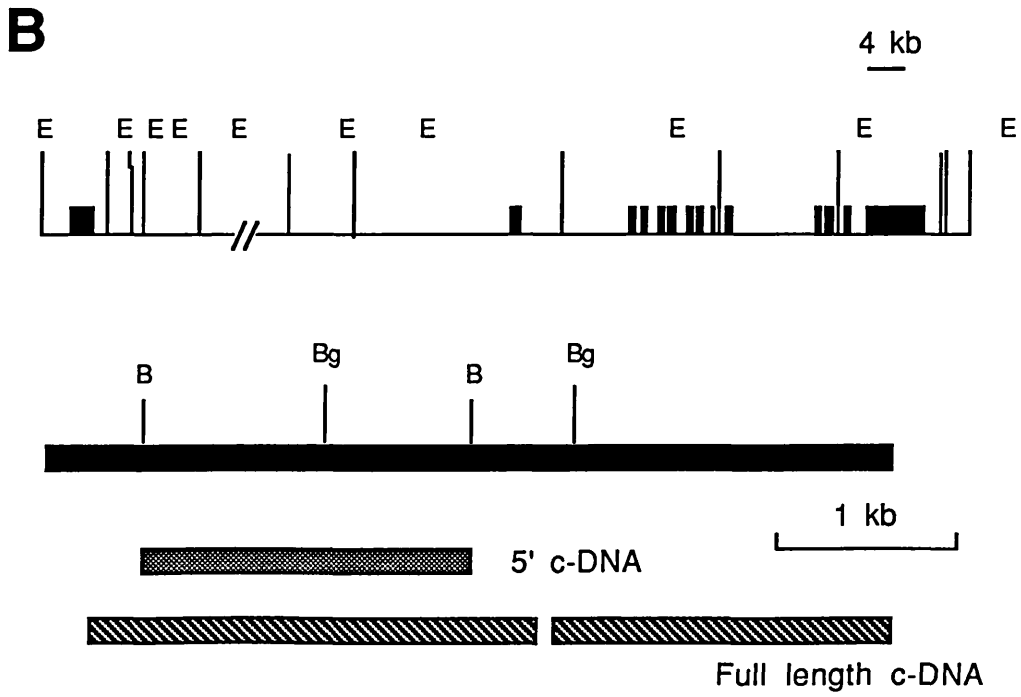
Fig 8 Schematic representation of cloned BCR DNA probes

(A) shows the positions of the cloned genomic probes which map to M-BCR: BCR-G (0.6 kb), 5' M-BCR (2 kb), Phl/bcr-3 (4 kb).

(B) shows the positions of the cloned cDNA probes, one of which maps 5' to M-BCR (1.4 kb) and the other is a 'full length' probe (4.5 kb) which lacks 300 bp at the 5' coding region and the 5' untranslated region and also lacks exon 5 of M-BCR.

Fig 8


A
4 kb
E E E E E E E E E E
M-BCR
Bg
E
Bg
Bg
1 kb
H
H
B
B
1
2
3
4
5
BCR-G
5' BCR
Phl / bcr-3


B
4 kb
E E E E E E E E E E
B
Bg
B
Bg
1 kb
5' c-DNA
Full length c-DNA

2.4.2.3 BCR-G

This was a 0.6 kb Eco RI/Pst I genomic fragment inserted into the pGem I plasmid vector. It corresponded to the intronic region between exons 3 and 4 of BCR.

2.4.3 **SIS**

This SIS probe was a 2.7 kb human cDNA Bst EII fragment inserted into the plasmid vector pAT 153. This DNA fragment corresponded to the last exon of the open reading frame of the SIS gene (Josephs et al, 1984).

2.4.4 **Probes for sequences distal to ABL (on chromosome 9q34)**

Two probes (L1424 and P111) mapping distal to the ABL gene (by classical linkage analysis) on chromosome 9q34 were obtained from Collaborative Research Incorporated (Boston, USA). These probes corresponded to regions of the genome that were polymorphic (according to Southern blotting criteria). L1424 (of unknown size) with the insert ends of Hae III and Alu I was cloned into a Phage vector (Charon 4A). P111 (of unknown size) with insert ends of Eco RI was cloned into a Phage vector (Charon 21A).

2.4.5 **Cλ**

This was an 8 kb human genomic EcoRI fragment subcloned into the plasmid vector pUC 122 (kindly provided by Dr. Terry Rabbitts).

2.4.6 **v-myb**

This was a 2kb viral cDNA Kpn I/Xba I fragment cloned into the plasmid vector Puc 18.

2.4.7 **v-fms**

This was a 3 kb cDNA Eco RI/Bgl II fragment containing most of the v-fms gene (described by Hanfe et al, 1984).

2.4.8 **MYC**

This was a human 1.5 kb Sac I genomic fragment cloned into M13 and corresponding to exon 2 of the MYC gene (Rabbitts, 1985).

2.4.9 **p53**

This is an 1.76 kb p53 cDNA clone PR4-2 that includes all the known exons of the p53 gene (Lamb and Crawford, 1986).

2.5 HANDLING OF DNA

2.5.1 **Extraction of high molecular weight DNA from peripheral blood**

DNA extracted from the mononuclear cell fraction of 10 mls of peripheral blood (as described above) was usually more than adequate for the molecular analyses. These cells were placed in a 50 mls conical tube

(Sterilin) and the sample made up to 50 mls with phosphate buffered saline (PBS). After resuspending, the cells were centrifuged at 2000 rpm for 10 mins at room temperature. This washing was repeated twice and finally the supernatant was poured off leaving the cell pellet. The cell pellet was broken up by gently shaking the tube, bearing in mind that in order to obtain high molecular weight DNA mechanical shearing of DNA should be avoided. The cells were then resuspended in 10 mls of glass distilled water (GDW) and left to stand at RT for 5 mins. The cell suspension was then centrifuged at 1500 rpm for 10 mins. The supernatant was discarded and the pellet resuspended (by gently inverting the tube) in up to 5 mls of lysis buffer (see appendix). The volume of lysis buffer added depended on the total number of cells present. 2.5 mls was used for less than 10^7 cells and 5 mls for between 10^7 and 10^8 cells. The solution was mixed and cells lysed by pipetting up and down vigorously with a wide-bore pipette. At this stage a moderately viscous cell suspension was obtained. Then 100 ul of 20% sodium dodecyl sulphate (SDS) per ml of lysis buffer was added to the cell suspension. The solution became more viscous as the DNA was released from the nuclei and it was mixed vigorously using a wide-bore pipette. The cell suspension was incubated at 37°C for 10 mins. The mixture was then transferred to a polypropylene tube (Sterilin) and phenol-chloroform extraction of proteins was carried out. This was done by adding one volume of phenol and one volume of chloroform and mixing vigorously until the solution resembled a milky emulsion. This mixture was centrifuged at 2000 rpm for 20 mins and the upper aqueous phase containing the DNA was removed into another polypropylene tube. The step of phenol-chloroform extraction was

repeated once and then the cell pellet was resuspended in one volume of chloroform. This mixture was also centrifuged at 2000 rpm for 20 mins and the aqueous phase removed into another polypropylene tube. Two volumes of cold (-20°C) absolute ethanol was added and mixed gently by inversion. A DNA precipitate were formed, fished out using a hooked pasteur pipette and transferred to a siliconised eppendorf tube (1.9 mls) containing 70% ethanol. The DNA precipitate was collected after centrifugation for 5 mins, and the ethanol removed with a pasteur pipette (and by vacuum pump suction if necessary). Approximately 100 ul of Tris disodium ethylene diamine tetraacetate (TE) (pH 8) was added to the DNA precipitate and left overnight at RT to allow the DNA to dissolve completely. The following day the concentration of the solution was measured by reading the absorbance on a spectrophotometer at 260 nm after an appropriate dilution. A DNA solution of 50 ug/ml has an absorbance of 1 at 260 nm.

2.5.2 **Ligation of DNA probes into plasmid vectors**

The cloning of the DNA sequences in some cases required the ligation of these probes into plasmid vectors. This was done as follows: 20 ng of linearised plasmid vector, a three-fold molar excess of probe DNA, 10 mM rATP (ribo-adenosine triphosphate), 100 mM DTT (dithiothreitol), 1 ul of 10x ligation buffer (see appendix), and 1 ul of T4DNA ligase (Boehringer) made up to 20 ul with GDW, were mixed together and incubated overnight at 15°C. These incubation conditions gave a ratio of DNA of approximately 4 containing vector only sequences for every one containing vector plus insert sequences.

2.5.3 **Preparation of DH1 (E coli) competent cells**

The DH1 cells were streaked out onto an L-broth agar (1.5%) (see appendix) plate from a frozen stock and incubated overnight at 37°C. A single colony was picked the following day and used to inoculate a 5 ml culture in LB (Luria-Bertani) broth (see appendix). This was grown at 37°C for 2 hours till the OD at 550 nm was 0.3. Then a 1 in 20 dilution subculture was made into a 100 mls of l-broth and grown for a further 2 hours at 37°C (optimal OD_{550}=0.48).

The culture was chilled for 5 mins on ice and spun in a Sorvall centrifuge in 30 mls Corex (Du Pont Instruments) tubes at 6000 rpm for 5 mins. The supernatant was discarded and the cells resuspended in 2/5 the volume of transformation buffer (Tfb1) (see appendix). The cells were left on ice for 5 mins and spun down at 6000 rpm for 5 mins. Thereafter the cells were again resuspended in 1/25 volume of Tbf1 and left on ice for 15 mins. Aliquots of suitable volumes (usually 200 ul) were placed in microfuge tubes and stored at -70°C.

2.5.4 **Transformation of DH1 cells**

An aliquot of DH1 competent cells was thawed at RT and then left on ice for 10 mins. DNA (up to 2/5 the volume of cells and no more than 100 ng/200 ul cells) was added and left on ice for up to 45 mins. The cells were then heat shocked for 90 secs at 42°C and returned to ice for

a further 1-2 mins. 2-3 volumes of SOB medium (see appendix) was added and incubated at 37°C for 45 mins with gentle shaking. This mixture was then plated onto an L-broth agar plate containing the appropriate antibiotic and incubated overnight at 37°C.

2.5.5 **Preparation of supercoiled DNA from bacteria**

The same protocol was followed for making small preparations (minipreps) as for making large preparations (maxipreps) of DNA from supercoiled bacteria. Naturally larger volumes were used in the maxipreps and solutions used were scaled up 200-fold. THe only difference in the protocol of maxipreps was that the final steps involved purification of DNA by banding on a caesium chloride (CsCl) gradient. Generally minipreps of supercoiled DNA were made first in order to test the quality of the DNA as well as to see if the correct DNA was extracted, and then only did we continue onto a large scale preparation.

A miniprep of supercoiled bacteria was made as follows:
A single colony was picked from the inoculated plate, placed in a sterile universal containing 2 mls of L-broth and antibiotics, and placed in a orbital shaking incubator (Gallenkamp) overnight. The next morning this turbid culture was placed in an eppendorf tube and spun down for 5 mins in a microfuge (MSE).

This cell pellet is resuspended in 500 ul Tris sodium chloride EDTA (TSE X1) and spun down as before. The cell pellet is resuspended in a lysozyme glucose Tris EDTA (GTE) solution (the lysozyme was added to the

GTE solution just before use) and left at RT for 10 mins. Alkaline SDS (200 ul) was added and mixed gently until the solution became clear and then left on ice for 10 mins. 5 M of 1% potassium acetate (KOAc) was added next, mixed by inversion and left on ice for 15 mins. This solution was then spun down as before and the pellet resuspended in 300 ul of isopropanol. This was then vortexed for a few seconds and then spun down after the supernatant was discarded the pellet was resuspended in RNase at a concentration of 2 g/ml solution and incubated at 37°C for 20 mins. Phenol/chloroform extraction of proteins (as mentioned above) was repeated and eventually the pelleted nucleic acids were dissolved in 160 ul GDW and mixed well. A 13% solution of polyethylene glycol (PEG) and 200 ul of 2 M sodium chloride (NaCl) was added and the solution left on ice for 1 hour. This was spun down in a microfuge, washed in 70% ethanol and then dissolved in TE (30 ul). DNA was finally precipitated in 0.3 M sodium acetate (NaOAc) and 2 volumes of ethanol at -20°C for 2 hours.

For a maxiprep of DNA the protocol above was followed (with scaled up volumes) but the final steps involved purification of bacterial DNA on a CsCl gradient. This procedure was as follows: The DNA precipitate was resuspended in 12.75 mls of TE, to which was added RNase at a final concentration of 100 g/ml and incubated for 15 mins at 37°C. Then about half a microspatula end of proteinase K was added and incubated for a further 15 mins. CsCl (15 g) was added, dissolved and transferred to a TU865 13 tube (Sorvall), and then finally 0.25 ml of ethidium bromide (EtBr) (10 mg/ml) was added. This was then centrifuged overnight at 45 000 rpm. The following day the band containing plasmid

DNA (below the band containing host DNA) was removed with a syringe and the help of a hand-held UV lamp. The EtBr was extracted 3-4 times with CsCl saturated isopropanol, dialysed against TSE for 1-2 hours at RT and the DNA ethanol precipitated (as above).

The concentration of DNA from a miniprep was estimated on an EtBr agarose plate. This was done by spotting DNA standards of known concentration (see appendix) next to a 1 ul spot of our sample DNA and comparing the intensity of fluorescence under a UV transilluminator (UV Product Inc.). The yield of DNA from a maxiprep, however was enough to estimate the concentration using a spectrophotometer (Cecil Instruments) (see above). Average yields of DNA for a miniprep and a maxiprep were approximately 5 g and 1 mg respectively.

To assess the purity of the plasmid preparation it was necessary to digest the DNA with restriction endonuclease and eventually to cut out the insert.

2.6 PREPARATION OF DNA PROBES IN LAMBDA PHAGE VECTORS

2.6.1 **Isolating single plaques**

The restriction fragment length polymorphic (RFLP) probes inserted into phage vectors were received from Collaborative Research Incorporated in the form of phage lysates. The phage lysates contained between 10^6 - 10^7 plaque forming units (pfu)/ml. Two dilutions of $1:10^3$ and $1:10^4$ were made with SM buffer (10mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$). 100 l of

diluted phage was added to an overnight culture of E coli (LE392) grown in LB medium shaking at 37°C. The phage and E coli were then incubated for 15 mins at 37°C and mixed with 3 mls of LB soft agar (see appendix), kept at 55°C, and poured onto an LB plate, swirling the plate to evenly spread the mixture. The LB plates were made previously by pouring LB hard agar (see appendix) onto 90 mm petri dishes (Sterilin) and stored in refrigerator at 4°C. This was incubated overnight at 37°C and the next day white plaques were visible on the surface of the LB agar plates.

2.6.2 **Phage plate lysates**

100 l of SM buffer was placed into a sterile eppendorf tube. A sterile short pasteur pipette attached to a rubber bulb was used to suck up a well isolated plaque from the plates incubated overnight. Mild suction was applied so that the plaque together with the underlying soft agar was drawn into the pipette. This was done because phage can diffuse considerable distances through the top agar layer. The agar and plaque was expelled into the SM buffer and allowed to stand at RT for at least 15 mins to allow the phage to diffuse out of the agar. These phage stocks were stored at 4°C or used immediately to make plate lysates.

2.6.3 **Plate lysates**

The plaque was added to 100 l of E. coli LE392 grown overnight in LBM which contained LB medium suplemented with 0.2% maltose and 1 mM and 10 mM magnesium sulphate ($MgSO_4$). A tube of 100 ul of E coli culture was

also prepared containing no phage as a control. The mixtures were incubated for 15 mins at 37°C, added to 3 mls of LBM agarose (55°C) and poured onto LBM plates. The soft agar was allowed to set and then the inverted plates containing phage was incubated at 37°C for approximately 9 hours. When nearly confluent and lysis had occurred the plates were chilled for at least 1 hour at 4°C.

2.6.4 **Harvesting phage**

5 mls of SM buffer was added to the surface of each plate and chilled overnight at 4°C. The buffer was thereafter sucked up with a pasteur pipette and collected into a sterile tube. This solution was centrifuged at 2500 rpm to remove the cell debris. Chloroform was added to the supernatant and then placed in a capped, sterile eppendorf ready for purification of phage. To obtain enough phage DNA (1 ug) to cut out the insert the volumes of the plate lysate preparations were scaled up three-fold and large 140 mm petri dishes (Sterilin) were used. The supernatants from approximately 5 large phage lysate plates were pooled for DNA extraction.

2.6.5 **Phage DNA preparation**

The supernatant from plate lysates was diluted by adding one half the volume of 10 M Tris-HCl (pH 8). A Biorad Econo disposable column was packed with 4 mls of DEAE-cellulose DE52 (see appendix). The lysate solution was poured onto the column and the liquid that flowed through the column was discarded. 5 mls of chase buffer (see appendix) was

added and the liquid that flowed through the column was discarded. 1 ml of elution buffer (see appendix) was added to the column and the flow through volume was once again discarded. 0.6 ml of elution buffer was then added to the column and the flow through volume now containing phage was collected in a sterile 1.5 ml eppendorf tube. 10 ul of 0.1 mg/ml proteinase K and 25 ul of 10% SDS was added to the 0.6 ml elution buffer 100 ul of 3 M KOAc was then added which formed a precipitate.The solution was mixed and heated to at least 65°C for 25 mins and cooled on ice for at least 20 mins. The solution was then centrifuged at 12 000 rpm at RT for 10 mins and the supernatant was poured into a sterile eppendorf containing 20 ul of herring-sperm DNA. 1 volume of isopropanol was added, mixed well and cooled to -20°C for an hour or more. This solution was thereafter centrifuged at 12 000 rpm for 30 mins and the supernatant was poured away. The remaining pellet of DNA was air dried and then resuspended in 20-50 μl of TE (pH 8).

The yield of DNA was assessed by running 1/20 of the total volume on a mini-agarose gel together with several phage DNA standards of known concentration. Thereafter the probe insert was excised from the phage vector by a method described below.

2.7 ANALYSIS OF DNA

2.7.1 Restriction endonuclease digestion

The restriction endonuclease digestion of approximately 1 μg of DNA from a plasmid miniprep was usually enough to be visualised on a gel. The

quality and size of the insert could then be verified. However, the endpoint of restriction digests on a maxiprep was to cut out the insert so much larger amounts of DNA (50-100 ug) were used.

For Southern blot analysis a typical DNA digestion mixture contained 10 ug of DNA, an appropriate buffer (see appendix) and restriction endonucleases (Boehringer). Restriction endonucleases work best at specific salt concentrations. The volume of the digestion mixture was calculated so that it would eventually all go into one well (approximately 40 ul) on an agarose gel. Restriction enzymes were normally stored in 50% glycerol; if the glycerol concentration in a reaction exceeded 5% many enzymes were inhibited, so the amount of enzyme in a 40 ul reaction was limited to less than 4 ul.

The composition of an endonuclease digestion mixture was:

Components	Volume (ul)
DNA	4-10
buffer (10X)	4
enzyme (10 u/ul)	2-3
GDW made up to a final volume of 40 ul	

The DNA, GDW and buffer were mixed together (on ice) in an eppendorf tube by gently flicking the tube. The restriction enzyme was added last after removal from the -20°C freezer (Foster) for as short a time as

possible due to its labile nature. The reaction mixture was then mixed gently, spun down for 1 sec in a microfuge and then incubated for 1 hour at 37°C.

2.7.2 DNA size markers

In order to determine the size of DNA fragments after restriction enzyme digestion of plasmid DNA or after Southern blot analysis each gel included molecular weight standards. The standards used depended on the size range of fragment expected. The phage molecular weight marker was used which had been cut by Hind III (see appendix). 0.5-1 ul of marker was loaded in one lane on a gel.

2.7.3. Horizontal non-denaturing gels

DNA fragments larger than about 100 bp were generally analysed on horizontal agarose gels. A 0.8% agarose gel was used for analysis of high molecular weight DNAs and a 1.4% agarose gel for analysis of small fragments (below 5 kb).

Agarose (1.4 g for a 1.4% gel) was melted in a 100 mls of gel buffer in a 250 ml conical flask in a microwave oven (Creda). The agarose was allowed to cool to about 60°C and 5 l of EtBr (10 mg/ml) was added. Yellow tape was stuck all the way around the edge of a glass gel plate, folding it in underneath so that it made a water-tight seal. A gel comb was placed about 2 cm from one end of the gel plate using bulldog clips and the gel was then poured onto the plate and allowed to set.

Thereafter the gel comb was carefully removed making sure that the slots were not damaged. The yellow tape was removed and the gel and plate was placed in a gel tank. The gel tank (RPMS workshop) was filled with gel buffer containing 50 l of EtBr up to the surface of the gel, making sure that there was good electrical contact at both ends. Each sample, containing 10% v/v of bromophenol loading dye, was loaded carefully into a well. At least one of the wells contained a DNA size marker (restriction enzyme digested DNA of known fragment sizes). The gel was then ready for electrophoresis. Genomic DNA was usually run at 50 V for about 1 hour and DNA fragments (below 5 kb) at 150 V for 1-2 hours. After electrophoresis the gel was lifted out of the tank and the DNA visualised under a UV transilluminator. A ruler was placed alongside the gel and a polaroid photograph was taken.

2.7.4 **Purification of insert DNA from vector DNA**

Insert was purified according to the method of Williams and Mason (1985): 20-50 g of DNA was digested with appropriate restriction enzymes. The digestion of the DNA was checked by running an aliquot (0.5 ug) on a gel by electrophoresis. The DNA was judged to be digested when the fragments were well separated. The rest of the DNA was run on a polyacrylamide gel (3 mm thick) in 0.5x Tris borate EDTA (TBE) buffer (see appendix) loading 10 g of DNA per lane. After electrophoresis the gel was placed onto a thin-layer chromatography plate. The gel was illuminated with a UV light (254 nm) in a dark room. The DNA fragment was then sliced out of the gel using a clean razor blade. The gel slice was placed in a length of dialysis tubing (5 mm longer than the gel

slice) containing approximately 200 ul of 0.5x TBE and sealed at both ends with a plastic clip. The dialysis bag (BDH) was placed on an electrophoresis tank bed so that the gel slice was at one end of the bag at right angles to the current flow. Electrophoresis took place at 100 v for 2 hours. The current was then reversed to detach any DNA that had become bound to the dialysis tubing. The buffer containing the eluted DNA fragment was removed and ethanol precipitated. The concentration and purity of the DNA fragment were determined by running an aliquot on an agarose gel with a DNA marker of known molecular weight.

2.7.5 **Assessment of genomic DNA digestion**

After each restriction endonuclease digestion 0.5 g of DNA was run on a 0.8% agarose mini-gel to assess whether the DNA has been cut to completion. Digestion of DNA was regarded as satisfactory if a smear of DNA fragments was seen ranging in size from approximately 30-30 000 bp. Each restriction enzyme generated its own particular pattern of smear, depending on the frequency of the particular cleavage sites in the genome. Some restriction digests generated discrete bands resulting from consistent cutting within repeat sequences.

2.8 SOUTHERN BLOTS

2.8.1 **Treatment of gels**

The gel was placed in a plastic tray (Raymond Lamb) and enough 0.25 M hydrochloric acid (HCl) was added to completely cover the gel. The tray

was placed on a horizontal shaker for 15-20 mins. This depurinated the DNA and facilitated the transfer of large DNA fragments. HCl was removed and replaced with a neutralising solution (see appendix) and allowed to shake gently for a 30 mins. This step was repeated once and then the gel was ready for transfer.

2.8.2 **Transfer of DNA to membrane filters**

Hybond-N (Amersham) filter was carefully cut to the size of the gel. The gel transfer apparatus was set up as follows: Two layers of Whatman 3 mm paper, saturated in 20X SSC, was placed across the solid base, dipping into the reservoir (containing 20X SSC) at either end.
The neutralised gel was placed face down on the 3 mm paper and any air bubbles removed by rolling a 10 ml pipette over the gel surface. The dry Hybond paper was placed on top of the gel and two pieces of Whatman 3 mm paper soaked in 3X SSC was placed on top of the filter. Paper towels cut to the size of the gel was then stacked on the Whatman paper about 10 cm high. A glass plate (the same size as the gel) was placed on the paper towels and weighed down with a 0.5 kg mass. The DNA was left to transfer overnight. The next day the weight, the glass and the paper towels were removed. The lefthand bottom corner of the gel and filter were cut off so as to identify the orientation of the filter later on. The filter was then gently removed onto a large piece of 3 mm (Whatman) paper and dried in an oven at 80°C for 2 hours.

2.8.3 **Radiolabelling of DNA probes**

2.8.3.1 Nick translation

This method of labelling probes was mainly used in Southern blotting procedures and also initially when the in situ hybridisation technique was being set up.

This procedure made use of two E. coli enzymes DNase 1 (Boehringer) and DNA polymerase 1 (Boehringer). The DNase 1 created single stranded nicks in the double stranded DNA and the DNA polymerase 1 (which has both 5' and 3' exonuclease activity) initiated the replacement of newly synthesised strands of DNA at the nicks. The reaction mixture was added as follows: 1-10 ul (100 ng) of DNA, 4 ul of nucleotide buffer solution, and 1 l ^{32}P-dCTP at a concentration of 3000 Ci/mM (Amersham) were gently mixed together in an eppendorf tube and spun down for 1 sec in a microfuge. 2 ul of enzyme solution was added and made up to a final volume of 20 l of GDW. After gently mixing and spinning down, the reaction mixture was incubated at 15°C for 1-2 hours.

2.8.3.2 Oligolabelling

This method was mainly used in the in situ hybridisation technique. This procedure made use of the hybridisation of short, random sequences of oligonucleotides to homologous sequences of denatured DNA. The oligonucleotides acted as primers for the synthesis of radiolabelled

complementary strands by the Klenow fragment of DNA polymerase 1 (Boehringer) in the presence of a nucleotide mix.

25-50 ng of DNA was made up to 15 µl with GDW and denatured by boiling for 5 mins and then rapidly cooling on ice. 10 µl of reagent mix (containing primer solution) and ^{32}P-dCTP / ^{125}I (20 µCi) were gently mixed together and spun down rapidly (for 1 sec) in a microfuge. 2 µl of Klenow enzyme was added, gently mixed and incubated at 37°C for 2 hours or overnight at RT.

2.8.3.3 Retrieval of radiolabelled DNA

The steps involving the retrieval of labelled DNA were the same for both oligolabelled probes and for nick-translated probes.

At the end of the incubation period 100 µl of TE/0.1% SDS buffer was added to stop the reaction. A G50 sephadex (AnalaR) column was made in a pasteur pipette and equilibrated with buffer. The reaction mixture was then loaded onto the column and rinsed through with 200 µl of buffer. Thereafter 15 fractions of 200 µl aliquots were collected into eppendorf tubes and their radioactivity counted on a mini scintillation counter (Mini-instruments). If the reaction was successful then the DNA with incorporated label was collected in fractions 4-7 and the unincorporated DNA was collected in fractions 9-12. The percentage of radiolabelled DNA was calculated as the scintillation counts of the first peak divided by the total number of scintillation counts and this value multiplied by 100. The specific activity (calculated from this

figure) was ideally between 10^8 and 10^9 cpm/ug. The incorporated DNA present in the first peak fraction was then ethanol precipitated overnight at -20°C and ready for use the next day.

2.8.4 **Hybridisation of Southern blots**

The purpose of this procedure was to produce a hybrid between the radioactively labelled probe and the filter bound DNA. This procedure was divided into four stages: prehybridisation, hybridisation, washing and autoradiography.

2.8.4 1 Prehybridisation

The baked Hybond-DNA bound filter was placed in a plastic bag containing 25 ml of prehybridisation mix consisting of 5X standard saline phosphate EDTA (SSPE), 50% formamide, 0.1% Denhardt's solution and 0.2% SDS, and sealed. The bag was then submerged in a shaking waterbath (Tecam) for 2 hours (or more) at 42°C.

2.8.4.2 Hybridisation

One corner of the bag was cut open and the prehybridisation solution discarded. The radiolabelled probe (see above) was denatured by boiling for 5 mins and rapidly cooling on ice for 10 mins and added to 10 mls of hybridisation mix which contained the same constituents as the prehybridisation mix but also included 1 g of dextran sulphate. This mixture was then introduced into the bag and resealed, making sure that

no air bubbles were present. The contents of the bag were mixed thoroughly to distribute the probe evenly over the filter and incubated at 65°C overnight in a shaking waterbath.

2.8.4.3 Washing procedure

The aim of the washing procedure was to remove all unbound and non-specifically bound probe from the filter. The filter was placed a plastic container containing 200 mls of 1X SSC, 0.2% SDS and placed in a shaking water bath at 65°C for 15 mins. The solution was discarded and the washing repeated once more. This was followed by a high stringency (low salt) wash in which the filter was washed for 15-30 mins in 0.1X SSC, 0.2% SDS at 65°C.

2.8.4.4 Autoradiography

The filter was removed from the tray, allowed to dry for 15-20 mins and then wrapped in Saran plastic. The blot was labelled appropriately with radioactive ink on sticky labels and stuck down in a light proof cassette. A sheet of XAR-5 Kodak film was placed over the blot in the darkroom, the cassette shut and placed in the -80°C freezer for several days. The length of time required to obtain a good signal depended on the total amount of DNA used and the specific activity of the probe.

2.8.4.5 Stripping filters

If a given filter was needed for rehybridisation with another probe, the blot was dehybridised and stripped of the previous probe. The probe was removed by incubating the filter in 0.4 M sodium hydroxide (NaOH) for 30 mins at 45°C. The NaOH was discarded and the filter washed at RT for 15 mins in neutralising solution. The filter was then ready to be reprobed. This method of dehybridisation of probe was quite effective although the subsequent hybridisations generally had higher backgrounds.

2.9 IN SITU HYBRIDISATION TO METAPHASE CHROMOSOMES

2.9.1 Preparation of metaphases

Slides of metaphase preparations were made essentially as mentioned above although special care was taken to have well spread metaphases (non-overlapping chromosomes). This was achieved by washing the cell suspension (more than 5 times) in fresh fix solution (see section 2.4.1.1). The microscope slides were prepared to give a better retention of cytological preparations. They were prepared as follows: Slides were soaked overnight in a 1% sulphuric acid (H_2SO_4) solution and rinsed thoroughly the next day in GDW. The slides were then placed in absolute ethanol for 3-4 hours and, after wiping with clean paper tissue, were ready to use.

2.9.2 **Labelling of DNA probes for in situ hybridisation**

DNA probes were oligolabelled with ^{125}I (Amersham) as mentioned above. The volume of target DNA used in the in situ hybridisation technique was small compared to Southern blotting so probes were usually labelled the day before and the incorporated DNA precipitated overnight. The next day the DNA precipitate was gently resuspended in deionised formamide.

2.9.3 **Prehybridisation procedures**

2.9.3.1 RNase treatment

Moist chambers were prepared by soaking three layers of tissue, lined in black boxes with tightly fitting lids (Raymond Lamb) in 2X SSC and placing these in 37°C waterbath. Meanwhile a solution of RNase in 2X SSC was made at a concentration of 100 g/ml. Glass coverslips previously soaked in absolute ethanol were removed and left in a vertical position to dry. 200 l of RNase solution was placed on each slide and a coverslip placed over it gently. The slides were then incubated in the moist chambers for 1 hour. Thereafter the coverslips were removed by soaking in 2X SSC for a few minutes and then washed twice in 2X SSC.

The slides were then dehydrated as follows: Slides were mounted into a tray which fitted neatly into 5 boxes containing 10%, 50%, 75%, 95%, and 100% of ethanol. The slide-containing tray was gently lowered into the

ethanols for 2 mins each starting from the most dilute solution (10%) to the most concentrated solution (100%). The slides (still within tray) were then left to air dry and placed in desiccator until needed.

2.9.3.2 Denaturation of chromosomal DNA and probe DNA

A box containing 250 mls of 70% deionised formamide, 5mM EDTA in 2X SSC was warmed to 65°C in a waterbath. The tray of slides was placed in the formamide solution for 4 mins. The slides were removed immediately, washed in 2X SSC and dehydrated (as above) and placed in a desiccator (Raymond Lamb).

The probe was added at a final concentration of 0.4 ng/ul to a solution of 50% hybridisation mix and 50% deionised formamide and denatured by boiling for 10 mins and then rapidly cooling on ice.

2.9.4 **Hybridisation**

Moist chambers were then prepared this time soaking paper tissue in 5X SSC at 42°C.

30 l of denatured probe was spread (one drop at each end) onto each slide of denatured chromosomal DNA and a coverslip gently placed over it. The slides were placed in moist chambers of 5X SSC at 42°C overnight to hybridise.

2.9.5 **Procedures post hybridisation**

2.9.5.1 Removal of coverslips

It was important to remove the coverslips post hybridisation in a particular way so as to prevent the lifting of cells from the slide. The removal of coverslips was achieved as follows: Slides were placed individually into a coplin jar containing 5X SSC. The coplin jar was placed in a waterbath at 37°C and the coverslips were allowed to drift off the slides. No force was applied to remove the coverslips.

2.9.5.2 Washing conditions

The slides were then put through a series of washes to remove excess unhybridised and nonspecifically bound probe molecules. The washing conditions depended on the type of probe used in the hybridisation reaction. If for example a probe was used which contained repetitive sequences present in other regions of the genome, then a particularly high stringency wash was used (0.1X SSC, 0.1% SDS at 65°C).
After the coverslips were removed then the slides were replaced in the tray. The washing procedure for probes with unique nucleotide sequences was as follows: The tray of slides was placed in a box containing 250 mls of 2X SSC for 1hour. A minimum of agitation was used and the washing of slides throughout was a gentle process. The solution was changed after 30 mins. Two boxes containing 0.5X SSC at 60°C were prepared meanwhile. The slides were removed from the 2X SSC and placed

in the 0.2X SSC solution for a further 30 mins (15 mins in each box). Finally the slides were washed in a solution containing 0.1X SSC at RT. The washing conditions for probe sequences containing repetitive sequences was as follows: Slides were washed in an SSC strength ranging from X0.5 to X0.1 at 65°C for 1 hour and the solutions changed every 15 mins. The slides were finally washed in 0.1X SSC for a further 30 mins with the solutions changed after 15 mins. After the slides were washed, they were dehydrated through a series of alcohols (as described above) and stored in a desiccator.

2.9.6 **Autoradiography**

Unlike the autoradiographic procedure involved in Southern blotting, the photographic film in the in situ hybridisation technique was replaced by a nuclear emulsion and the whole autoradiographic process was carried out on the microscope slide containing metaphases hybridised with specific probe sequences.

The hybridised slides were coated in Ilford nuclear emulsion (L4) in the darkroom (without the use of safelights). The process of emulsion dipping was carried out as follows: All necessary items were placed in accessible positions so that they could be handled in complete darkness. L4 nuclear emulsion contained within an opaque bottle and in gel form at RT was liquefied in a 55°C waterbath (Grant) for approximately 15 mins. The emulsion was stirred continuously using a glass rod to ensure complete liquification. Then it was diluted 1:1 with GDW also heated to 55°C in a coplin jar and again stirred with a glass rod so

that the emulsion was thoroughly mixed. The emulsion was left for a minute or so to allow bubbles that might have formed to disperse. Each slide was dipped into the emulsion and left to dry standing vertically against a clean surface in a large light-tight box. The emulsion usually formed an even ultra-thin coating over the slide. After 2 hours the slides were removed into slide trays and placed in small light tight boxes containing desiccant (silica gel). The boxes were covered with a layer of foil and then placed in a refrigerator at 4°C (away from any other radioactive material) for a period of 4-7 days.

2.9.7 **Developing of slides**

The slides were usually divided among several boxes so that the correct time of exposure could be assessed. Four days after slide incubation one box was removed from the refrigerator and left to equilibrate at RT for 30 mins. Boxes containing developing solutions were prepared and left in the cold room to reach 15°C. The developer (Kodak D19) was made up in GDW, from the powder, as directed. The stop solution was a 1% acetic acid solution made up in GDW. The fixing solution (Ilford Hypam) was diluted 1:4 with GDW with the recommended hardener added at a dilution of 1:20. Slides were developed for 5 mins (agitating slides every 30 secs), the developing reaction stopped in 1% acetic acid for 1 min, and then fixed for a further 5 mins (agitating slides every 1 min). The slides were washed under gently running water for 20-30 mins.

2.9.8 **Staining of slides**

Slides were stained using the modified protocol of Le Beau et al (1983) (if cultures were unsynchronised) and the modified protocol of Perry and Wolf (1974) (if cultures were synchronised with BrdU). Initially, however, 1 or 2 slides were stained immediately to assess the general level of background and the specific grain size. If the background was quite high (an average more than 5 grains per metaphase was unacceptable), then the slides were washed for a further period and the rest of the slides developed the same day. If the grain size was small and could just about be visualised the rest of the slides were usually left for a few more days before they were developed.

Whether the metaphases were synchronised or unsynchronised these test slides were usually stained in a Wright's stain (EM Science) for 5-8 mins. The Wright's stain was made up as follows: A glass bottle was set aside only for mixing the stain. 500 mls of methanol (anhydrous, acetone free) was poured into a glass bottle, and the liquid was stirred by means of a magnetic stirrer (Gallenkamp). 1.25 g of Wright stain was added to the methanol slowly while constantly stirring. The solution was stirred for a period of 2 hours. The stain was then filtered through Whatman number 1 filter paper of double thickness and the filter paper changed every second funnel-full. The bottle containing stain was capped tightly and stored in the dark at RT for a period of 14 days. A working solution of Wright's stain diluted with phosphate buffer (0.06 M) was made up for use. The phosphate buffer consisted of 0.06 M

potassium dihydrogen phosphate (KH_2PO_4) and 0.06 M disodium hydrogen phosphate (Na_2HPO_4).

Banding of unsynchronised chromosomes after hybridisation was carried out as follows: 3 mls of phosphate buffer was placed into a disposable plastic centrifuge tube and 1 ml of stain was added to the buffer. The solution was pipetted up and down a few times and poured onto a slide. The slide was stained for 10 mins and rinsed with GDW. The slide was then dried in air. The slide was then destained in 95% ethanol for 90 secs, in 95% ethanol containing 0.25%, 0.5% or 0.1% 10 M hydrochloric acid (HCl) for 35 secs, and finally in 100% methanol for 90 secs. During the destaining process the slides were agitated continuously by dipping the slides in and out of the solutions. The slides was dried thoroughly between solutions. The slides were then restained as described above. At this stage the chromosomes should still appear uniformly stained but some banding should appear after destaining and restaining a second time. Sometimes a faint background staining due to the photographic emulsion was visible after this procedure. A quick rinse in 95% ethanol followed by restaining the slides often helped this problem. This procedure was repeated for the rest of the slides and procedure resulted in faint G-banding or C-banding.

Banding of synchronised chromosomes after hybridisation was as follows: The slides were treated essentially as for conventional banding of synchronised cultures (see section 2.3.3) but with slight modifications. Slides were mounted in GDW and sealed with a rubber solution so that the Hoechst 33258 stained cells remained moist during exposure to UV light.

Slides were exposed to 5-6 hours of UV light. Thereafter the rubber solution was carefully removed and the cover slips removed by soaking in GDW for 10-15 mins. The slides were rinsed in GDW once more and then stained with 10% Giemsa solution for 10 mins.

In general the quality of chromosome banding post hybridisation was poorer than the banding obtained using conventional cytogenetic techniques. However the banding (post hybridisation) of metaphases derived from synchronised cultures was better than for that obtained with the Wright's stain. Often chromosomes were difficult to identify and karyotyping required a combination of cytogenetic skills acquired with experience.

2.9.9 **Analysis of in situ hybridisation data**

2.9.9.1 Gene localisation

Hybridised preparations were analysed by adding the total number of grains, on all chromosomes in multiple mitotic spreads. The banding pattern of the metaphase spreads were best visualised when a green interference filter (Zeiss) was used. However the grains were best visualised without this filter. The grain locations were scored in 30-100 metaphases containing a complete set of chromosomes which were identifiable. Grains were scored only if they were located on the chromosome or touching at least one chromatid. Generally labelling resulted in one grain on a chromosome or sometimes one grain on each chromatid and rarely multiple grains at one location. Specific grain

hybridisation was usually found on one chromosome homologue in any one metaphase and seldom on both homologues. The level of nonspecific background was relatively low and ranged from 2-4 grains per metaphase. Assigned probe localisations were based on obvious sites of labelling throughout the karyotype. Histograms of grains plotted against chromosomes showed a clear clustering in one or two regions of of the karyotype.

2.9.9.2 Calculation of statistical significance of probe localisation

The significance of clustering of grains was found by calculating deviation from the 'expected' number of grain hybridisations to chromosomes using the Chi squared (^{2}) statistical test. The number of grains expected to lie on each chromosome, if grains were distributed evenly over all 23 chromosome pairs, was calculated allowing for the fact that chromosomes vary in their size and DNA content. This was done using the data on relative lengths of chromosomes given by Mendelsohn et al (1973). Each of these expected numbers of grains was then compared to those observed by a ^{2} test with one degree of freedom.

2.9.9.3 Sensitivity of the in situ hybridisation technique for detecting multiple copies of genes

The sensitivity of the in situ hybridisation technique for detecting multiple copies of genes was assessed. The results stated here in brief are based on three independent series of experiments. The increase in the number of grains concomitant with the increase the number of copies

of the gene was calculated. A strain of the cell line HL60 with a 4 to 8 fold amplfication of the MYC oncogene was studied. The grain localisation of MYC in the metaphase chromosomes from HL60 was compared with that for normal lymphocytes. The number of grains hybridising to the MYC oncogene in HL60 was approximately twice that seen for MYC localisation in normal lymphocytes. This suggested that an increase in grain count was seen only when there was an approximately 4-fold increase in copy number. The in situ hybridisation technique was therefore not sensitive enough to pick up duplication or triplication of genes.

CHAPTER 3: Ph-NEGATIVE, M-BCR-POSITIVE CML: RELATIONSHIP TO Ph-POSITIVE M-BCR-POSITIVE DISEASE

3.1 LITERATURE

In a study of 50 Ph-positive CML patients Spiers et al (1977) concluded that the presence of a Ph chromosome could have been inferred from the specific clinical and haematological features in each case. These 50 patients had a median age at diagnosis around 45 years, a median survival of three to four years, and splenomegaly. The peripheral blood picture showed a leucocytosis with marked basophilia with the presence of granulocytes at all stages of maturation. The bone marrow showed a marked hypercellularity with hyperplasia of the granulocytic and megakaryocytic lineages.

In contrast features of patients with CML lacking a Ph chromosome are heterogeneous (Kardinal et al, 1976). Some cases resemble Ph-positive disease while others showed striking differences. These differences include a shorter clinical course, older age at diagnosis (median around 65 years) and lower initial white cell and platelet counts. There may also be an excess of monocytes, a virtual absence of basophils and the presence of dysplastic features from the myelocytes to the more mature stages. The classification of these patients has been controversial and some authors have disputed the existence of Ph-negative CML as an entity. For example Pugh et al (1985) and Travis et al (1986) suggested that the majority of patients classified as Ph-negative CML had been misdiagnosed and were instead examples of chronic myelomonocytic leukaemia, myelodysplastic syndromes or myeloproliferative disorders.

Although the majority of Ph-negative CML patients have a normal karyotype, some Ph-negative CML patients have visible rearrangements of chromosome 9q34. With the growing awareness that 9q34 was often involved in Ph-positive CML, Lawler (1982) suggested that a group of Ph-negative CML patients might exist who shared essentially the same pathogenetic mechanism(s) as the Ph-positive leukaemia.

Whereas the molecular features of Ph-positive CML have been well documented the molecular study of Ph-negative CML has been undertaken by few groups. An early report by Bartram et al (1985a) seemed to show that Ph-negative CML had a different molecular basis from Ph-positive CML. They identified a Ph-negative CML patient whose leukaemic cells exhibited rearrangement of M-BCR without juxtaposition of ABL sequences. However more recent molecular genetic evidence supports the hypothesis of Lawler (1982). Bartram et al (1985b) studied a patient with a translocation between chromosomes 9 and 12 without apparent involvement of chromosome 22. They demonstrated a rearrangement within M-BCR and a translocation of both BCR and ABL sequences, presumably as a chimeric BCR/ABL gene, onto the derivative chromosome 12. Kurzrock et al (1986) reported M-BCR rearrangement and expression of the P210 BCR-ABL chimeric protein in one patient with a translocation between chromosome 9 and 11 but a normal chromosome 22.

In investigations of Ph-negative CML cases with normal karyotypes, Bartram (1985), Bartram et al (1986), Bartram and Carbonell (1986), Morris et al (1986), Ganesan et al (1986) found that a total of 13 of the 28 patients studied had rearrangement of M-BCR as in Ph-positive

CML. Morris et al (1986) and Dreazen et al (1987) studied a total of 12 Ph-negative patients (with normal karyotypes) and M-BCR rearrangement. Localisation of the ABL in four cases by in situ hybridisation showed that the ABL oncogene had moved from its normal position on chromosome 9 to chromosome 22.

The formation of the BCR/ABL chimeric gene is thought to play a central role in Ph-positive CML. However it is still unclear to what extent other factors influence the clinical phenotype. The molecular studies of patients whose disease resembled CML but who did not have a Ph chromosome might shed more light on this question. I therefore studied 8 patients classified as CML who lacked the Ph chromosome mainly by cytogenetics and in situ hybridisation. Five patients had clinical and haematological features indistinguishable from Ph-positive CML while 3 patients had some atypical features.

3.2 CLINICAL AND HAEMATOLOGICAL FEATURES

The clinical and haematological findings of one Ph-positive CML patient and eight Ph-negative CML patients are shown in Table 2. The features of the eight Ph-negative CML patients resembled the Ph-positive CML patient to a greater or lesser extent. Their leucocyte counts varied from 22.9 to 506 X 10^9/l at diagnosis. In each case the bone marrow was hypercellular and showed gross granulocytic hyperplasia with maturation and no excess of blasts. The neutrophil alkaline phosphatase was low in 7 of the 8 patients studied at diagnosis. The morphology in patients 1, 2, 3, 5, and 7 was indistinguishable from that characteristic of Ph-

Table 2 Clinical and haematological details of one Ph-positive and eight Ph-negative CML patients.

	Patient	Age/ sex	Spleen (cm)	Peripheral blood at diagnosis: Hb (g/dl)	WBC ($\times 10^9$/l)	Plat's ($\times 10^9$/l)	Differential (% of 300 cells): Bl	Pm	My & Met	Neut	Eos	Baso	Mono	Lymph	NRBCs	NAP	Treatment & response	Survival (months)
Ph-pos, M-BCR pos		M/35	0	13.8	96.5	217	1	2	8	71	0	2	1	9	0	0	HU.PR	24+
Ph-neg, M-BCR pos	1	M/24	25+	8.3	174	941	6	6	21	41	6	14	0	4	0	0	HU.PR	24+
	2	M/58	0	11.2	170	53	1	3	28	61	0	0.5	0.5	6	0	0-12	HU.PR.	16
	3	F/43	12	10.3	506	319	2	4	28	59	2	3	1	1	0.3	0	HU.PR.	13+
*	4	F/4	8	10.9	29	384	1	0	9	39	5	2	16	22	3	24	HU.CR.BMT.	15
*	5	M/49	12	12.9	170	283	0	4	25	61	2	4	1	4	0	16	BU.CR.	24+
	6	M/81	6	10.9	74	300	0	0	0	74	2	11	0	13	0	16	NT	24+
*	7	M/39	0	11.3	298	210	1	6	10	35	4	0	0	4	x	x	BU.CR	24+
Ph-neg, M-BCR neg	8	M/60	0	15.7	22.9	290	0	0	0	82	0	6	10	27	2	94	BU.HU.PR	24+

HU = hydroxyurea BU = busulphan NT = not treated CR = complete haematological response

PR = poor or partial haematological response BMT = bone marrow transplantation
+ indicates survival at the time of diagnosis

NAP = neutrophil alkaline phosphatase

* indicates patients studied further in Chapter 4

positive CML, although minor dysplastic features were present in the granulocyte series of patient 2, including hypogranulation of granulocytic precursors with a decrease in secondary granulation and a persistence of cytoplasmic basophilia. Patients 4, 6, and 8 had some features not typical of Ph-positive CML. These patients had absent or very low numbers of myelocytes and metamyelocytes. Patient 4 was treated by allogeneic bone marrow transplantation and died 6 months later. Patients 4, 5 and 7 were described further in Chapter 4 and are indicated in Table 2 by asterisks.

3.3 CYTOGENETICS

3.3.1 Ph-positive CML patient

30 G-banded metaphases from the bone marrow of one patient with typical Ph-positive CML were analysed. All the metaphases had a Ph translocation, 46,XY, Ph, t(9;22) (q34;q11) with no other chromosomal abnormalities (Fig 9).

3.3.2 Patient 1

40 G-banded metaphases were analysed from peripheral blood cultures (without PHA) at 48 and 72 hours. 38 metaphases had a normal karyotype (46,XY) and 2 metaphases had a 22q- with no apparent change in chromosome 9 (46,XY, 22q-) (Fig 10)

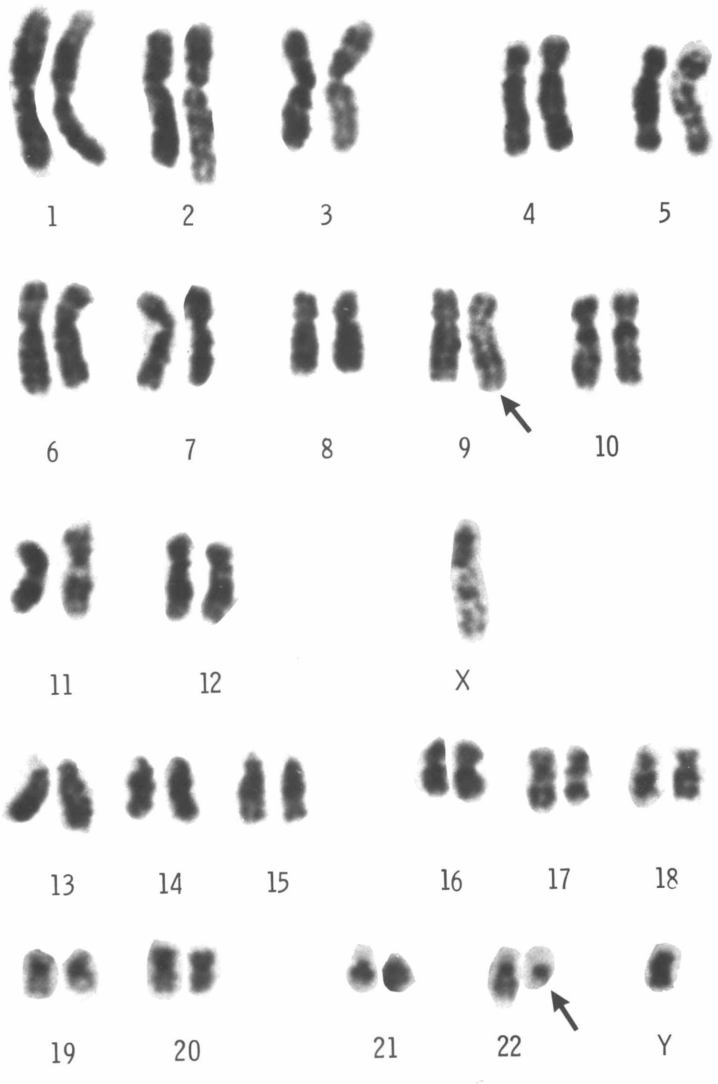


Fig 9 **Karyotype of a G-banded metaphase from a Ph-positive CML patient showing translocation between chromosomes 9q34 and 22q11, t(9;22)(q34;q11).**

This reciprocal translocation results in the formation of chromosomes 9q+ and 22q-

Fig 10 Karyotype of a G-banded metaphase from patient 1 showing a deletion of chromosome 22 (22q-), but with two normal chromosomes 9.

3.3.3 **Patient 2**

30 G-banded metaphases derived from bone marrow cultured for 48 hours were analysed. All metaphases showed normal chromosomes 9 and 22 but had a terminal deletion of chromosome 16 at band q22, 46,XY del(16)(pter q22:) (fig 11).

3.3.4 **Patient 3**

35 G-banded metaphases were analysed. 25 metaphases were derived from peripheral blood cultures at 72 hours and 10 metaphases were derived from bone marrow cultures at 48 hours. All 35 metaphases analysed had a normal male karyotype, 46,XY.

3.3.5 **Patient 4**

25 G-banded metaphases were analysed from fresh and cryopreserved samples of peripheral blood cultured for 48 hours. All metaphases had a normal female karyotype, 46,XX.

3.3.6 **Patient 5**

20 G-banded metaphases were analysed from unstimulated bone marrow cultured for 48 hours. All the metaphases had a normal male karyotype, 46,XY (Fig 12).

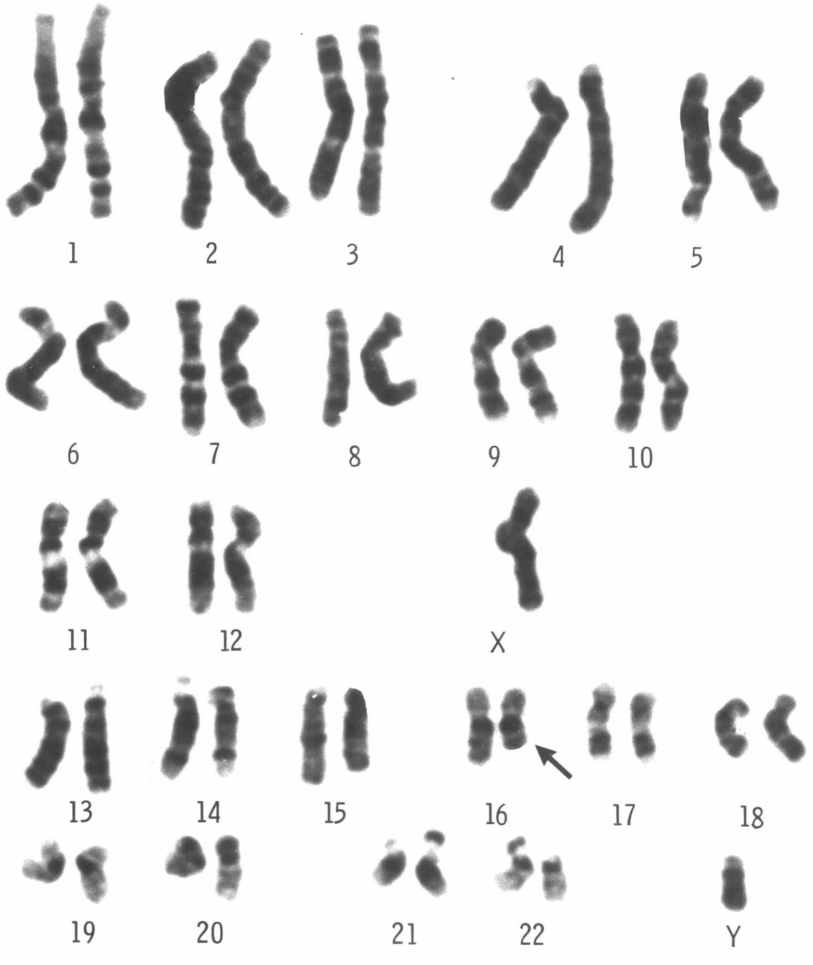


Fig 11 **Karyotype of a G-banded metaphase from patient 2 showing normal chromosomes 9 and 22, but a terminal deletion of the q arm of chromosome 16 (16q-).**

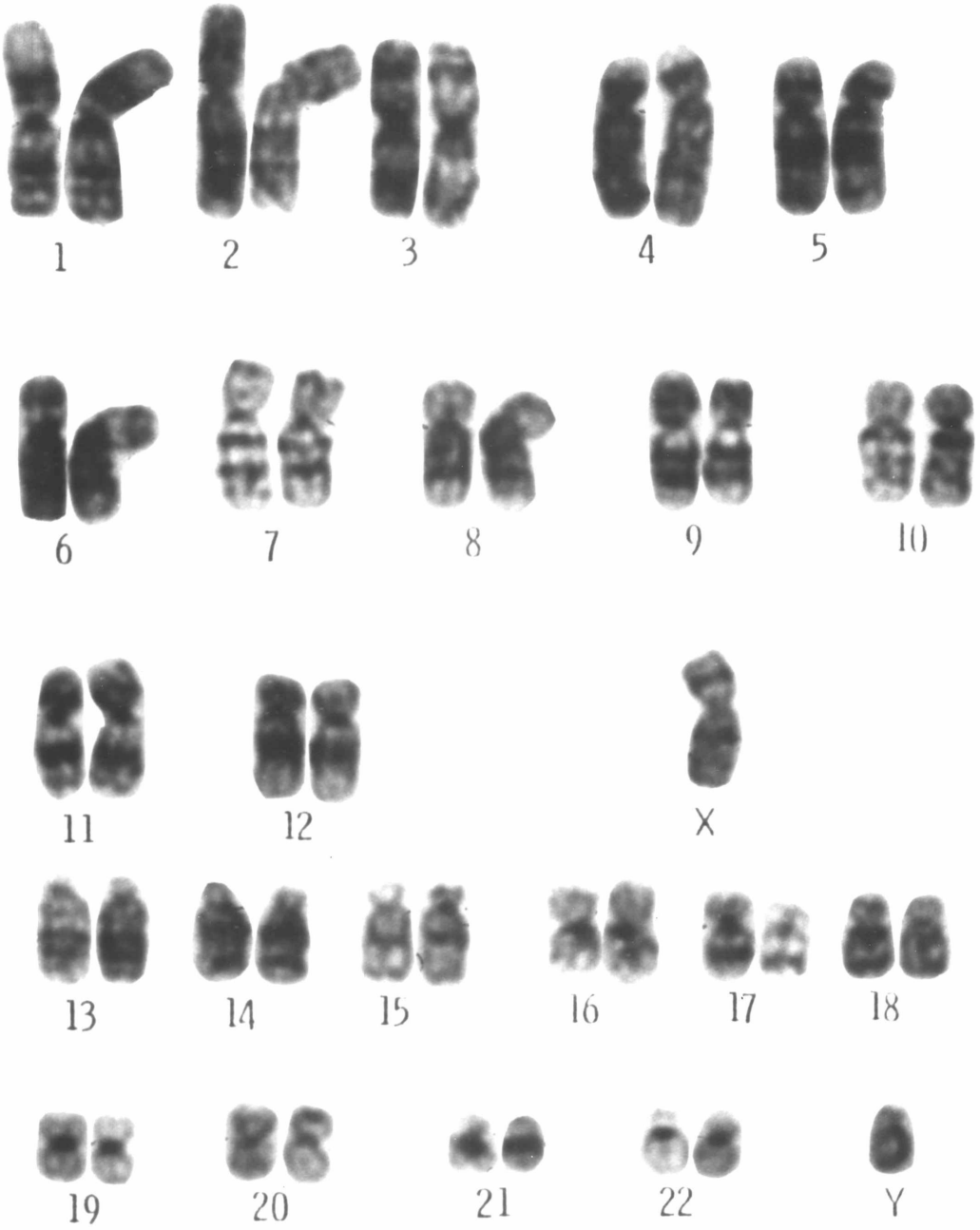


Fig 12 **Karyotype of a G-banded metaphase from Ph-negative CML patient 5 showing normal chromosomes.**

3.3.7 **Patient 6**

15 G-banded metaphases were analysed from unstimulated bone marrow cultured for 24 hours. All metaphases had a normal male karyotype, 46,XY.

3.3.8 **Patient 7**

20 G-banded metaphases were analysed from bone marrow cultured for 24 and 48 hours. All metaphases had a normal male karyotype, 46,XY.

3.3.8 **Patient 8**

20 G-banded metaphases were analysed from bone marrow cells cultures for 24 hours. All the metaphases had a normal male karyotype, 46,XY.

3.4 **SOUTHERN BLOT ANALYSIS**

Genomic rearrangements of M-BCR were analysed by Southern blotting with the cooperation of Drs P Martiat and L M Gao. High molecular weight DNA extracted from the leukaemic cells of patients was digested with the restriction endonucleases Bam HI, Eco RI, Hind III, Bgl II and Xba I and probed with the 0.6 kb genomic M-BCR probe (BCR-G) and the 4 kb genomic BCR probe (Phl/bcr-3) (Fig 8). We detected rearrangements in the Ph-positive CML patient and in 7 of the 8 Ph-negative patients studied (Fig 13).

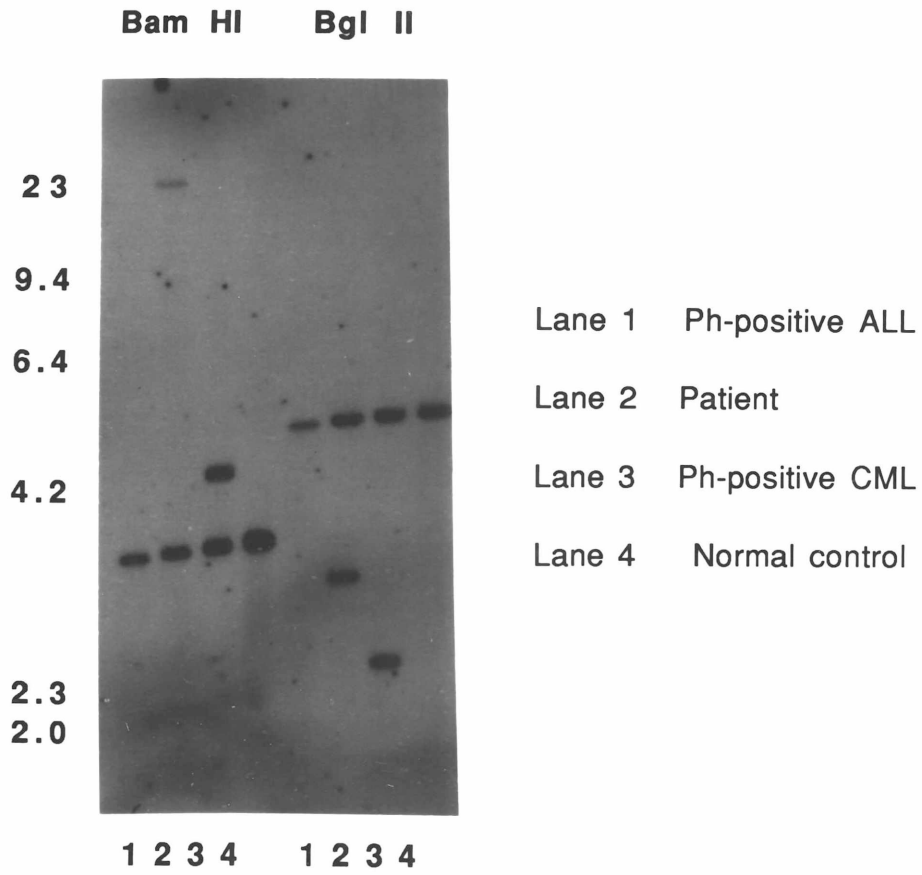


Fig 13 **Southern blot analysis of DNA from Ph-negative CML patient 5 showing genomic rearrangement of M-BCR**

High molecular weight DNA was restriction endonuclease digested with Bam HI and Bgl II and probed with the 0.6 kb M-BCR probe, BCR-G.

The DNA from patient 5 (lane 2), like the Ph-positive control (lane 3), shows a band in the germline configuration of M-BCR and another band in the rearranged configuration using both restriction endonucleases.

3.5 IN SITU HYBRIDISATION

3.5.1 Analysis of in situ hybridisation data

Metaphase spreads were analysed for grain localisation. The number of grains localising to chromosomes 9 and 22 are indicated below. The long arm of chromosome 22 represents approximately 1.3% and the long arm of chromosome 9 approximately 4% of the haploid genome (Mendelsohn et al, 1977). The deviation from the expected number of 'background' hybridisations was calculated on the assumption of random distribution of grains proportional to the length of the chromosome arms. The significance of differences between the observed and expected hybridisation pattern is indicated below.

3.5.2 **ABL localisation**

A 1.5 kb human cDNA Pst I fragment was used as a probe for the in situ localisation of the ABL oncogene (for details see Chapter 2).

3.5.2.1 **Ph-positive CML patient**

In 40 metaphases analysed from a Ph-positive CML patient with a total of 79 grains, 12 (15%) grains hybridised to the normal chromosome 9 and 8 (10.1%) grains hybridised to the Ph chromosome (Fig 14, Table 3). The clustering of grains on chromosome 9 and the Ph chromosome was significant ($p = 0.0003$ and $p = 0.0001$ respectively).

Table 3 **Localisation of the ABL proto-oncogene in metaphases from a Ph-positive CML patient**

The grain localisation of the ABL proto-oncogene to chromosomes 9q34 and 22q- in 40 bone marrow metaphases by in situ hybridisation.

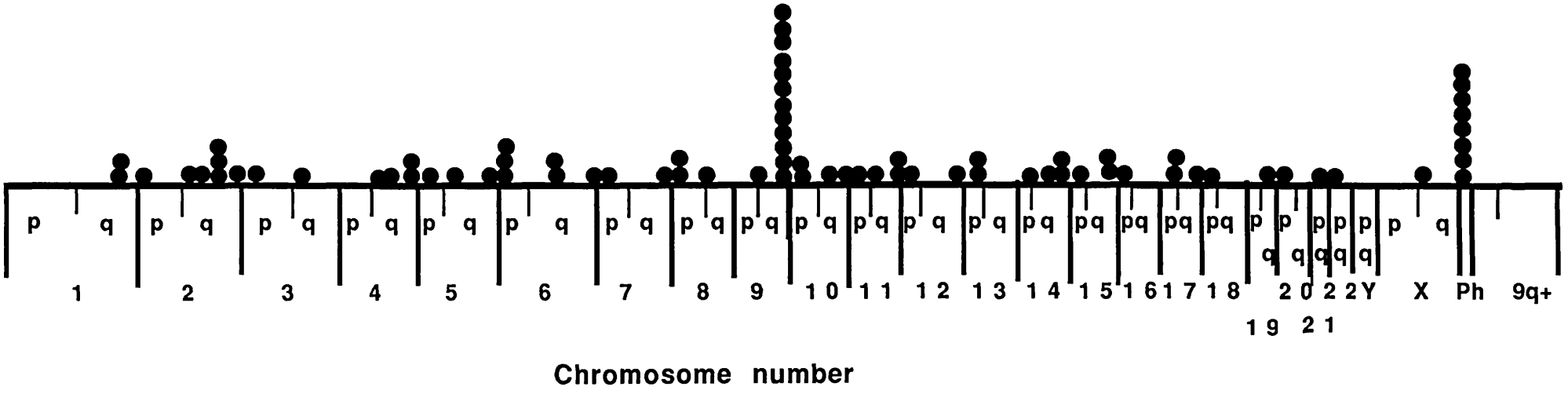




Fig 14 **Localisation of the ABL proto-oncogene to chromosome 22q- (Ph) in a metaphase from Ph-positive CML patient.**
The black grain on the chromosome 22q- (indicated by an arrow) represents localisation of the ABL probe to complementary ABL proto-oncogene sequences in situ. The normal chromosome 22 is also indicated.

3.5.2.2 **Patient 1**

In 40 metaphases analysed with a total number of 123 grains 20 (16.2%) grains mapped to chromosome 9 and 18 (14.6%) grains mapped to chromosome 22 (Fig 15, Table 4). The clustering of grains on both chromosome 9 and the chromosome 22 was significant ($p < 0.0001$).

3.5.2.3 **Patient 2**

In 50 metaphases analysed 24 with a total number of 136 grains, 18 (13.2%) grains mapped to chromosome 9, 2 (1.2%) grains mapped to chromosome 22 and 15 (11.0%) mapped to chromosome 1q24 (Fig 16, Table 5). The clustering of grains on chromosome 9 and chromosome 1 was significant ($p = 0.0005$ and $p = 0.03$ respectively).

3.5.2.4 **Patient 3**

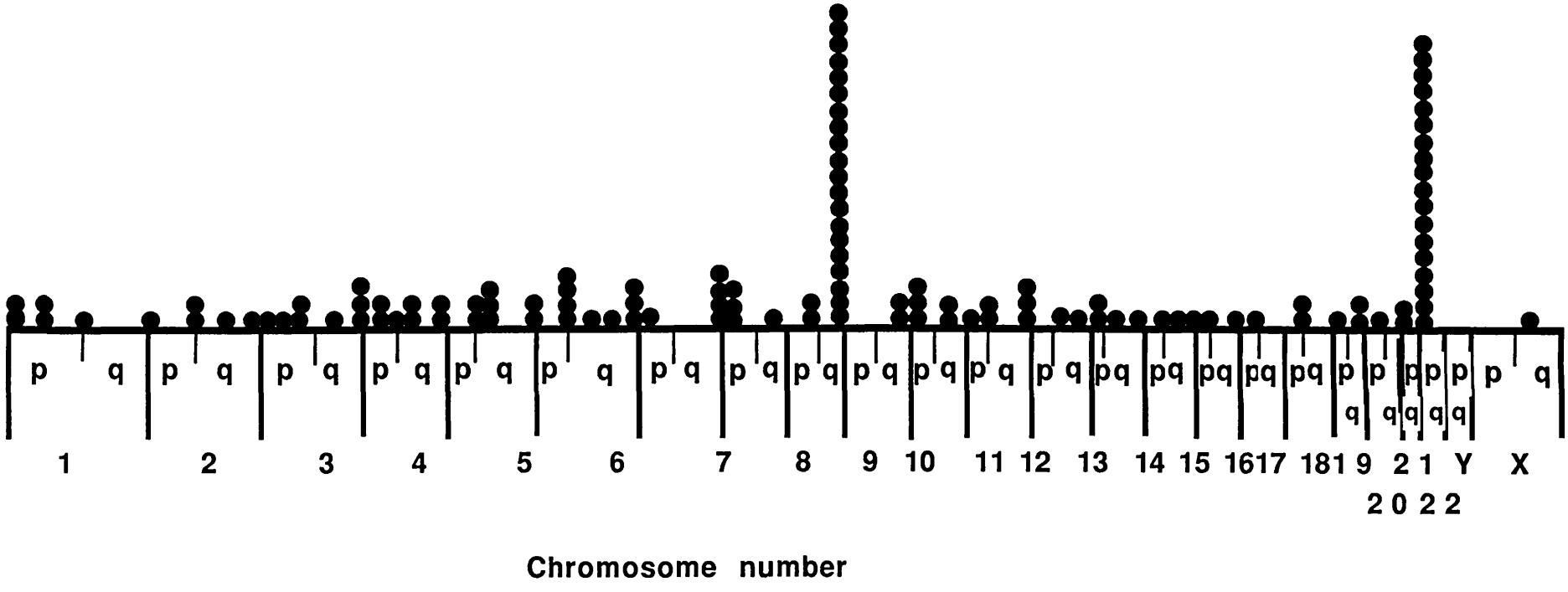
In 45 metaphases analysed with a total number of 118 grains 20, (16.9%) grains mapped to chromosome 9 and 21 (17.8%) grains mapped to chromosome 22. The clustering of grains on chromosome 9 and chromosome 22 was significant ($p < 0.0002$).

3.5.2.5 **Patient 4**

In 50 metaphases analysed with a total number 143 grains, 16 (11.9%) mapped to chromosome 9 and 17 grains (11.8%) mapped to chromosome 22.

Table 4 **Localisation of the ABL proto-oncogene in metaphases from Ph-negative CML patient 1.**

The grain localisation of the ABL proto-oncogene to chromosome 9q34 and 22q11 in 40 bone marrow metaphases by in situ hybridisation.



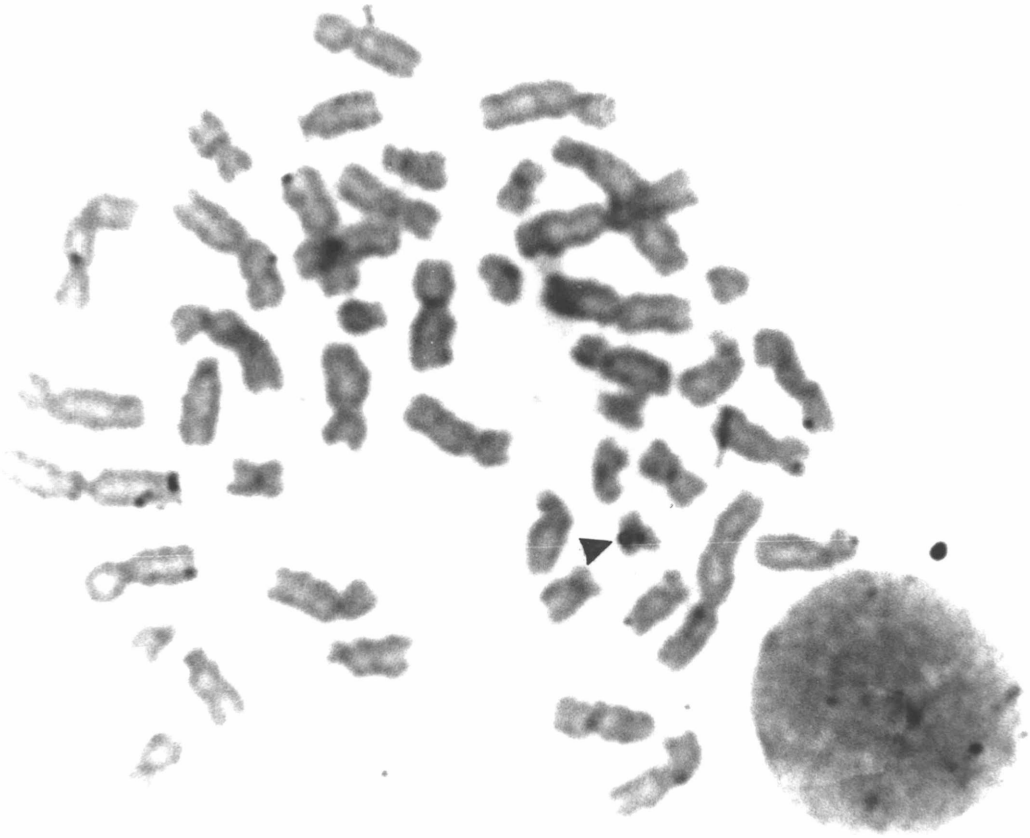
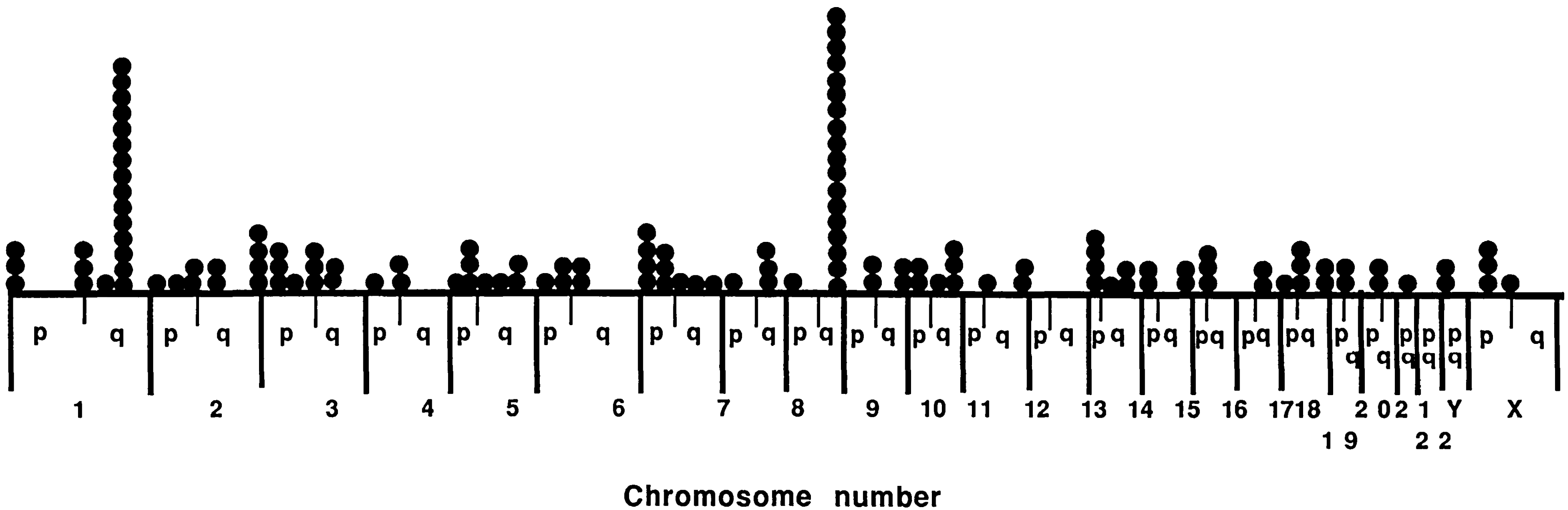


Fig 15 Localisation of the ABL proto-oncogene to a cytogenetically normal chromosome 22 in a metaphase from Ph-negative CML patient 1.

The black grain on chromosome 22q11, indicated by an arrow, represents localisation of ABL proto-oncogene in situ.

Table 5 **Localisation of the ABL proto-oncogene in metaphases from Ph-negative CML patient 2**

The grain localisation of the ABL proto-oncogene to chromosome 1q24 and 9q34 in 50 bone marrow metaphases by in situ hybridisation.



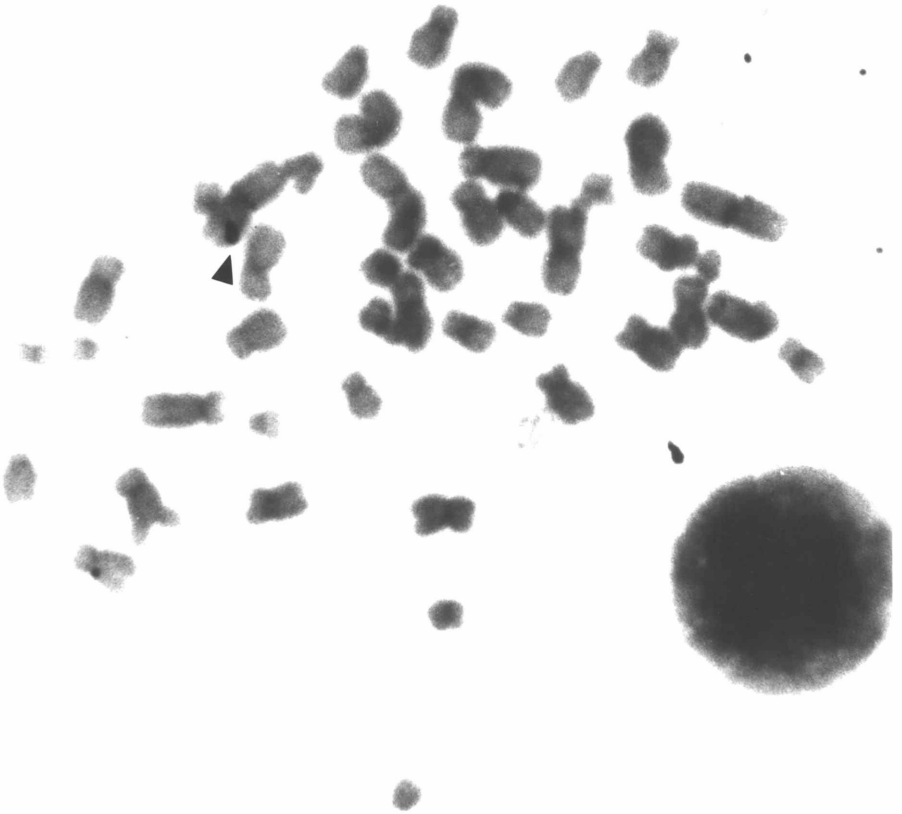


Fig 16 **Localisation of the ABL proto-oncogene to chromosome 1q22-24 in a metaphase from a Ph-negative CML patient 2 with a deletion of chromosome 16q.**

The black grain on chromosome 1q, indicated by an arrow, represents localisation of the ABL proto-oncogene in situ.

The clustering of grains on both chromosome 9 and 22 was significant ($p = 0.001$ and $p < 0.0002$ respectively).

3.5.2.6 **Patient 5**

In 48 metaphases analysed with a total number of 115 grains, 20 (17.3%) grains mapped to chromosome 9 and 16 (13.9%) grains mapped to chromosome 22 (Fig 17, Table 6). The clustering of grains on both chromosome 9 and 22 was significant ($p < 0.0001$ and $p < 0.0002$ respectively).

3.5.2.7 **Patient 6**

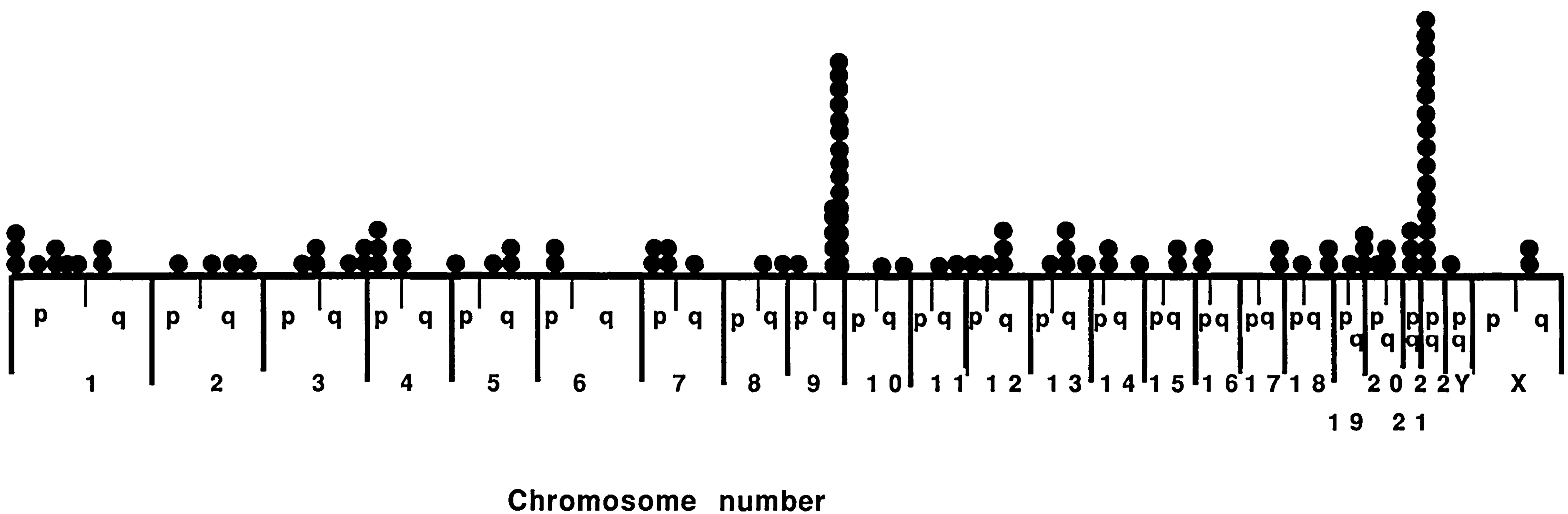
In 50 metaphases analysed with a total number of 111 grains 13 (11.7%) grains and 16 (14.4%) grains mapped to chromosome 22. The clustering of grains on chromosome 9 and 22 was significant ($p < 0.0024$ and 0.0026 respectively).

3.5.2.8 **Patient 7**

In 50 metaphases analysed with a total number of 125 grains 15 (12%) grains mapped to chromosome 9 and 14 (11.2%) grains mapped to chromosome 22. The clustering of grains on chromosome 9 and 22 was significant ($p < 0.0009$ and 0.0001 respectively).

Table 6 **Localisation of the ABL proto-oncogene in metaphases from Ph-negative CML patient 5**

The grain localisation of the ABL oncogene to chromosome 9q34 and 22q11 in 48 bone marrow metaphases by in situ hybridisation.



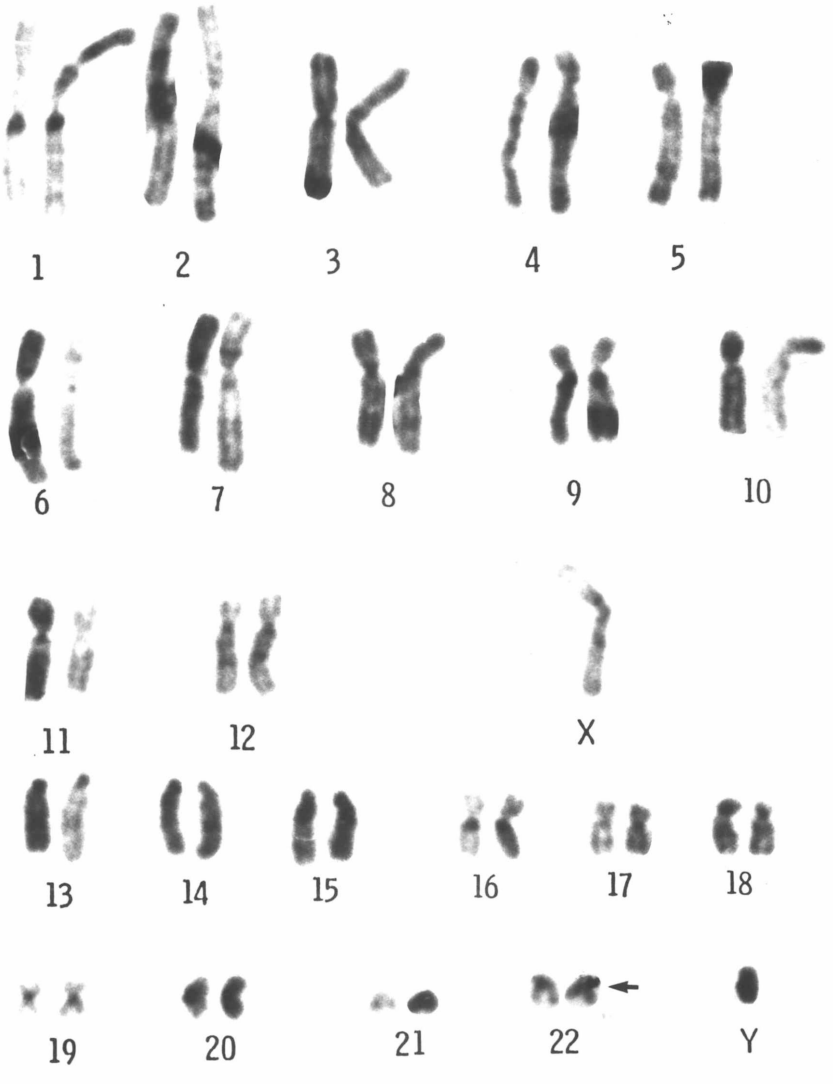


Fig 17 Localisation of the ABL proto-oncogene to chromosome 22 in a karyotyped metaphase from Ph-negative CML patient 5.

The black grain on chromosome 22, indicated by an arrow, represents the localisation of ABL proto-oncogene sequences in situ.

3.5.2.9 **Patient 8**

In 50 metaphases analysed with a total number of 116 grains 12 (10.3%) grains mapped to chromosome 9 and 2 (1.7%) grains mapped to chromosome 22. The clustering of grains on chromosome 9 was significant ($p < 0.009$) but no significant clustering of grains on chromosome 22 was demonstrated.

3.5.3 **BCR LOCALISATION**

The 4 kb 'full length' cDNA Eco RI fragment was used as a probe for the in situ localisation of the BCR gene (Fig 8).

3.5.3.1 **Ph-positive CML patient**

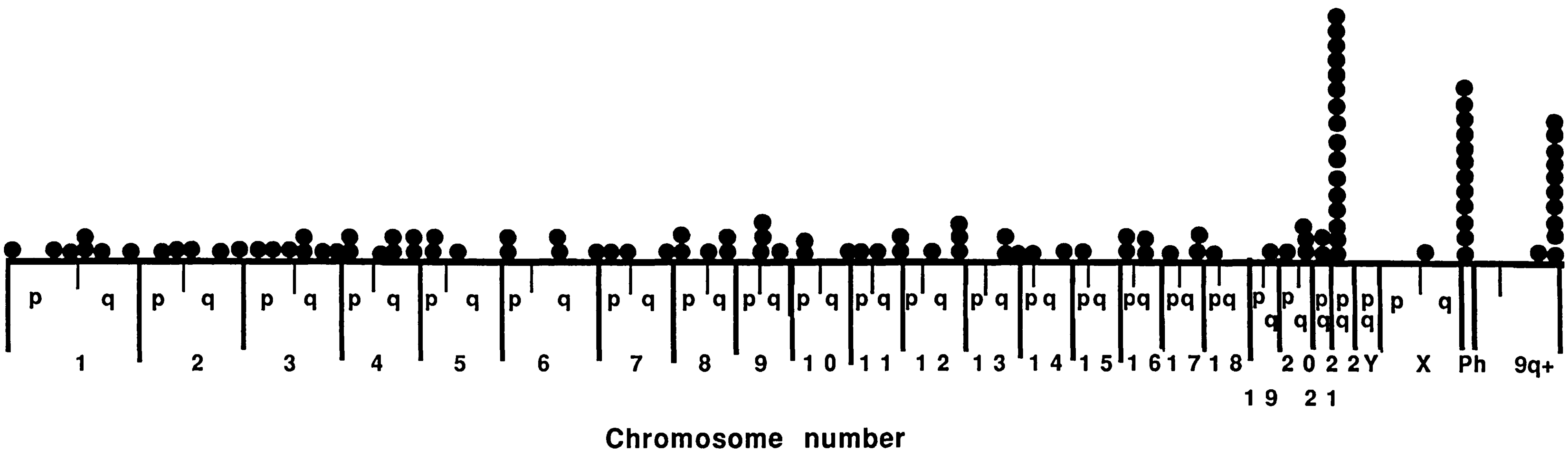
In 50 metaphases analysed with a total of 118 grains, 16 (13.5%) grains hybridised to the normal chromosome 22, 12 (10.1%) grains hybridised to the Ph chromosome and 10 (8.4%) grains hybridised to chromosome 9q+ (Fig 18, Table 7). The clustering of grains on chromosome 22, chromosome 9q+ and the Ph was significant ($p < 0.0002$, $p < 0.0002$ and $p = 0.024$ respectively)

3.5.3.2 **Patient 5**

In 100 metaphases analysed with a total of 97 grains, 17 (17.8%) grains hybridised to chromosome 22. The clustering of grains on chromsome 22

Table 7 Localisation of the BCR gene in metaphases from a Ph-positive CML patient

The grain localisation of the BCR gene to chromosomes 22q11, 22q- and 9q+ in 50 bone marrow metaphases by in situ hybridisation.



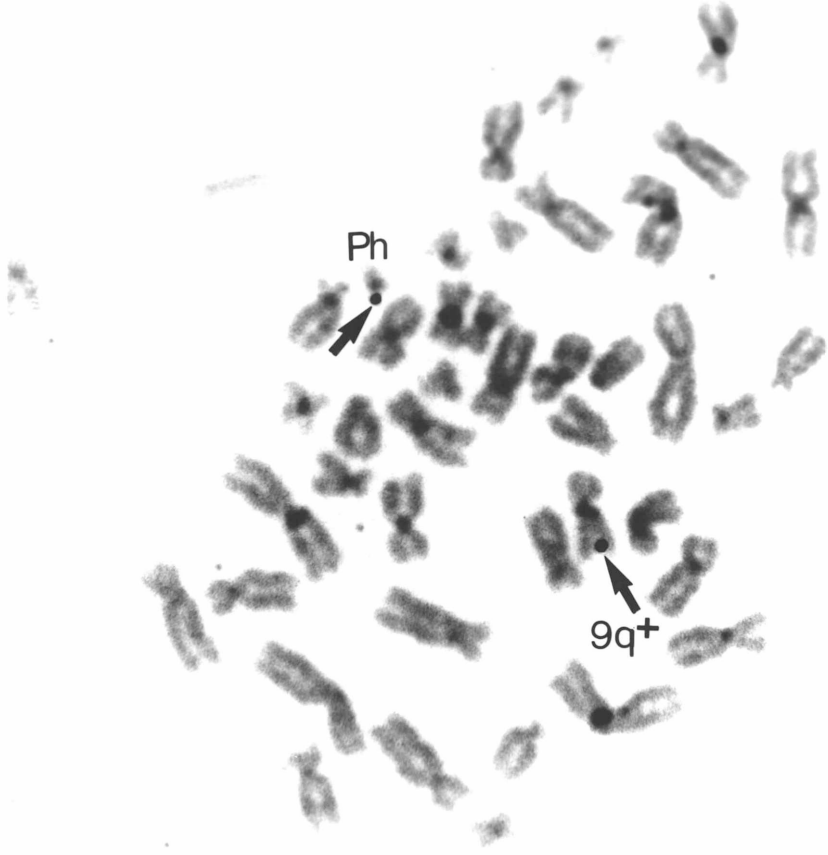


Fig 18 Localisation of the BCR gene to both the chromosomes 9q+ and 22q- (Ph) in a metaphase from Ph-positive CML patient.

The black grains on the chromosomes 9q+ and Ph, indicated by arrows, represent hybridisation of the BCR probe both to the BCR gene sequences on chromosomes 22q- and those translocated to the 9q+.

was significant ($p < 0.0003$). We did not find any clustering of grains on the distal portion of chromosome 9 or any other chromosome.

3.5.3.3 **Patient 7**

In 65 metaphases analysed with a total number of 101 grains 3 (2.9%) grains hybridised to chromosome 9 and 11 (10.8%) grains hybridised to chromosome 22 (Fig 19, Table 8). The clustering of grains to chromosome 22 was significant ($p < 0.0001$).

3.5.4 SIS LOCALISATION

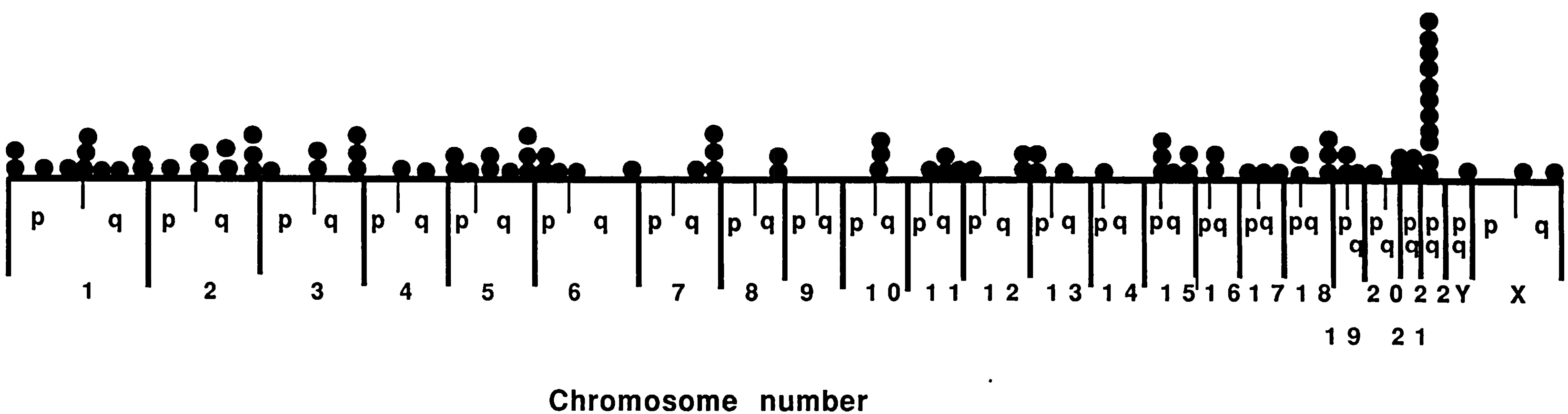
A 2.7 kb human cDNA Bst EII fragment was used as a probe for the in situ localisation of the SIS oncogene. A summary of the results of SIS localisation is shown in table 8.

3.5.4.1 **Ph-positive CML patient**

In 40 metaphases analysed with a total number of 87 grains, 10 (11.4%) grains hybridised to the normal chromosome 22 and 9 (10.3%) grains hybridised to chromosome 9q+ (Fig 20, Table 9). The clustering of grains on chromosome 22 and chromosome 9q+ was significant ($p < 0.0001$ and $p < 0.002$ respectively).

Table 8 Localisation of the BCR gene in metaphases from Ph-negative CML patient 7

The grain localisation of the BCR gene to chromosome 22q11 only in 65 bone marrow metaphases by in situ hybridisation.



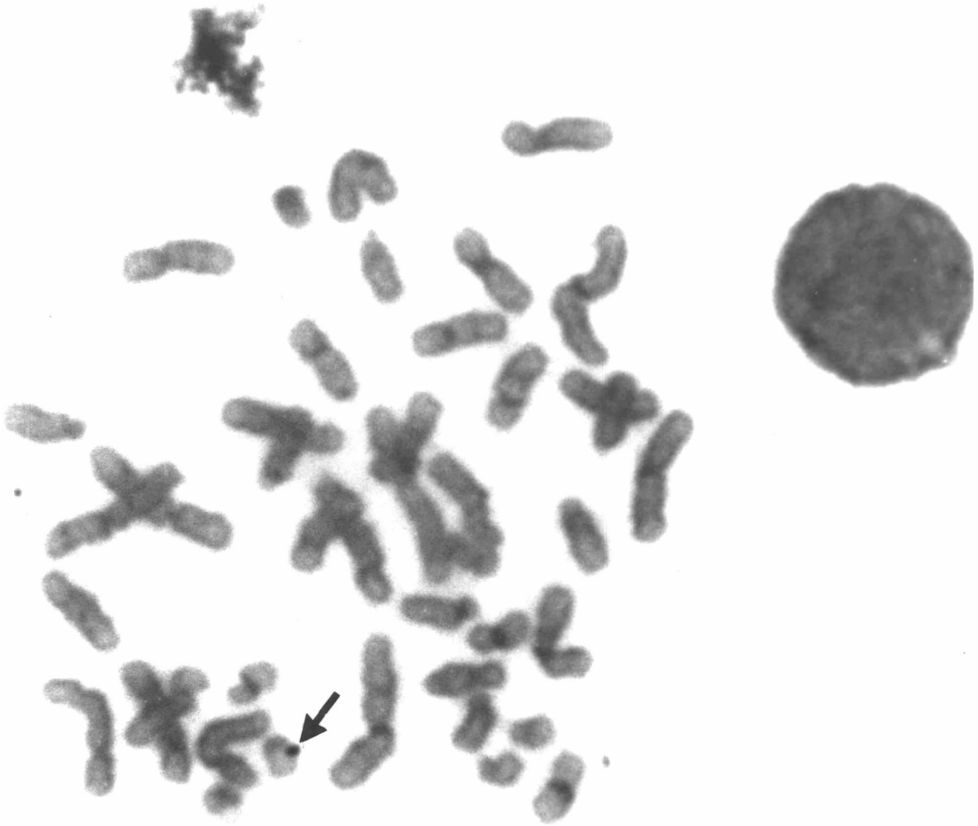
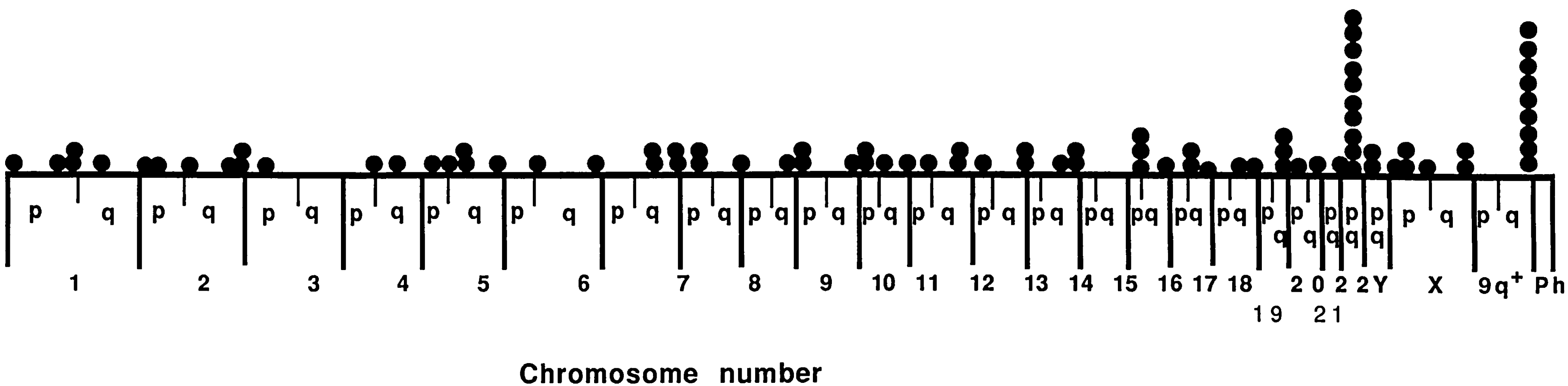


Fig 19 **Localisation of the BCR gene to chromosome 22 in a metaphase from Ph-negative CML patient 7.**

The black grain on chromosome 22, indicated by an arrow, represents localisation of BCR probe to the BCR gene in its normal position on chromosome 22q11.

Table 9 Localisation of the SIS proto-oncogene in metaphases from Ph-positive CML patient

The grain localisation of the probe for the SIS proto-oncogene to chromosomes 22q13 and 9q34 in 40 bone marrow metaphases by in situ hybridisation



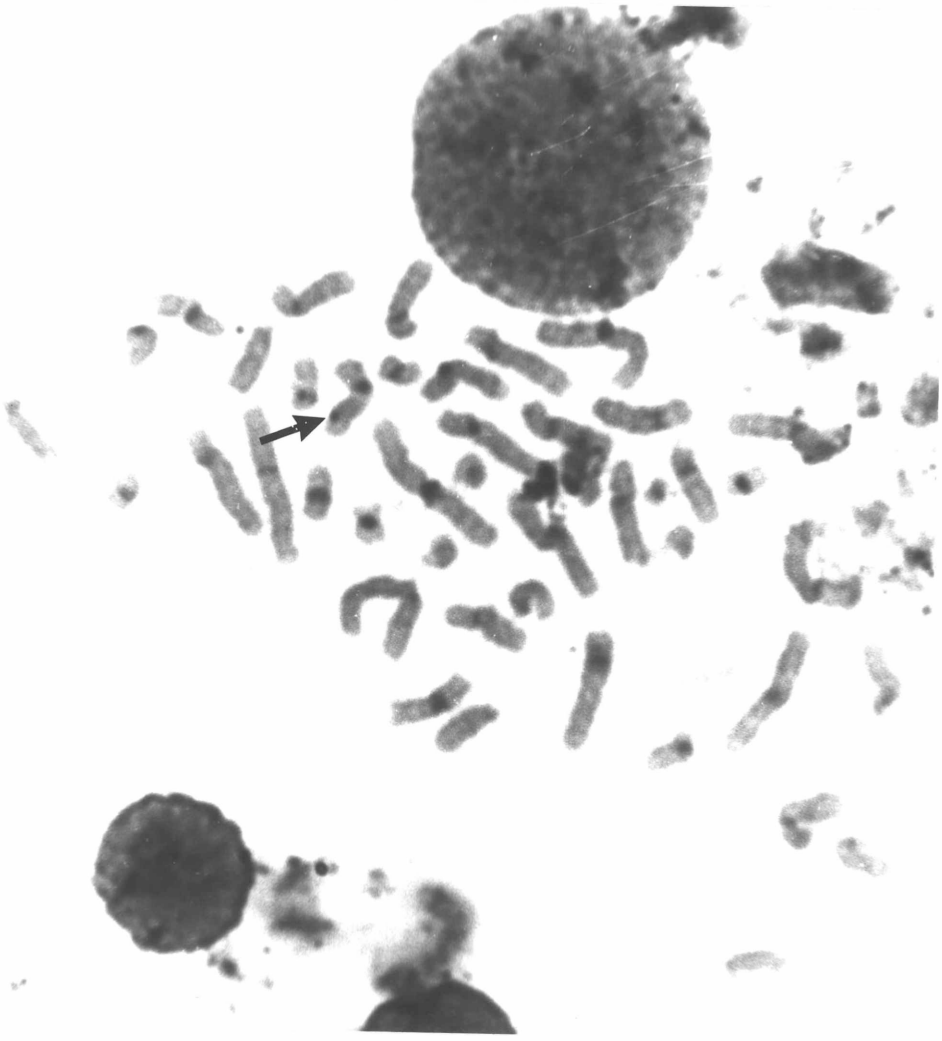


Fig 20 Localisation of the SIS proto-oncogene to chromosome 9q+ in a metaphase from Ph-positive CML patient.

The black grain on the terminal end of chromosome 9q+, indicated by an arrow, represents the localisation of SIS proto-oncogene sequences in situ.

3.5.4.2 **Patient 1**

In 40 metaphases analysed with a total number of 81 grains 2 (2.4%) grains mapped to chromosome 9 and 10 (12.3%) grains mapped to chromosome 22. The clustering of grains to chromosome 22 was significant ($p < 0.0049$).

3.5.4.3 **Patient 3**

In 50 metaphases analysed with a total number of 122 grains 1 (0.8%) grains mapped to chromosome 9 and 16 (13.1%) grains mapped to chromosome 22. The clustering of grains to chromosome 22 was significant ($p < 0.0001$).

3.5.4.4 **Patient 4**

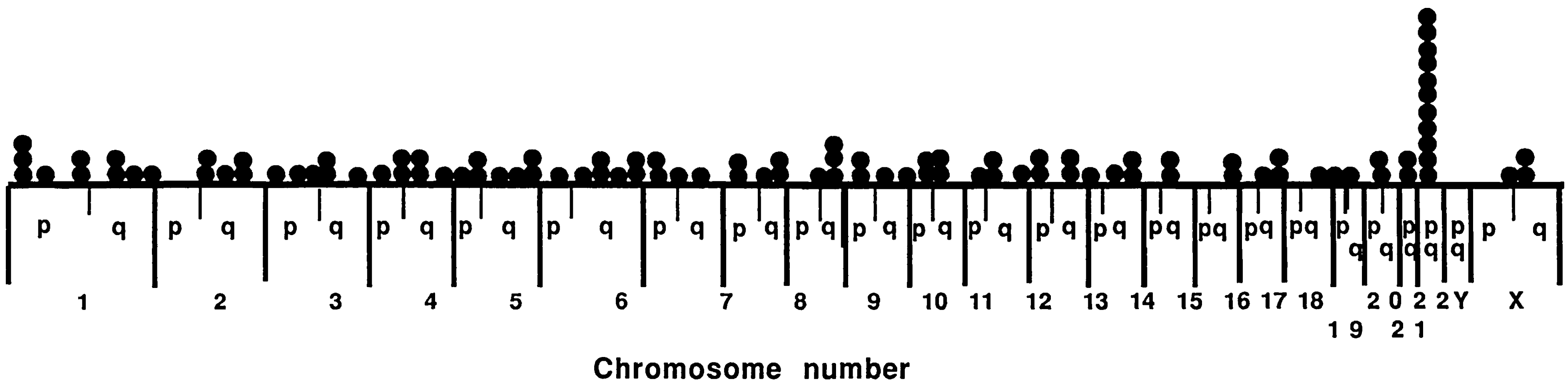
In 43 metaphases analysed with a total number of 102 grains 2 (1.9%) grains mapped to chromosome 9 and 11 (10.7%) grains mapped to chromosome 22 (Fig 21, Table 10). The clustering of grains on chromosome 22 was significant ($p < 0.0001$).

3.6 **mRNA ANALYSIS**

Having detected BCR rearrangement in 7 of the 8 patients Dr Orna Dreazen looked for expression of the chimeric BCR/ABL mRNA. The presence of a chimeric BCR/ABL mRNA was determined by hybridisation of mRNA from patients 1, 3, and 6 to radiolabelled RNA probes representing the two

Table 10 Localisation of the SIS proto-oncogene in the metaphases from Ph-negative CML patient 4.

The grain localisation of the probe for the SIS oncogene to chromosome 22 q13 in 43 bone marrow metaphases from patient 4 by in situ hybridisation.



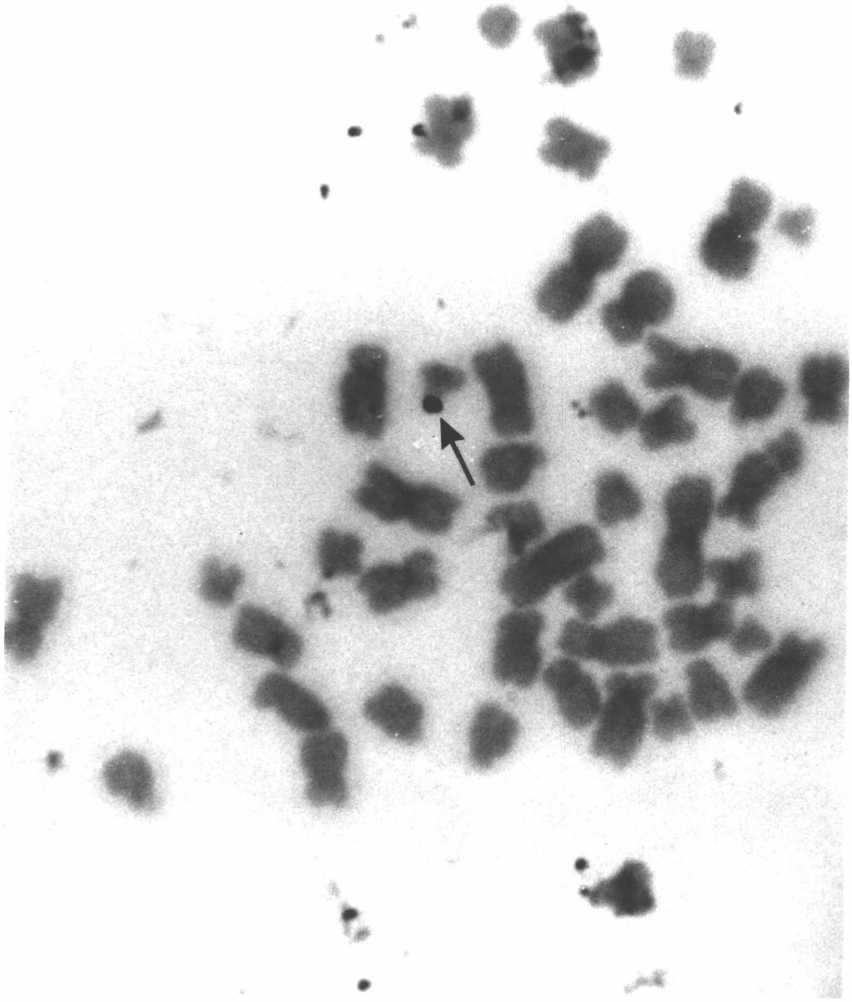


Fig 21 **Localisation of the SIS proto-oncogene to chromosome 22q13 in a metaphase from Ph-negative CML patient 4.**

The black grain on chromosome 22q13, indicated by an arrow, represents localisation of SIS proto-oncogene sequences to its normal position in situ.

alternative BCR/ABL splice junctions typical in CML (Shtivelman et al, 1986). The probes complementary to the two BCR/ABL junctions, designated b2a2 and b3a2, were used to 'protect' polyadenylated RNA from digestion with RNase. The RNA from approximately 50-70% of individuals with Ph-positive CML protects probes for the b2a2 junction and the RNA from 30-50 % of Ph-positive CMLs protects the b3a2 junction. The probes for the b2a2 junction consisted of a 450 b probe in which 172 b are contributed by BCR exons 2 and 3 and 278b of ABL sequences contributed by ABL exon 2. The b3a2 junction was composed of 30 b of BCR sequences from exon 2 and 250 b of ABL sequences. RNA from individuals in whom the BCR break occurs between exons '3' and '4' can potentially protect both the b2a2 and the b3a2 junctions by a mechanism of alternative splicing (Fig 22). All three patients had evidence the BCR/ABL transcript. In patient 1 only the b3a2 junction was protected, indicating a break between exons '2' and '3'. In patient 3 alternative splicing resulted in protection of both the b2a2 and the b3a2 junction probes, which was consistent with a break between exons '3' and '4'. In patient 6 the b2a2 junction was protected which was also consistent with a break between exon '3' and '4'.

3.7 SUMMARY

A summary of all the cytogenetic, in situ hybridisation and molecular findings is shown in Table 11.

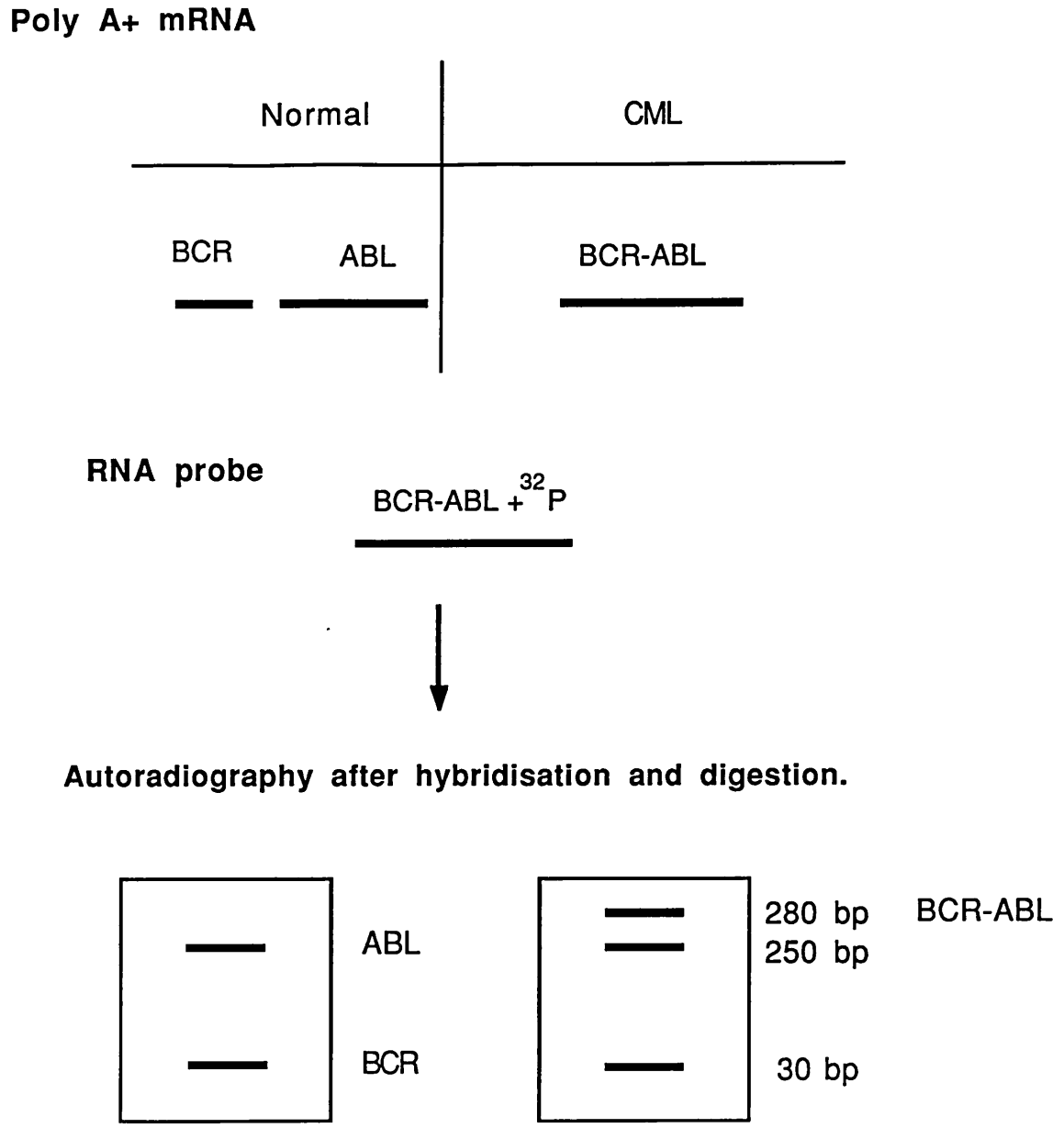


Fig 22 **Principles of the RNA protection assay**

RNA probes complementary to the BCR/ABL chimeric message are hybridised with poly A+ RNA extracted from the leukaemic cells of patients. Thereafter the total RNA is subjected to RNase digestion. The ds RNA remains intact and is displayed on a polyacrylamide gel. In normals only fragment sizes corresponding to the normal ABL and BCR genes will be protected. In Ph-positive CML larger fragments corresponding to the BCR/ABL chimeric mRNA as well as the normal ABL and BCR mRNAs will be protected.

Table 11 Summary of molecular features in one Ph-positive and eight Ph-negative CML patients

	Patient	Cytogenetics	M-BCR rearrangement	Chromosomal localisation			Chimeric mRNA
				ABL	BCR	SIS	
Ph-pos, M-BCR pos		46,XY, Ph, t(9;22)	+	9q34/22q11	22q11/9q34	22q13/9q34	NT
Ph-neg, M-BCR pos	1	46,XY, 22q- 46,XY	+	9q34/22q11	NT	22q13	b2a2
	2	46,XY, 16q-	+	9q34/1q24	NT	NT	NT
	3	46,XY	+	9q34/22q11	NT	22q13	b2a2/b3a2
	4	46,XX	+	9q34/22q11	NT	22q13	NT
	5	46,XY	+	9q34/22q11	22q11	NT	NT
	6	46,XY	+	9q34/22q11	NT	NT	b3a2
	7	46,XY	+	9q34/22q11	22q11	NT	NT
Ph-neg, M-BCR neg	8	46,XY	-	9q34	NT	NT	NT

3.8 DISCUSSION

Reports of molecular studies in Ph-negative CML have covered a cytogenetically heterogeneous population. Some of these patients had apparently normal chromosomes while others had abnormalities of chromosomes 9 or 22 without formation of the common derivative chromosome 9pter 9q34::22q11 22qter (Bartram et al,1984, Kurzrock et al, 1986, Morris et al, 1986, Bartram and Carbonell, 1986, Ganesan et al, 1986). None of the eight patients studied by G banding had a classical t(9;22). One patient had a minority of metaphases which with a 22q- without a corresponding chromosome 9q+ and one patient had a deletion of the terminal end of chromosome 16. Both these patients had haematological features indistinguishable from Ph-positive CML. The 7 remaining patients all had apparently normal chromosomes.

Results of Southern analysis showed that the M-BCR was rearranged in 7 of the 8 Ph-negative patients studied. All the patients who resembled Ph-positive CML had rearrangement of M-BCR. However of the three patients who showed atypical features patients 4 and 6 showed M-BCR rearrangement and patient 8 did not.

In situ hybridisation studies can be summarised as follows:
In 6 patients with rearrangement of M-BCR one allele of the ABL oncogene was translocated from chromosome 9 to chromosome 22, as in Ph positive CML. In M-BCR positive patient 2, the ABL oncogene had been translocated to the q arm of chromosome 1. In the one patient lacking

M-BCR rearrangement the ABL oncogene remained in its normal position on chromosome 9. Using the long BCR probe in M-BCR positive patients I found no evidence of reciprocal translocation, i.e. the 3' portion of BCR remained in its normal position on chromosome 22. I also localised the SIS oncogene in three M-BCR positive patients found it in its normal position on chromosome 22.

The mRNA from three patients was studied and all had evidence of a BCR/ABL transcript. This result suggests the formation and transcription of the BCR/ABL chimeric gene on the apparently normal chromosome 22. The localisation of SIS and BCR to chromosome 22 suggests that Ph-negative CML can result from interstitial insertion of a small ABL-containing DNA sequence from the tip of chromosome 9 into BCR on chromosome 22. Hecht et al (1985) reported the first case of the genesis of the Ph chromosome by interstitial deletion of chromosomal material from chromosome 22 and insertion of this deleted material into another chromosome. The translocation in this patient showed no involvement of chromosome 9. With hindsight this 'simple' translocation was probably 'complex', involving chromosome 9 at a submicroscopic level. Interstitial insertion events in CML are being reported with increasing frequency (Hagemeijer et al,1985, Morris et al 1988). This almost certainly reflects the improvement of cytogenetic technology in conjunction with the use of high resolution banding and in situ hybridisation.

The significance of the minority clone of cells with 22q- in patient 1 is not known. There have been reports of CML patients who were Ph-

negative at presentation and who after varying periods of time acquired a Ph translocation (Lisker et al, 1982). One interpretation of this finding is based on the multistep theory of pathenogenesis, according to which a primary 'neoplastic' mutation occurs in a pluripotent stem cell and is followed at a later stage by acquisition of the Ph chromosome. In patient 1 however the cytogenetically normal cells appeared to have the same genetic change as in Ph-positive CML and the cells containing 22q- did not have a classical chromosome 9q+. Southern analysis of M-BCR in this patient detected just one rearranged band and therefore the 22q- containing cells do not represent a second breakpoint within M-BCR. It is probable that these cells represent a clonal evolution of the original Ph-negative 'leukaemic' population.

Abnormalities involving the band q22 of chromosome 16 have been reported in acute myeloid leukaemia patients with abnormal eosinophils (Arthur and Bloomfield, 1983, Le Beau et al, 1983, Larson et al, 1986). The metallothionein (MT) gene cluster has also been mapped to chromosome 16q22 (Harper and Saunders, 1981, Le Beau et al, 1984b). Le Beau et al (1985a) studied an acute myelomonocytic leukaemia patient with a rearrangement of chromosome 16q22 and suggested that MT genes or their regulatory regions may function as an 'activating' sequence for an as yet unidentified cellular gene. There are several mechanisms by which MT genes may be involved in the pathogenesis of leukaemia affecting the myeloid series. For example mature eosinophils and neutrophils have a high zinc content in their granules (Barnes and Sato, 1980). MT functions in the intracellular binding and storage of zinc and may therefore have an important role in granulocyte and monocyte

differentiation. The dysplastic features in the granulocytic series of patient 2 with a deletion of chromosome 16q22 were hypogranulation of granulocytic precursors with an increase in secondary granulation and persistence of cytoplasmic basophilia. Therefore a genomic alteration of the MT genes in this patient may be responsible for these dysplastic features.

The finding of ABL on chromosome 1q in a patient with an apparently normal chromosome 1 and M-BCR rearrangement suggests two possible sequences of events. ABL sequences may have juxtaposed with unknown gene sequences on chromosome 1q to make a different chimeric gene also with enhanced tyrosine kinase activity. The alternative explanation (if we accept that M-BCR rearrangement is not an independent phenomenon) is that a complex chromosomal rearrangement occurred which involved chromosomes 1, 9, and 22. This rearrangement may have involved initially the juxtaposition of ABL with BCR and then subsequent translocations placed the chimeric BCR/ABL gene on chromosome 1q. Proof of the latter hypothesis would have been to localise 5'BCR sequences in the metaphases of our patient. The lack of stored material and the early death of our patient made this study impossible. However Bartram et al (1985b) demonstrated that the BCR/ABL chimeric gene had been relocated to chromosome 12 in a Ph-negative CML patient and Hagemeijer et al (1984) demonstrated that as a result of complex chromosomal rearrangements, the BCR/ABL chimeric gene can be relocated to another chromosomal site. Recently Morris et al (1988) reported the involvement of an apparently normal chromosome 1 in a simple variant translocation using in situ hybridisation. The significance of the

involvement of chromosome 1q in CML is unknown. Abnormalities of chromosome 1 occur frequently in human tumours (Brito-Babapulle and Atkin, 1981). Kruh et al (1986) demonstrated the presence of ABL-like sequences on chromosome 1q24 so translocation of ABL may have been facilitated through recombination of homologous sequences.

In the one M-BCR rearrangement-negative patient whom I studied ABL remained in its normal position on chromosome 9. This finding probably reflects the fact that Ph-negative CML patients (they may be 60%) exist in whom the key genetic change does not involve the formation of a BCR/ABL chimeric gene.

The finding of M-BCR rearrangement and movement of ABL to chromosome 22 in two patients with atypical features seems to contradict the reports of Morris et al (1986), Wiedemann et al (1988) and Ohyashiki et al (1988). They failed to detect BCR rearrangement in cases classified as atypical CML. Their result suggested that the molecular events associated with M-BCR rearrangement directly determined the morphology, the types of cells and the relative proportions in which they appeared in the peripheral blood. Our conflicting results may reflect a bias in selection and classification of CML patients. It is possible that a combination of aberrant values in differential counts led to a diagnosis of atypical CML or that minor alterations in the morphological criteria would permit inclusion of particular atypical cases into the classical CML category. However it is equally likely that phenotypic diversity seen in CML with M-BCR rearrangement may be generated by other genetic

changes as yet unidentified. The confusion will be clarified by further molecular investigation of Ph-negative CML.

I conclude that a significant proportion (i.e. 40%) of patients with Ph-negative CML superficially resembling the Ph-positive disease have an identical lesion at the molecular level. Some patients have a disease otherwise indistinguishable from Ph-positive CML, whereas in others some of the clinical and laboratory findings are distinct (Fig 23). The basis of this heterogeneity must involve factors other than the abnormal expression of BCR/ABL.

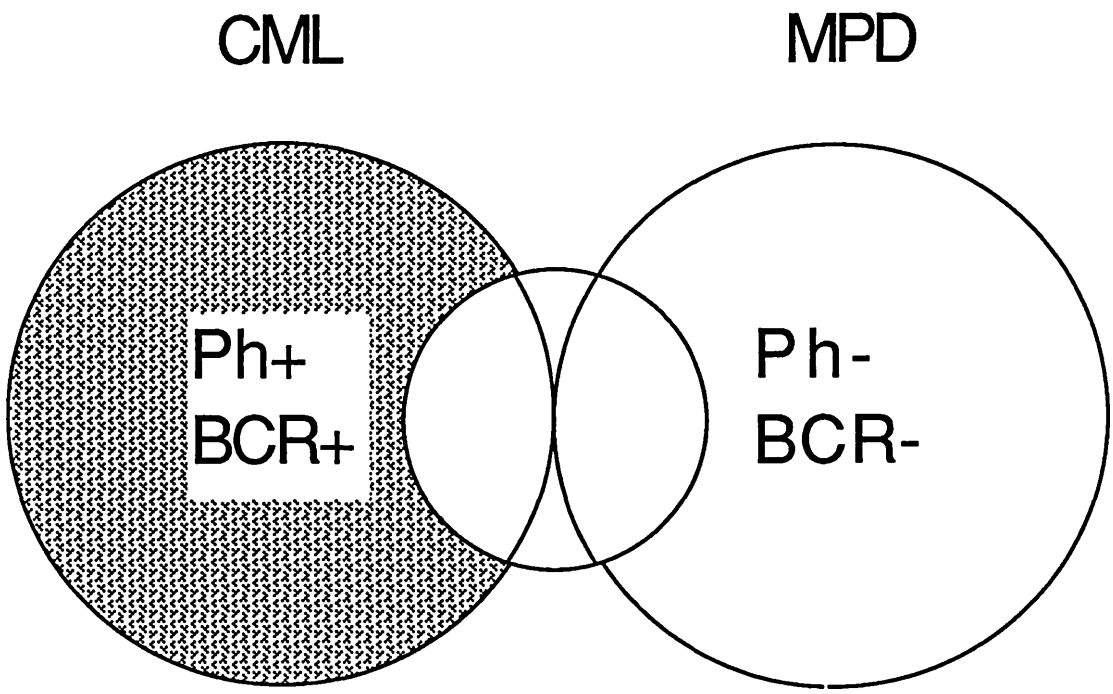


Fig 23 Relationship between Ph-positive, M-BCR-positive CML and Ph-negative, M-BCR-negative myeloproliferative diseases.

The left hand circle represents those patients with Ph-positive, M-BCR-positive CML. The right hand circle represents those patients with Ph-negative, M-BCR negative myeloproliferative diseases. The smaller, intersecting middle circle represents those patients that are M-BCR positive, some of whom are indistinguishable from the Ph-positive disease and some who show atypical features.

CHAPTER 4: WHERE IS THE SECOND BREAKPOINT IN Ph-NEGATIVE CML?

4.1 Ph-NEGATIVE CML

I studied further the molecular features of a selected group of patients whose disease closely resembled CML at the clinical and haematological level. Southern analysis using high molecular weight DNA revealed genomic alterations of M-BCR. The use of BCR/ABL sequences defining the two common (alternative) junctions in an mRNA protection assay confirmed the presence in the leukaemic cells of the same abnormal mRNA that characterises Ph-positive CML. I discussed in the previous chapter studies to identify the position of ABL, SIS and BCR genes in the metaphase preparations from these patients to understand the mechanism of formation of the BCR/ABL gene. I found that the ABL oncogene had moved from its normal position on chromosome 9 to chromosome 22. Both BCR and SIS genes remained in their normal position on chromosome 22, unlike the situation in the Ph-positive CML where 3'BCR and SIS move to chromosome 9. These results suggested that in these patients the BCR-ABL chimeric gene was created not as a result of reciprocal translocation but by interstitial insertion. If a functional BCR/ABL gene was created in this manner the ABL-containing fragment inserted into 22q11 might contain only ABL sequences or could include a very variable quantity of material distal to ABL on chromosome 9q34.

In order to establish how much ABL-containing genetic material was inserted into chromosome 22q11 I studied by in situ hybridisation the

localisation of the probes CRI-P111 and CRI-L1424 in the metaphases of 3 patients (see previous chapter). These probes recognise distinct polymorphic regions distal to the ABL locus on chromosome 9q34.

4.2 RESTRICTION FRAGMENT LENGTH POLYMORPHISMS AND CLASSICAL LINKAGE ANALYSIS

Sequence polymorphisms occur once every few hundred nucleotides in the normal human genome. They are predominantly single nucleotide substitutions, which may change the site of cleavage by specific restriction endonucleases. These polymorphisms can be detected by Southern analysis as changes in the length of DNA fragments. These are termed restriction fragment length polymorphisms (RFLP) and represent valuable genetic linkage markers (Kan and Dozy, 1978). The analysis of genetic linkage is based on the exchange of genetic information (recombination) between the pairs of maternally and paternally derived chromosome homologues during the meiotic cell division that gives rise to the germ cells. Information about the genetic distance between two markers is obtained by analysing the frequency of recombination between markers in large families (White and Lalouel, 1987). The 'genetic' distance between two markers is defined as 1 centiMorgan (cM) when the markers have a probability of 1 in 100 of recombining in each generation and this corresponds to an average physical distance of 1×10^6 base pairs. A human linkage map with an average marker spacing of 10 cM has been constructed (Donis-Keller et al, 1987).

The relationship between the 'genetic' and physical distances in different parts of the map is non-linear due to the existence of preferential sites of recombination in the human genome. Studies in a number of organisms suggest that these 'hotspots' of recombination occur preferentially in the distal portions of the autosomes and that as a result the 'genetic' map is expanded at these points. In humans, detailed studies of the distribution of chiasmata (cytological manifestations of recombination) show a significant excess near the telomeres. The most completely studied case is chromosome 1, in which the distal 15% of the chromosome contains 40% of all the chiasmata (Hulten et al 1982).

4.3 IN SITU HYBRIDISATION STUDIES

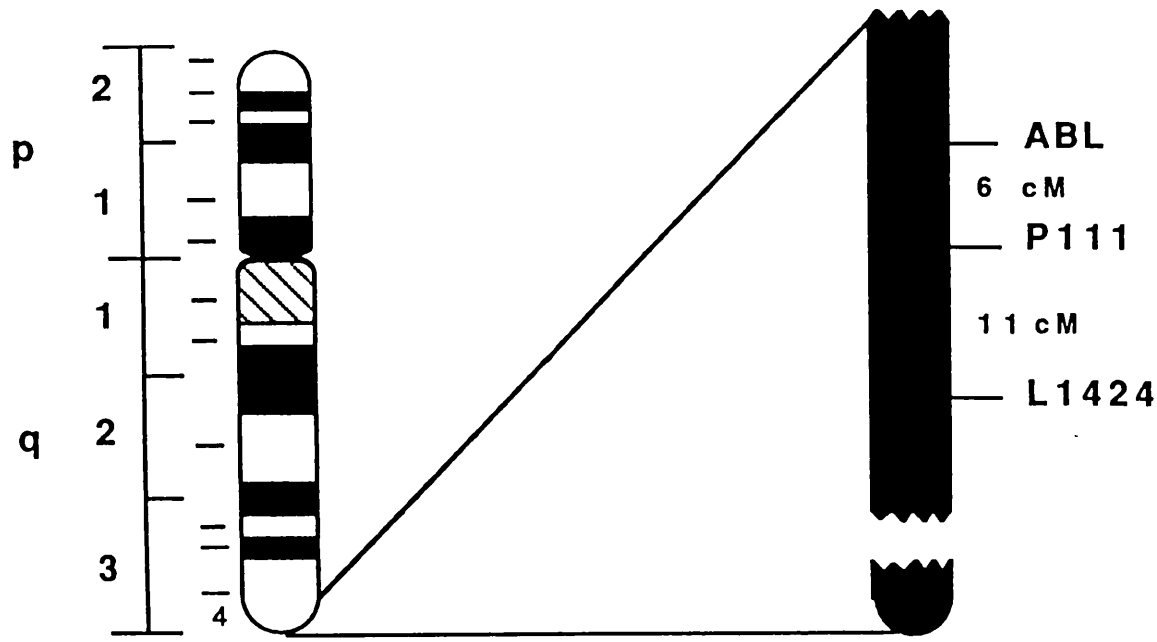
4.3.1 Localisation of CRI-P111 and CRI-L1424 in normal metaphases

The estimated distance between ABL and CRI-P111 based on classical linkage analysis is 6 cM and between CRI-P111 and CRI-L1424 is 11 cM (Donis-Keller et al, 1987) (Fig 24).

To confirm that these RFLP probes did indeed localise to chromosome 9q34 I performed an in situ hybridisation to the metaphase chromosomes from normal female lymphocytes.

4.3.1.1 CRI-P111 localisation

In 60 metaphases analysed with a total number of 96 grains, 17 (15 %) grains mapped to chromosome 9. The hybridisation of grains to



Normal chromosome 9

Fig 24 **Relative positions of ABL and the probes CRI-P111 and CRI-L1424 on the normal chromosome 9q34.**

Restriction fragment length polymorphic (RFLP) probe, CRI-P111, mapped by classical linkage analysis 6 cM 3' of ABL. RFLP probe, CRI-L1424, mapped by classical linkage 11 cM 3' of CRI-P111 and 17 cM 3' of ABL.

chromosome 9 was significant ($p < 0.0002$). No other clustering of grains was observed.

4.3.1.2 CRI-L1424 localisation

In 60 metaphases analysed with a total number of 112 grains 20 (20.8%) grains mapped to chromosome 9. The hybridisation of grains to chromosome 9 was significant ($p < 0.0001$). No other clustering of grains was observed.

4.3.2 Localisation of CRI-P111 and CRI-L1424 in Ph-positive CML metaphases

Although the translocation t(9;22) is known to be reciprocal and balanced the transposition of chromosomal material from 9q to 22q is not visible by standard cytogenetic techniques. However de Klein et al (1982) and Groffen et al (1983) confirmed the reciprocal nature of the translocation when they demonstrated that the ABL oncogene was translocated from chromosome 9 to the Ph chromosome and that SIS was translocated from chromosome 22 to 9. Thus genetic material occurring distal to ABL on chromosome 9q34 is assumed, but not conclusively proved, to translocate along with ABL to the Ph chromosome. I therefore proceeded to localise by in situ hybridisation the probes CRI-P111 and CRI-L1424 to the metaphase chromosomes of a Ph-positive CML patient.

4.3.2.1 Clinical and haematological features

The clinical and haematological features of this patient is shown in Table 13.

4.3.2.2 CRI-P111 localisation

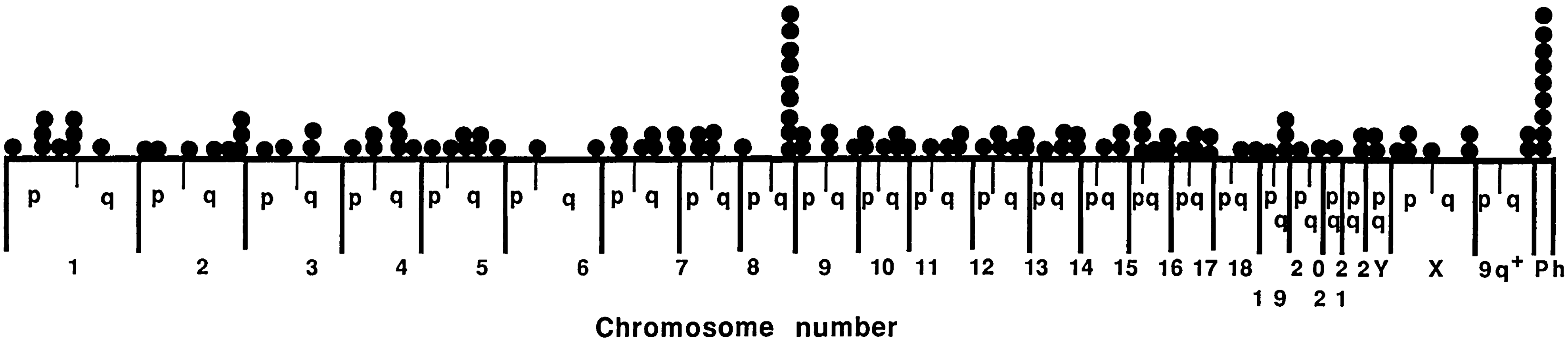
In 60 metaphases analysed with a total number of 112 grains, 11 (9.8%) grains mapped to the normal chromosome 9 at 9q34, 13 (11.6) grains to the 22q- and none to the 9q+. The hybridisation of grains to chromosomes 9 and 22q- was significant ($p = 0.007$ and $p < 0.0001$ respectively).

4.3.2.3 CRI-L1424 localisation

In 75 metaphases analysed with a total number of 128, 9 (7%) grains mapped to the normal allele at chromosome 9q34, 9 (7%) grains to the chromosome 22q- and no grains mapped to the 9q+. The hybridisation of grains to chromosome 9 and 22q- was significant ($p = 0.0836$ and $p < 0.0001$ respectively) (Fig 25, Table 12).

Table 12 Localisation of the RFLP probe CRI-L1424 in metaphases from a Ph-positive CML patient

The grain localisation of CRI-L1424 to chromosomes 9 and 22q- in 75 bone marrow metaphases by in situ hybridisation.



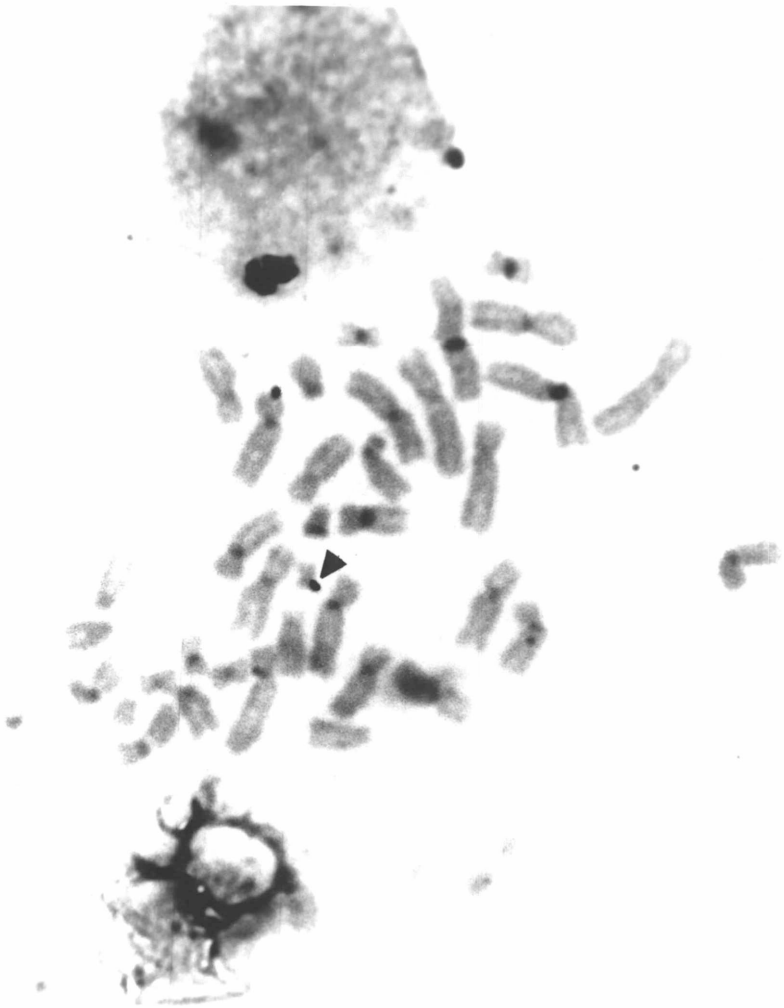


Fig 25 **Localisation of CRI-L1424 to chromosome 22q- in a metaphase from Ph-positive CML patient.**

The black grain on chromosome 22q-, indicated by an arrowhead, represents the localisation of CRI-L1424 sequences normally found on chromosome 9q34.

4.3.3 **Localisation of CRI-P111 and CRI-L1424 in Ph-negative CML metaphases**

4.3.3.1 Clinical and haematological features

The clinical and haematological features of the three Ph-negative patients studied with the CRI probes are shown in Table 13 and also indicated by asterisks in Table 2 in Chapter 3.

4.3.3.2 **Patient 4**

CRI-P111 localisation

In 50 metaphases analysed with a total number of 82 grains, 9 (11%) grains mapped to chromosome 9 and 2 (2.4%) grains mapped to chromosome 22. The hybridisation of grains to chromosome 9 was significant with a p value of 0.015. The hybridisation of grains to chromosome 22 was not significant.

CRI-L1424 localisation

In 70 metaphases analysed with a total of 110 grains, 13 (11.8%) grains mapped to chromosome 9 and 2 (1.8%) mapped to chromosome 22 . The hybridisation of grains to chromosome 9 was significant ($p = 0.002$) whereas hybridisation to chromosome 22 was not.

Table 13 Clinical details of 3 Ph-negative and one Ph-positive CML patient

	Patient	At presentation					Response to standard therapy
		Age/ sex	Spleen (cm)	WBC ($X10^9$/l)	Plats	Blasts (%)	
Ph-neg	4	4/F	8	17	384	0	CR
	5	49/M	12	170	283	0	CR
	7	39/M	0	298	210	1	CR
Ph-pos		77/M	3	35	374	0	CR

CR = complete haematological response

4.3.3.3 **Patient 5**

CRI-P111 localisation

In 40 metaphases analysed with a total number of 77 grains, 10 (12.9%) grains mapped to chromosome 9 and 9 (11.6%) grains mapped to chromosome 22. The hybridisation of grains to chromosomes 9 and 22 was significant ($p = 0.0034$ and $p < 0.0001$ respectively).

CRI-L1424 localisation

In 75 metaphases analysed with a total number of 134, 13 (9.7%) grains mapped to chromosome 9q34 and 14 (10.4%) grains mapped to chromosome 22q11 (Fig 26, 27). The hybridisation of grains to chromosomes 9 and 22 was significant ($p = 0.011$ and $p < 0.0001$ respectively).

4.3.3.5 **Patient 7**

CRI-P111 localisation

In 53 metaphases analysed with a total number of 112 grains, 9 (8%) grains mapped to chromosome 9 and 2 (1.7%) grains mapped to chromosome 22. The hybridisation of grains to chromosome 9 was significant ($p = 0.085$) whereas the hybridisation of grains to chromosome 22 was not.

CRI-L1424 localisation

In 80 chromosomes analysed with a total of 126 grains, 17 (13.4%) grains mapped to chromosome 9 and 3 (2.2%) grains mapped to chromosome 22 (Fig

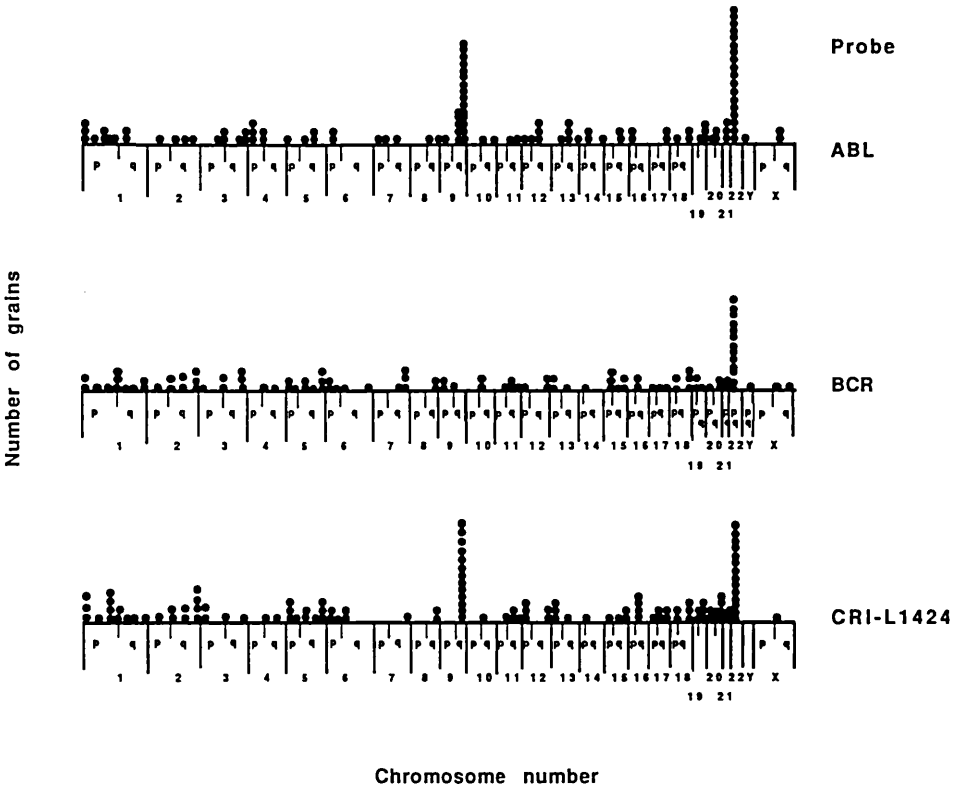


Fig 26 Composite diagram showing grain distribution for the three probes ABL, BCR and CRI-L1424 in 40 to 70 metaphases from Ph-negative CML patient 5.

The x axes represent the chromosomes in proportion to their sizes; along the y axes are shown the number of grains hybridising to each chromosome. There was obvious clustering of grains either in one or two distinct chromosome regions for each probe used; non-specific 'background' grains were observed on all chromosomes.



Fig 27 **Localisation of the probe CRI-L1424 to chromosome 22q11 in a metaphase from Ph-negative CML patient 5.**

The black grain on chromosome 22q11, indicated by an arrow, represents localisation of CRI-L1424 DNA sequences in situ.

28, Table 14). The hybridisation of grains to chromosome 9 was significant (p = 0.015) whereas the hybridisation of grains to chromosome 22 was not.

4.4 Summary of Results

A summary of the molecular findings in these three Ph-negative and one Ph-positive patient is shown in table 15.

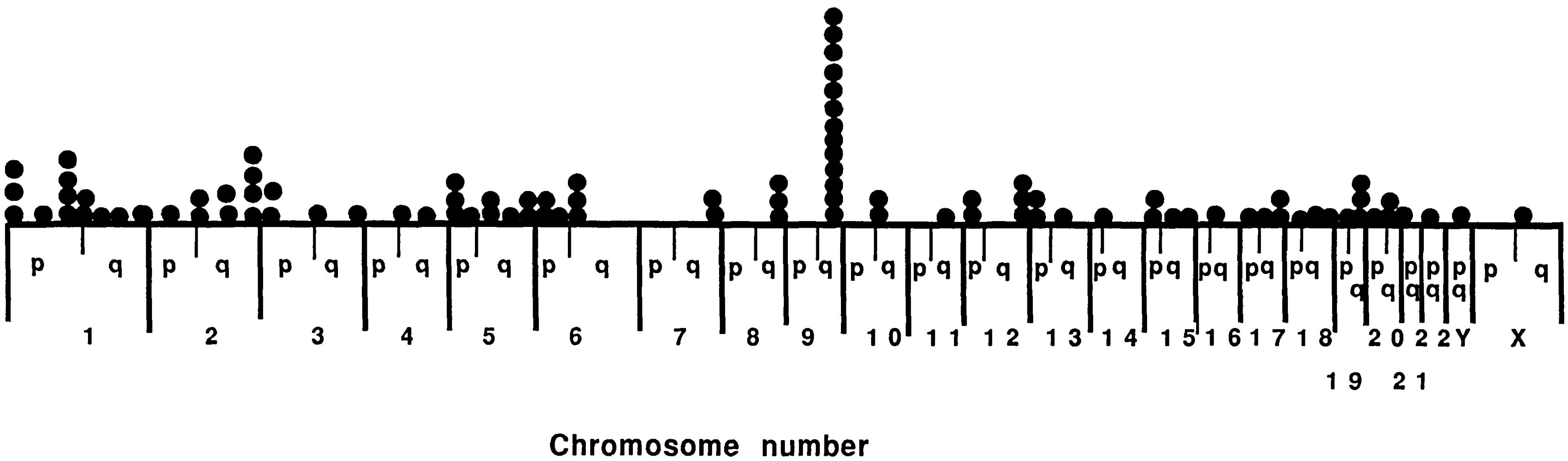
4.5 DISCUSSION

I studied by in situ hybridisation the position of the polymorphic probes CRI-P111 and CRI-L1424 in the metaphases of normal, Ph-positive and Ph-negative CML cells. I confirmed that both CRI-P111 and CRI-L1424 were located on the normal chromosome 9q34. In the patient with Ph-positive CML both CRI-P111 and CRI-L1424 were translocated to the Ph chromosome together with the ABL oncogene. This finding is consistent with the conclusion derived from classical linkage analysis that both probes recognise genetic sequences distal to ABL on chromosome 9q but the resolution of grain localisation by the in situ hybridisation technique made it impossible to distinguish the positions of the two probes relative to each other.

In the Ph-negative patients studied here we had previously shown that there was genomic rearrangement of BCR. We also demonstrated by in situ hybridisation that ABL had moved from chromosome 9 to chromosome 22 and furthermore that in patient 5 3'BCR had remained in its normal position

Table 14 Localisation of RFLP probe CRI-L1424 in metaphases from Ph-negative CML patient 7

The grain localisation of CRI-L1424 to chromosomes 9 only in 70 bone marrow metaphases by in situ hybridisation



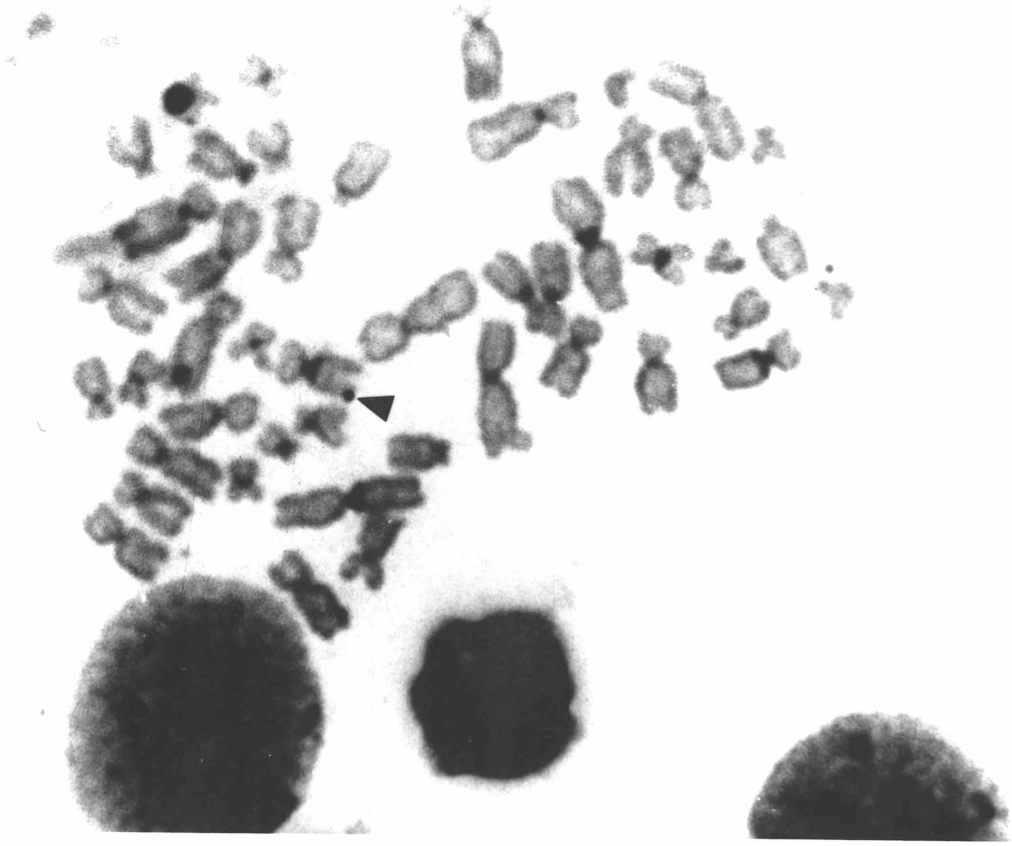


Fig 28 Localisation of the probe CRI-L1424 to chromosome 9q34, its normal position, in a metaphase from Ph-negative CML patient 7.

The black grain on chromosome 9q34, indicated by an arrow, represents the localisation of CRI-L1424 to its normal position.

Table 15 Results of molecular studies in three Ph-negative and one Ph-positive CML patient

	Patient	Rearrangement of M-BCR by Southern analysis					Breakpoint localisation	Chromosomal localisation by in situ hybridisation				
		Bam HI	HindIII	BglII	EcoRI	XbaI		ABL	BCR	SIS	P111	L1424
Ph-neg	4	NT	NT	+	+	+	3'	9 & 22	22 [a]	22	9	9
	5	+	NT	+	+	NT	3'	9 & 22	NT	NT	9 & 22	9 & 22
	7	NT	+	+	+	NT	3'	9 & 22	22 [a]	NT	9	9
Ph-pos		+	+	+	+	NT	3'	NT	NT	NT	9 & 22q-	9 & 22q-

a In Ph-positive CML this probe localises to chromosomes 22, 22q- and 9q+

Within M-BCR the position of the breakpoint has been classified 5' or 3' in relation to the Hind III site immediately downstream of exon 3 of the M-BCR (sometimes designated b3)

on chromosome 22. In situ hybridisation with the probes CRI-P111 and CRI-L1424 showed that in patient 5 both probes localised both to chromosome 9 and to chromosome 22 while in the two other patients they were localised exclusively to chromosome 9. The translocation of ABL to chromosome 22 without the concurrent movement of CRI-P111 and CRI-L1424 from their normal positions in patients 4 and 7 implies that in these patients the 'second' breakpoint must occur upstream of CRI-P111 within a region of approximately $6x10^6$ bp. In contrast the movement of ABL, CRI-P111 and CRI-L1424 sequences to chromosome 22 in patient 5 implies either that all of the genetic material on chromosome 9 distal to ABL is inserted into chromosome 22 or that the second breakpoint has occurred downstream of CRI-L1424. Due to the increase in the frequency of recombination towards the telomeric ends of chromosomes these polymorphic probes may be physically much closer to ABL than is indicated by genetic linkage maps. Nevertheless our results suggest that in these Ph-negative patients the amount of ABL-containing genetic material that is inserted into M-BCR to create a functional BCR-ABL chimeric gene is variable. Similar findings have been demonstrated in DNA from retinoblastoma patients where the deletions of chromosome band 13q14 are of variable size (Howard et al, 1974, Knudson et al, 1976, Wilson et al, 1977). Such variability in the position of the second or 'distal' breakpoint implies that the precise mechanism of insertion must be complex.

In order to elucidate the nature of the DNA sequences that makes insertion of different amounts of DNA possible the next step is to clone the 'distal' breakpoint on chromosome 9q34 in these patients. The

cloning and ordering of these 'distal' breakpoints in different Ph-negative CML patients may identify any 'hotspots' of molecular recombination and define the nature of the sequences which make this translocation event probable.

CHAPTER 5: DO VARIANTS OF THE STANDARD Ph TRANSLOCATION DETERMINE ANY OF THE CLINICAL FEATURES IN CML?

5.1 INTRODUCTION

Variants of the standard Ph translocation have been classified as 'simple' when chromosome 22 and a chromosome other than 9 were involved, or 'complex' when chromosome 9, 22, and one or more additional chromosomes were involved (Sandberg, 1980). However several years ago the validity of this classification was challenged. Some investigators suggested that 'simple' variants were in fact 'complex' translocations that could not be seen microscopically. The breakpoints in 'simple' exchanges involved G band negative terminal bands that were difficult to visualise, whereas 'complex' exchanges involved the positively-staining interstitial chromosomal bands (Heim et al, 1985, Masarati et al, 1986). Hagemeijer et al (1985) among other authors using high resolution R (reverse) banding and T (telomeric) banding subsequently demonstrated that the chromosomal material 9q34 qter was deleted in apparently 'simple' variants and in situ hybridisation to metaphase chromosomes confirmed that ABL was translocated to 22q11 in both 'simple' and 'complex' variants including those resulting in 'masked' Ph translocations (Ishihara et al, 1985, Ohyashiki et al,1987).

A group of investigators reported clinical differences in CML patients with variant Ph translocations. Potter et al (1981) reported that patients with variant Ph translocations had a significantly shorter

survival in chronic phase. However recent evidence based on several hundred cases suggests that the disease does not deviate significantly from cases of CML with a standard Ph chromosome (reviewed by De Braekeleer, 1987). Moreover Southern blotting data including the results from De Klein and Hagemeijer (1984) demonstrated the same type of rearrangement within M-BCR in CML patients with standard and variant Ph translocations.

It seemed probable therefore that the chromosomes involved in variant translocations other than chromosome 9 and 22 were not important in the pathogenesis of CML. In accord with the notion that involvement of other chromosomes in CML with variant translocations is irrelevant, Heim et al (1985) found no obvious correlation between chromosome breakpoints (excluding chromosome 9 and 22) and fragile sites or oncogene locations or consistent breakpoints in other malignancies. However, the results of three studies have contradicted this conclusion (De Braekeleer et al, 1987, Huret et al, 1986, Maserati et al, 1986). These investigators showed that a limited number of chromosome bands were involved more frequently than expected by chance and that these bands were also the locations of proto-oncogenes or fragile sites

Various theories have been put forward to explain the mechanism by which these variant translocations arose. Some have suggested that these translocations were formed by three-or four-way rearrangements, depending on the number of chromosomes participating in each translocation (Sandberg et al,1980; Oshimura et al, 1982; Borgstrom, 1981). According to this theory breaks in all chromosomes involved in

the translocation occurred simultaneously, and the translocation between chromosomes proceeded in one direction and not reciprocally. Others have postulated that complex variants are formed by further chromosomal evolution of the standard Ph translocation (Alimena et al, 1987; Ishihara et al, 1985; Ohyashiki et al, 1987; Pedersen, 1984). Evidence in favour of this hypothesis comes from reports describing a standard Ph chromosome and later a variant Ph translocation in the same patient (Ohyashiki et al, 1987, Ishihara and Minamihisamatsu, 1987). Hagemeijer et al (1985) demonstrated the relocation of the BCR/ABL chimeric gene to another chromosome in one CML patient with a 'complex' translocation implying the Ph translocation preceded the other chromosomal rearrangements.

I investigated four cases of CML with variant Ph translocations, three 'complex' and one 'simple'. I looked for M/BCR rearrangement in these patients by Southern blotting. I performed cytogenetic analysis and in situ hybridisation studies on leukaemic cells from the patient with the 'simple' translocation to find out whether 9q34 and hence the ABL oncogene was involved in the translocation. The objective was to clarify by in situ hybridisation the sequence of chromosomal rearrangements in two 'complex' translocations where the banding pattern after G and R banding analysis was ambiguous. I looked by in situ hybridisation for translocation of the FMS proto-oncogene in one patient and the MYB proto-oncogene in another. These patients respectively had translocations with chromosomal breakpoints located in the general regions of these oncogenes.

5.2 CLINICAL AND HAEMATOLOGICAL FEATURES

The clinical and haematological features at presentation of the four CML patients with variant Ph translocations are presented in Table 16. All four patients had features typical of Ph-positive CML. The differential leucocyte count showed two peaks involving neutrophils and myelocytes. Granulocytes were present at all stages of maturation with no dysplastic features. Three patients were treated with busulphan and hydroxyurea and one patient was treated with -interferon. All four patients achieved complete haematological remission. One patient received an allogeneic bone marrow transplant but relapsed 12 months after transplant.

5.3 M-BCR REARRANGEMENT

Southern analysis of high molecular weight DNA digested with restriction endonucleases Bam HI, Hind III and Eco RI and hybridised with the 0.6kb probe for M-BCR (Fig 8) demonstrated genomic rearrangement in all four patients (Fig 29).

5.4 Patient 1

5.4.1 Cytogenetics

Cytogenetic analysis of 15 G-banded metaphases with poor chromosome morphology demonstrated a deletion of chromosome 4p and a 9q+ chromosome but normal chromosomes 22 (46,XX, 4p-, 9q+). This patient was therefore

Table 16 Clinical and haematological details in four CML patients with variant translocations

Patient	Age/ sex	Spleen (cm)	Peripheral blood at diagnosis												Treatment & response	Survival (months)
			Hb (g/dl)	WBC ($\times 10^9$/l)	Plat's	Differential (% of 300 cells)								NAP		
						Bl	Pm	My & Met	Neut	Eos	Baso	Mono	Lymph	NRBCs		
1	54/F	3	7.0	163	545	2	0	22	58	2	9	1	1	0.3	0	BU,HU,CR	51
2	18/F	5	11.8	148	426	0	0	10	68	0	2	4	5	0	0	HU,CR,BMT	13
3	52/F	0	14.8	7.3	459	3	2	26	10	33	3	1	1	16	0	HU,CR	36
4	28/M	6	12.0	152	348	0	3	30	46	5	2	2	4	0	0	HU,BU,CR	42

HU = hydroxyurea BU = busulphan CR = complete haematological response

NRBCs = nucleated red blood cells NAP = neutrophil alkaline phosphatase

BMT = bone marrow transplantation

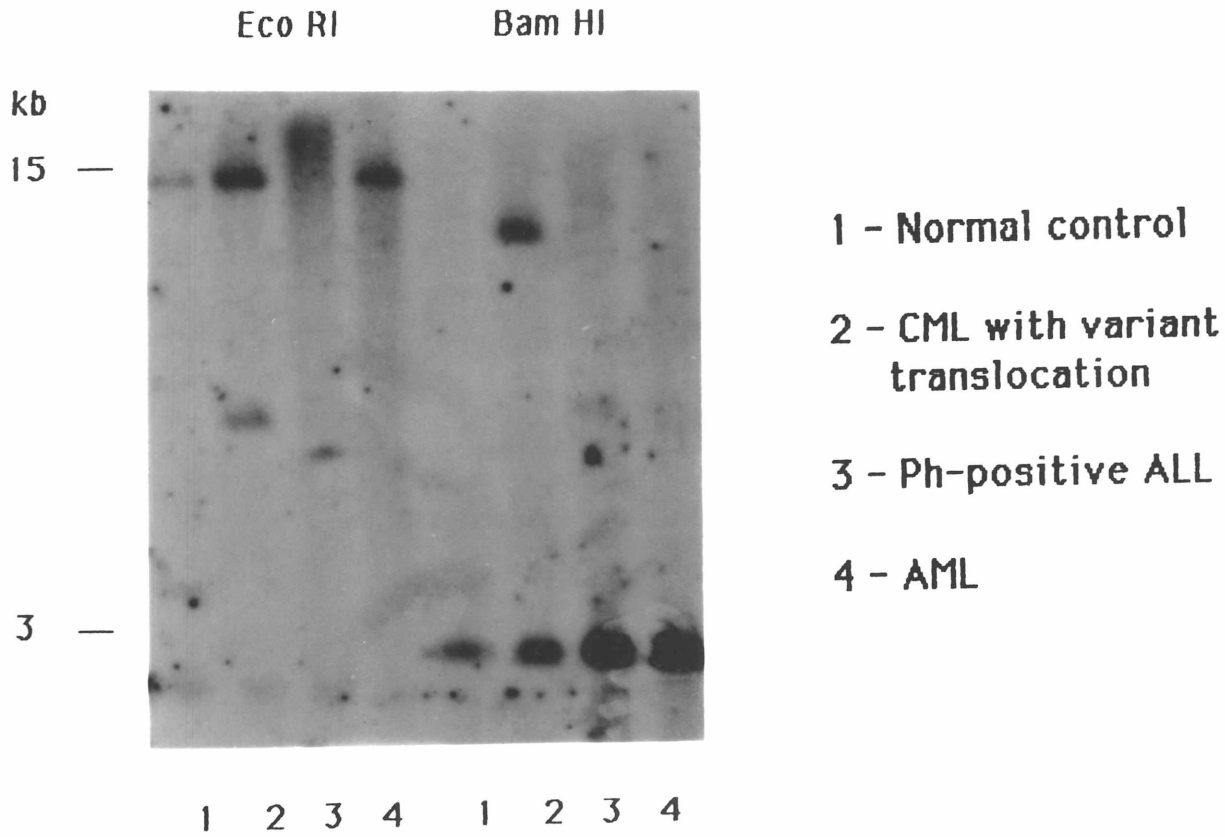


Fig 29 **Southern blot analysis of DNA from CML patient with variant translocation t(4;9;22) showing genomic rearrangement of M-BCR.**

High molecular weight DNA was restriction endonuclease digested with Eco RI and Bam HI and the Southern blot probed with the 0.6 kb M-BCR probe, BCR-G.
The DNA from CML variant patient 1, lane 2, shows a band in the germline configuration of M-BCR and a rearranged band using both restriction endonucleases.
Other negative controls include patients with Ph-positive acute lymphoblastic leukaemia (ALL) and acute myeloid leukaemia (AML).

categorised as Ph-negative. However, cytogenetic analysis of 30 G-banded metaphases from BUdr synchronised cultures of cryopreserved peripheral blood demonstrated a complex translocation involving chromosomes 4, 9, and 22 resulting in a 'masked' Ph chromosome. Material from chromosome 22 (22q11 22qter) had been translocated to chromosome 9 to give a standard 9q+ chromosome and chromosome 4p11 4pter had been translocated to chromosome 22, t(4;9;22) (9pter 9q34::22q11 22qter;22pter 22q11::4p11 4pter;4qter 4p11:) (Fig 30). A translocation of chromosome material from 4p to 22q was evident but I were not certain whether there had been a reciprocal movement of material from chromosome 22q to chromosome 4p.

5.5 **Patient 2**

5.5.1 Cytogenetics

Cytogenetic analysis of 15 G-banded metaphases from bone marrow after 48 hours of culture demonstrated a 'simple' variant translocation involving chromosome 5, 17 and 22 with breakpoints at bands q35, q21 and q11 respectively. Analysis of poorly banded chromosomes suggested that a translocation had occurred between chromosome 5 and 17 (5pter 5q35::17q21 17qter;17pter 17q21::5q35 5qter) and chromosomal material was deleted from 22q [der(22) (22pter 22q11:)]. However, analysis of G-banded mitoses derived from synchronised cultures demonstrated an abnormality of chromosome 9 at band q34 and a discrepancy in the amount of chromosomal material translocated from chromosome 17q to chromosome 5. There was a slight increase in the G-

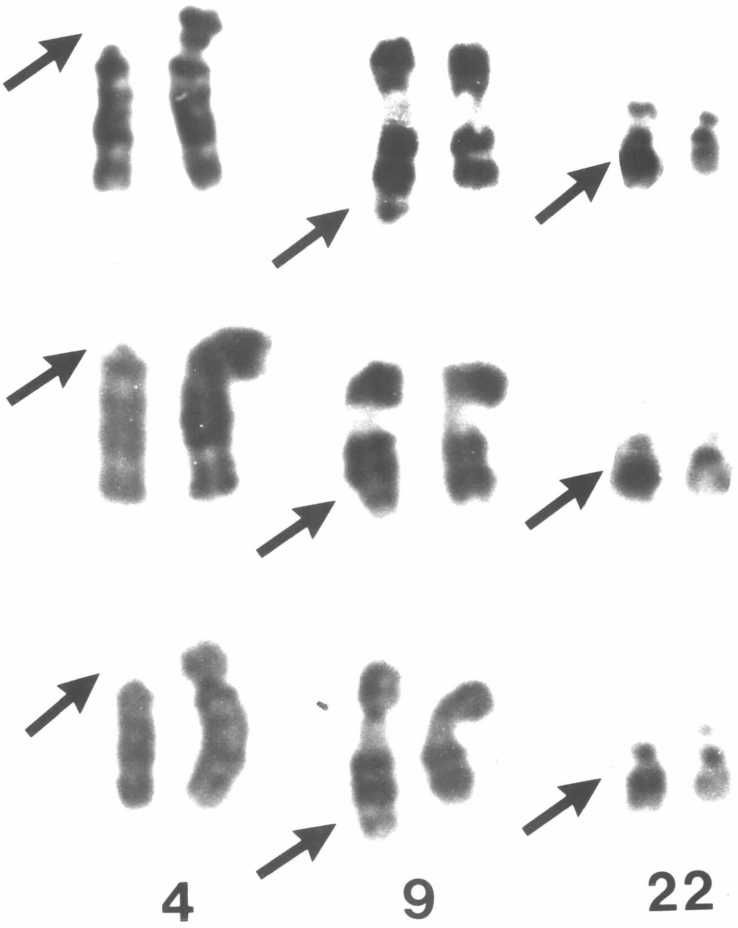


Fig 30 **Partial karyotypes of three G-banded metaphases from a CML patient with the 'complex' variant translocation t(4;9;22)(p11;q34;q11)**

Chromosomal material has been reciprocally translocated between chromosomes 9 and 22 resulting in chromosome 9q+. Material from the short arm (p) of chromosome 4 has been translocated to the chromosome 22q-, thereby 'masking' the Ph chromosome.

band negative chromosomal material at the terminal band of chromosome 9 (q34) and chromosome 5q which resembled the material from chromosome 22q (22q11 22qter) (Fig 31). However, the increase in the G-band negative material on either 9q34 or 5q did not account for the material deleted from chromosome 22. Therefore the interpretation of chromosome rearrangements from cytogenetic analysis was ambiguous.

5.5.2 In situ hybridisation

To confirm that the minor abnormality in chromosome 9 involved the translocation of ABL sequences to chromosome 22 I localised the ABL oncogene in the metaphases of this patient. I found that in 65 metaphases analysed with a total number of 101 grains, 13 (12.5%) grains hybridised to chromosome 9 and 11 (10.8%) grains hybridised to the 22q- (Table 17). The hybridisation of grains to chromosomes 9 and 22q- was significant ($p < 0.0003$ and $p < 0.0001$ respectively). The ABL proto-oncogene had been translocated to chromosome 22q-. The result of ABL localisation allowed me to interpret the sequence of chromosomal rearrangements in this patient. A schematic representation of the sequence of chromosomal rearrangments is shown in Fig 32 as t(5;9;17;22) (9pter 9q34::22q11 22qter; 22pter 22q11::9q34 9qter; 17pter 17qter::9q34 9qter; 5pter 5qter::17q21 17qter).

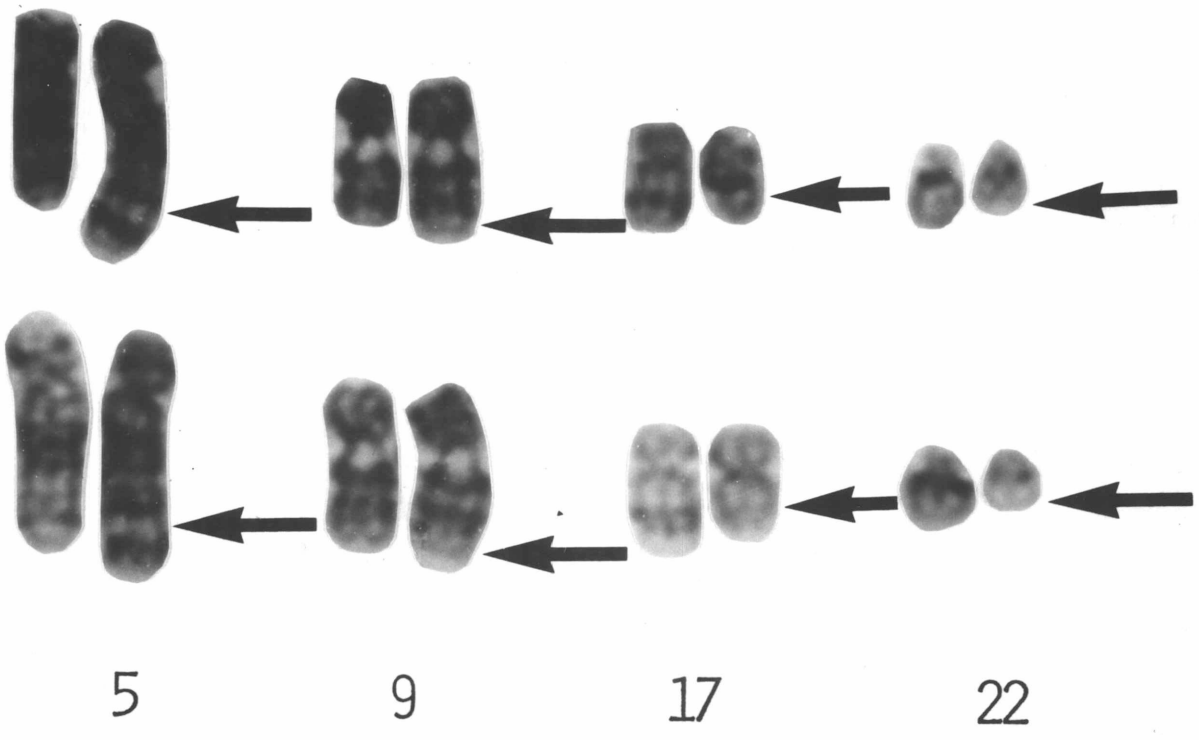
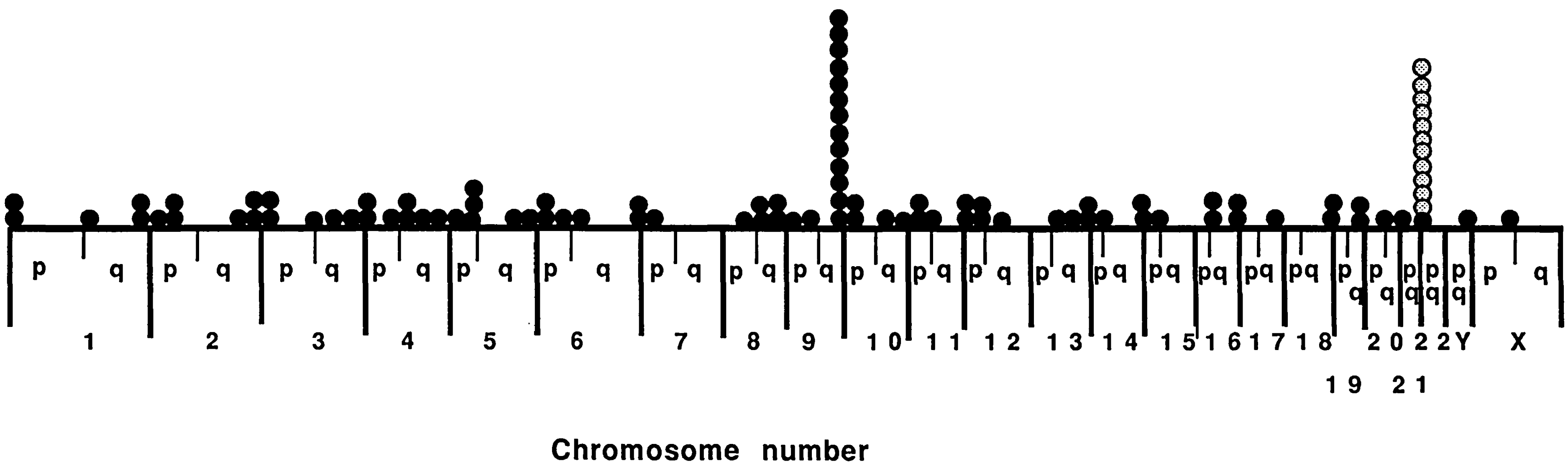


Fig 31 Partial karyotype of two G-banded metaphases from CML patient with 'simple' variant translocation t(5;17;22)(q35;q21;q11).

A translocation occurred between chromosomes 5, 17 and 22 with breakpoints, indicated by arrows, at q35, q21 and q11 respectively. The chromosomes 9, however appear to be morphologically normal.

Table 17 **Localisation of the ABL proto-oncogene in metaphases from a CML patient with variant translocation t(5;17;22)(q35;q21;q11)**

The grain localisation of the ABL proto-oncogene to chromosomes 9q34 and 22q- in 50 bone marrow metaphases by in situ hybridisation. The stippled circles indicate hybridisation to the chromosome 22q-.



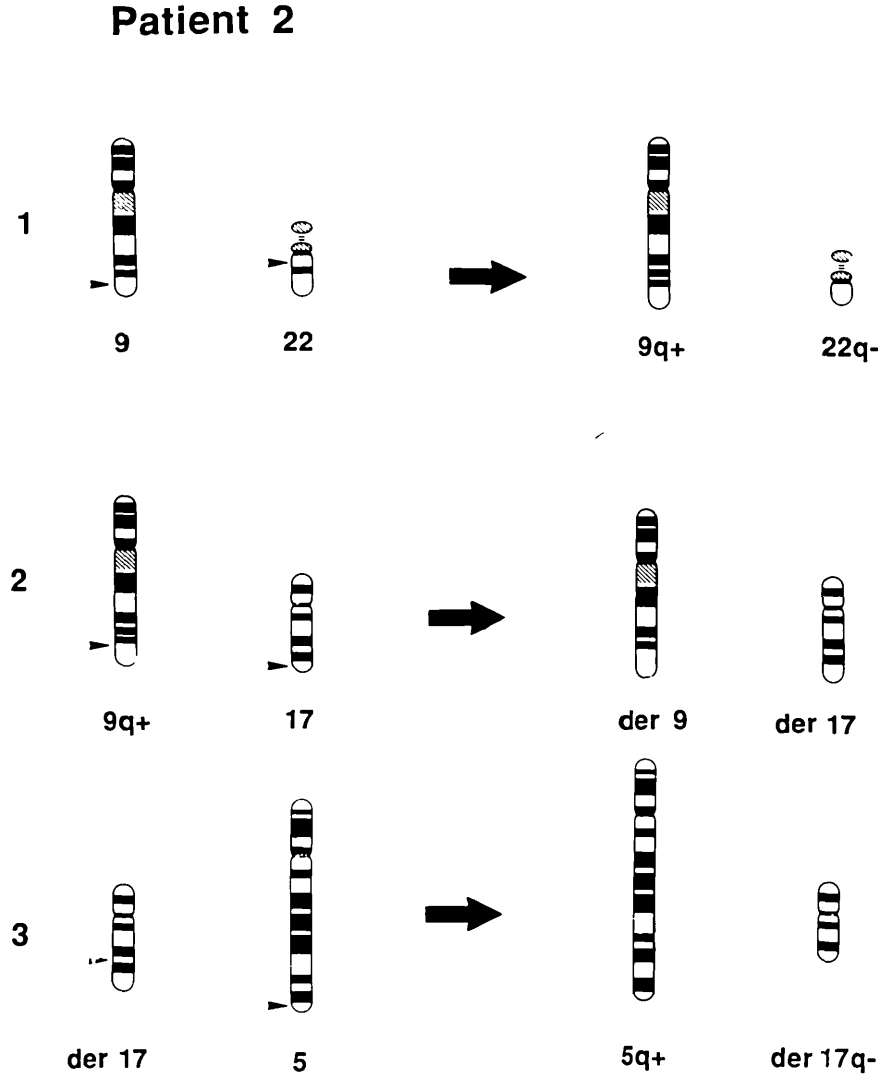


Fig 32 **Proposed sequence of events leading to variant translocation t(5;9;17;22)(q35;q34;17q21;22q11) in CML patient.**

Stage 1 - Ph translocation
Stage 2 - translocation between chromosomes 9q+ and 17
Stage 3 - translocation between chromosomes der(17) and 5
The arrowheads indicate the chromosome breakpoints.

5.6 **Patient 3**

5.6.1 Cytogenetics

Cytogenetic analysis of 10 G-banded metaphases derived from bone marrow after 24 hours of culture demonstrated a 'complex' translocation involving chromosomes 6, 9, and 22 with breakpoints at bands q23, q34 and q11 respectively, t(6;9;22) (q23;q34;q11) (Fig 33). The G-band negative chromosomal material 22q11 22qter seemed to be present on chromosome 6q rather than chromosome 9q as would be expected with a standard Ph translocation. Therefore an initial interpretation of cytogenetic data was as follows: A 'break and join' event involving chromosomes 6q and 9q (9pter 9q34::6q23 6qter) occurred and then a 'break and join' event occurred between chromosome 6q and 22q (6pter 6q24::22q11 22qter). I was unable by cytogenetic analysis to establish whether a translocation between chromosome 9 and 22 had previously occurred.

5.6.2 In situ hybridisation

To establish whether a translocation had occurred between chromosome 9 and 22 and hence whether the ABL oncogene had moved to chromosome 22 I localised ABL in the metaphase chromosomes of this patient. In 50 metaphases analysed with a total number of 88 grains, 9 (10.2%) grains hybridised to the normal chromosome 9, 7 (7.9%) grains hybridised to the Ph chromosome and no grains hybridised to the 9q+ or the 6q+ derivative

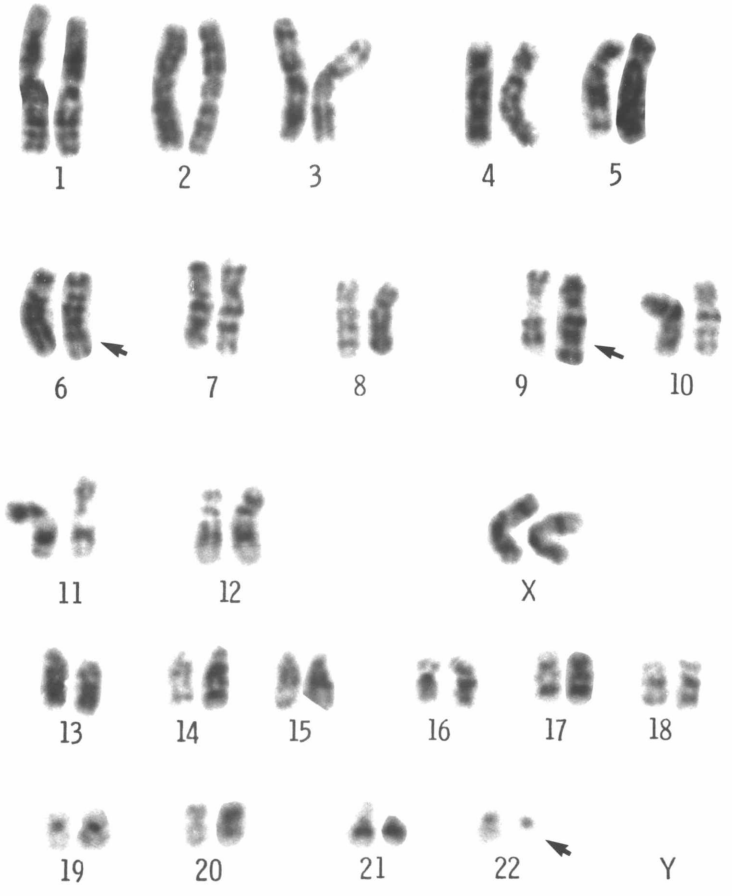


Fig 33 **Karyotype of a G-banded metaphase from CML patient with 'complex' translocation t(6;9;22)(q23;q34;q11)**

A translocation in this patient occurred between chromosome 6, 9 and 22 with breakpoints at q23, q34 and q11 respectively.

chromosomes (Table 18). The hybridisation of grains to chromosomes 9 and the 22q- was significant (p = 0.008 and p = 0.0001). Therefore the 'complex' translocation in this patient was interpreted as: t(6;9;22) (9pter 9q34::22q11 22qter) (22pter 22q11::9q34 9qter) (der[9pter] der[9q34]::6q24 6qter) (6pter 6q24::der[9q34] der[9qter]) A schematic diagram of the proposed changes is shown in Fig 34.

5.7 **Patient 4**

5.7.1 Cytogenetics

Cytogenetic analysis of 30 metaphases from synchronised cultures R-banded with acridine orange demonstrated a 'complex' translocation involving chromsomes 6, 9, 9, 10 and 22 at breakpoints q24, q34, p11, p15, q11 respectively, t(6;9;9;10;22) (q24; q34;p11;p15;q11) (Fig 35). R-banding from synchronised cultures did not permit a definite interpretation of the sequence of chromosomal exchanges which gave rise to this 'complex' translocation. For example, I could not determine with certainty to which chromosome the material 22q11 22qter was translocated.

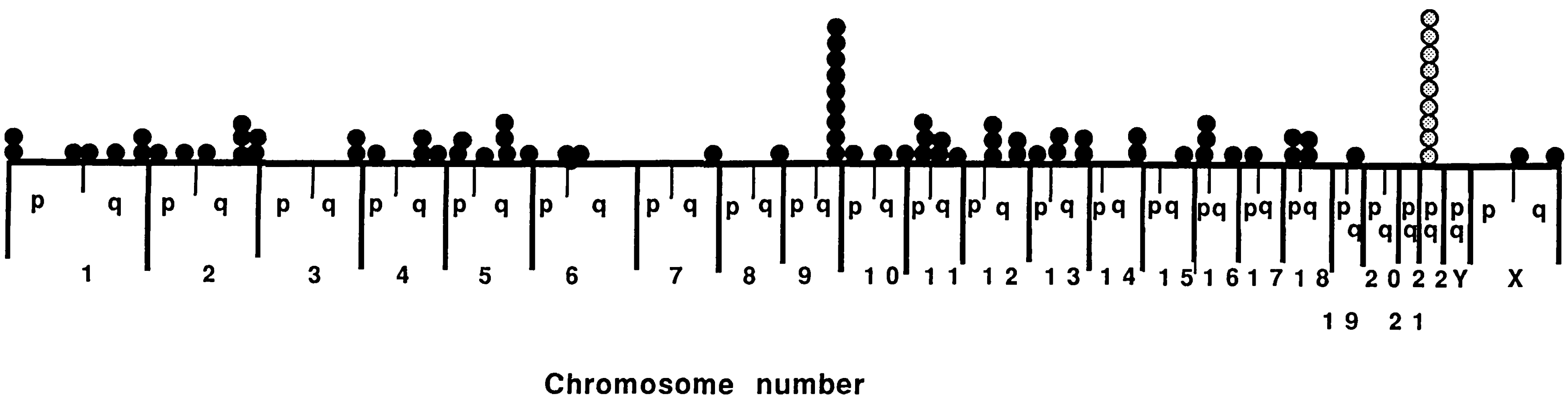
5.7.2 In situ hybridisation

5.7.2.1 M-BCR localisation

I demonstrated rearrangement within M-BCR in the DNA of patient 4 by Southern blotting (as in Ph-positive CML) but was uncertain of the

Table 18 Localisation of the ABL proto-oncogene in metaphases from a CML patient with variant translocation t(6;9;22)(q23;q34;q11).

The grain localisation of the ABL proto-oncogene to chromosomes 9 and 22q- in 50 bone marrow metaphases by in situ hybridisation. The stippled circles indicate hybridisation to chromosome 22q-.



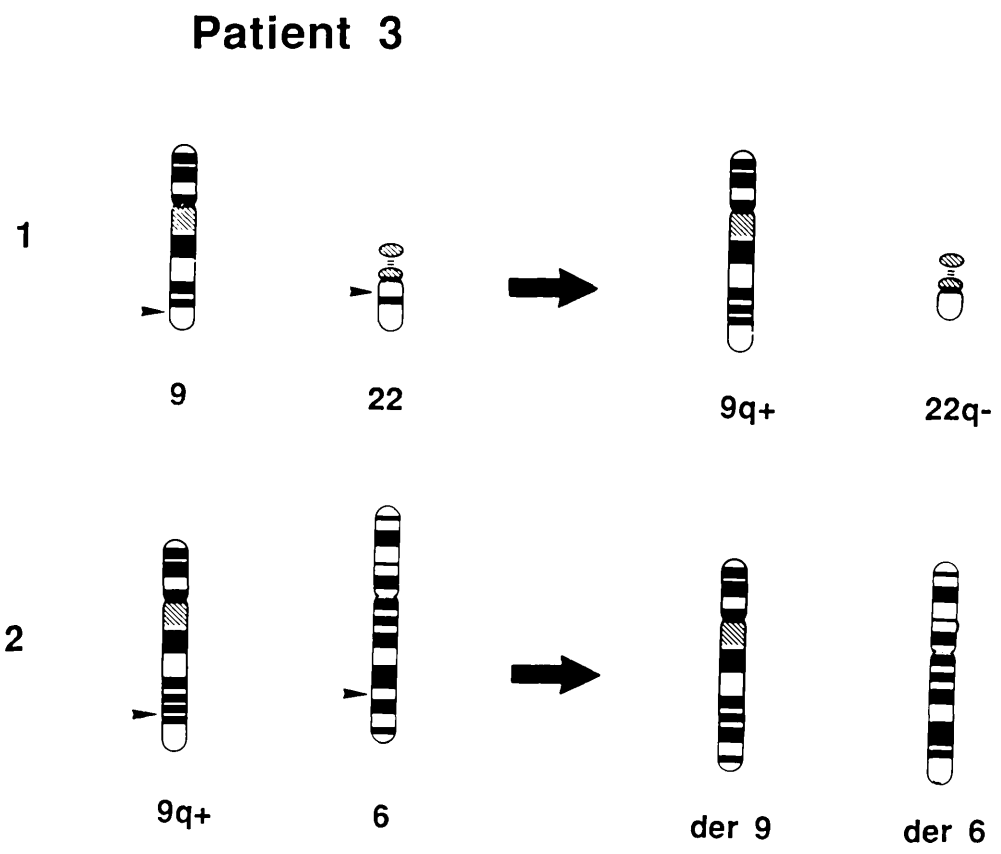


Fig 34 Proposed sequence of events in CML patient leading to variant translocation, t(6;9;22) (q23;q34;q11)

Stage 1 - Ph translocation
Stage 2 - translocation between chromosomes 6 and 9q+
The arrowheads indicate chromosome breakpoints.

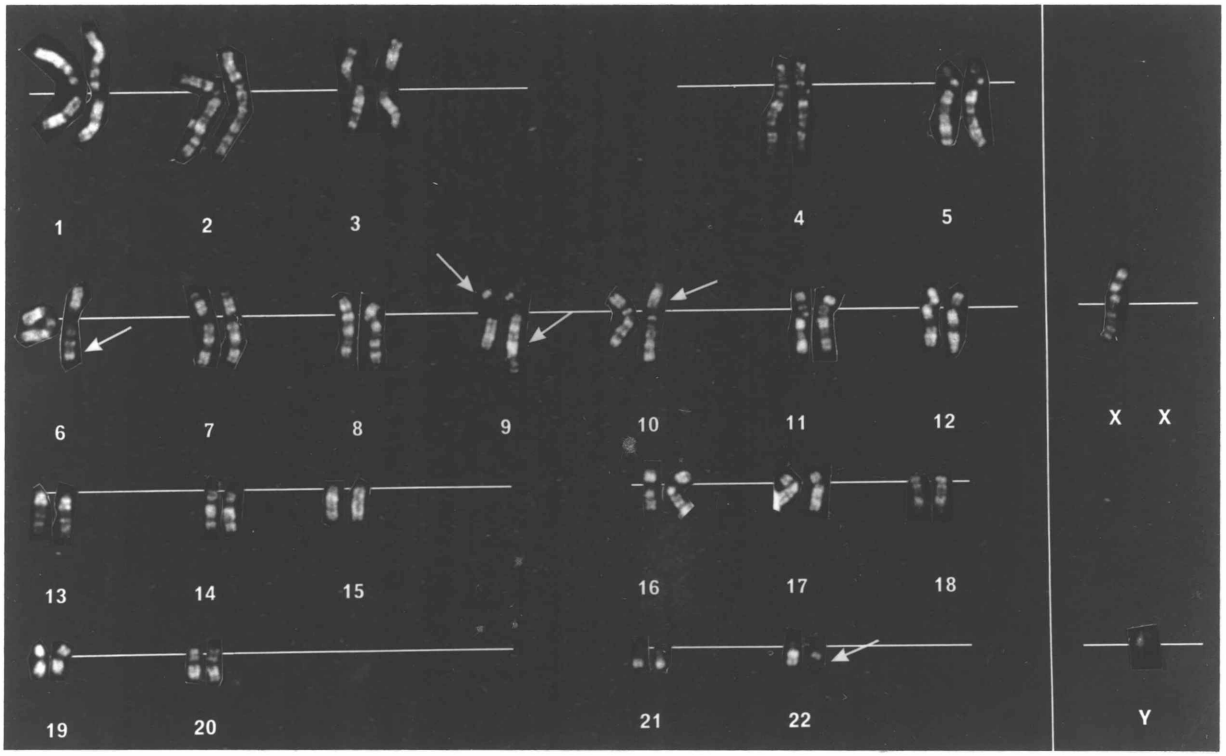


Fig 35 **R-banded karyotype of a metaphase from CML patient with 'complex' variant translocation t(6,9,9,10,22)(q24,q34,p11,p15,q11)**

A translocation occurred between chromosomes 6, 9, 9,10 and 22 with breakpoints q24, q34, p11, p15, q11 respectively.

chromosomal location of 22q11 22qter by conventional R-banding analysis. In the classical Ph translocation the chromosome material 22q11 22qter contains part of the BCR gene (3' of the breakpoint in M-BCR) and is normally translocated to the chromosome 9q+. I therefore localised the BCR gene in the metaphase chromosomes of our patient with a 4 kb cDNA Eco RI probe (see chapter 2 for details). In 50 metaphases analysed with a total number of 115 grains, 12 (10.4%) grains hybridised to chromosome 22, 14 (12.1%) grains hybridised to the derivative chromosome 9 and 11 (9.5%) grains hybridised to the Ph chromosome. Hybridisation of grains to chromosomes 9q+, 22 and 22q- was significant ($p < 0.0001$, $p < 0.0002$ and $p < 0.0002$ respectively).

5.7.2.2 SIS localisation

The SIS oncogene is normally located on band q12-q13 of chromosome 22. In the formation of the classical Ph chromosome it is translocated to the chromosome 9q+ together with 3' BCR sequences. I localised 3' BCR on chromosome 9q+ in patient 4 (see above) but was uncertain whether DNA sequences located in another band (q12-q13) of chromosome 22, probably several megabases away from the BCR gene, were also on the chromosome 9q+. I therefore localised the SIS gene in the metaphase chromosomes of patient 4 by in situ hybridisation with the 2.7 kb Bst EII cDNA SIS probe. In 50 metaphases analysed with a total grain count of 128, 14 (10.9%) grains hybridised to chromosome 22, 11 (8.5%) grains hybridised to chromosome 10p and 3 (2.3%) grains were found on chromosome 9q+. The hybridisation of grains to the normal chromosome 22 and chromosome 10p were significant ($p < 0.0001$ and $p = 0.018$ respectively) but the

hybridisation of grains to the chromosome 9q+ was not. A composite diagram of BCR and SIS localisation is shown in Fig 36.

5.7.3 Interpretation of the 'complex' translocation

The results of localisation of the BCR and SIS genes enabled me to interpret the chromosomal rearrangements that occurred in this 'complex' translocation. The proposed sequence of chromosomal rearrangements, shown schematically in Fig 37, is as follows: A translocation initially occurred between chromosome 9 and 22 (9pter 9q34::22q11 22qter; 22pter 22q11::9q34 9qter), and a further translocation occurred between the derivative chromosome 9 [der(9)] and chromosome 10p (10qter 10p15::9q34 9qter). R-banding, however, showed that the material on chromosome 10p was derived from chromosome 6q, so a subsequent translocation must have occurred between the derived chromosome 10p [der(10p)] and 6q, (10qter 10p15::6q24 6qter). Finally a translocation probably occurred between chromosome 9p and the der(9), (9pter 9q34::9p11 9pter).

5.8 **Localisation of other proto-oncogenes in variant Ph translocations**

5.8.1 Localisation of the FMS proto-oncogene in patient 2

The breakpoint on chromosome 5 band q34 in the complex translocation t(5;9;17;22) occurred in the region of the FMS proto-oncogene. FMS, the

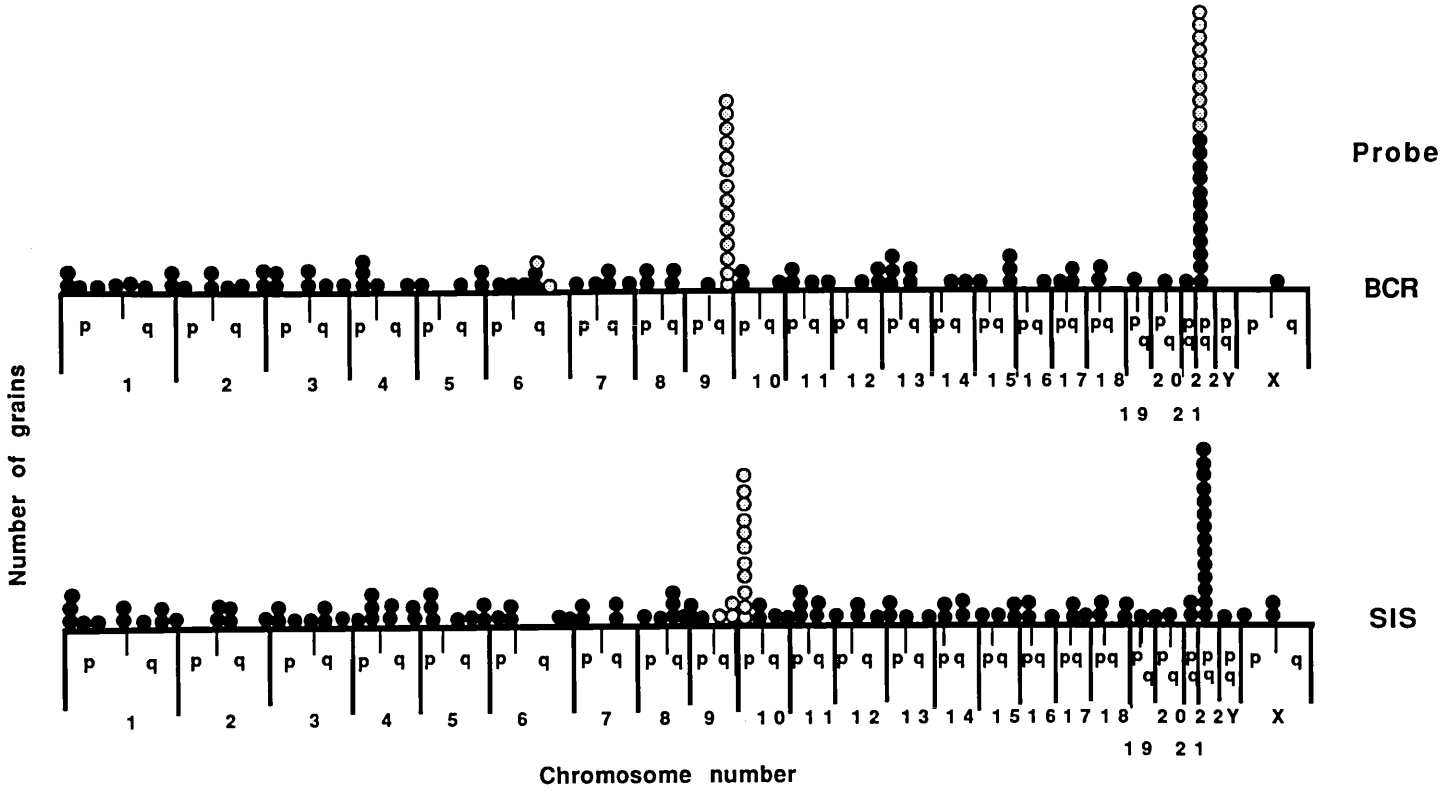


Fig 36 Composite diagram showing grain distribution in 50 metaphases for each of the gene probes: BCR and SIS in CML patient with variant translocation t(6;9,9;10;22).

The x axes represent the chromosomes in their proportion; the y axes show the number of grains. There was localisation of BCR to chromosomes 22q11 and the 22q-. The SIS proto-oncogene localised to chromosomes 22q13 and 10p. The stippled circles indicate hybridisation to abnormal chromosomes 22q- and der (9) using the BCR probe, and der (10) using the SIS probe.

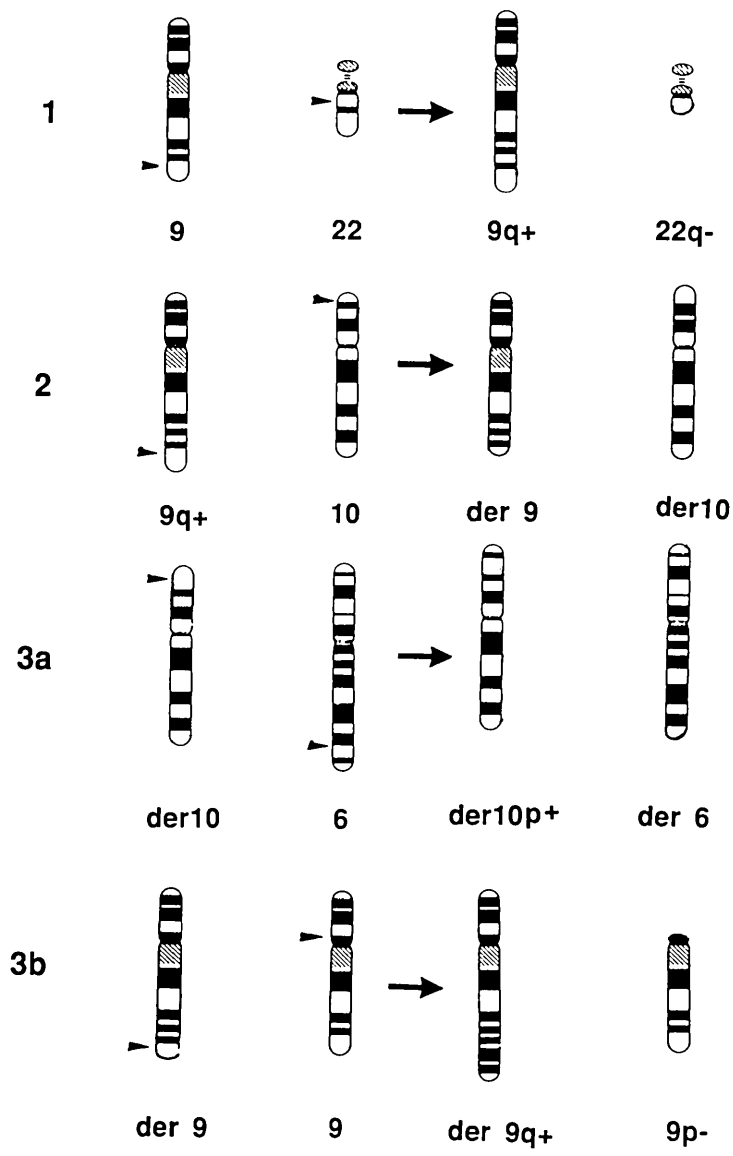


Fig 37 **Proposed sequence of events in CMLleading to the variant translocation, t(6;9;9;10;22)(q24;q34;p11;p15;q11) in a CML patient**

Stage 1 - Ph translocation
Stage 2 - translocation between chromosomes 9q+ and 10
Stage 3a - translocation between chromosomes der(10) and 6
Stage 3b - translocation between chromosomes der(9) and normal 9.
The arrowheads indicate chromosome breakpoints.

human homologue of the transforming gene of the feline sarcoma virus, is normally localised to chromosome 5q34 (Groffen et al, 1983). I localised the FMS oncogene in the metaphases from patient 2. I wondered whether a chromosomal break at 5q34 involved the movement of FMS to another chromosomal site and hence its possible activation.
For localisation of the FMS oncogene in the metaphases of patient 2 I used a 3 kb cDNA Eco RI/Bgl II viral fms fragment (v-fms).
In 45 metaphases analysed with a total number of 101 grains, 10 (10%) grains hybridised to the normal chromosome 5 and 12 (11.8%) grains hybridised to the abnormal chromosome 5 (Table 19). The clustering of grains on chromosomes 5 and 5q+ was significant ($p = 0.0004$ and $p = 0.059$ respectively). There was no other significant clustering of grains on any of the other metaphase chromosomes.

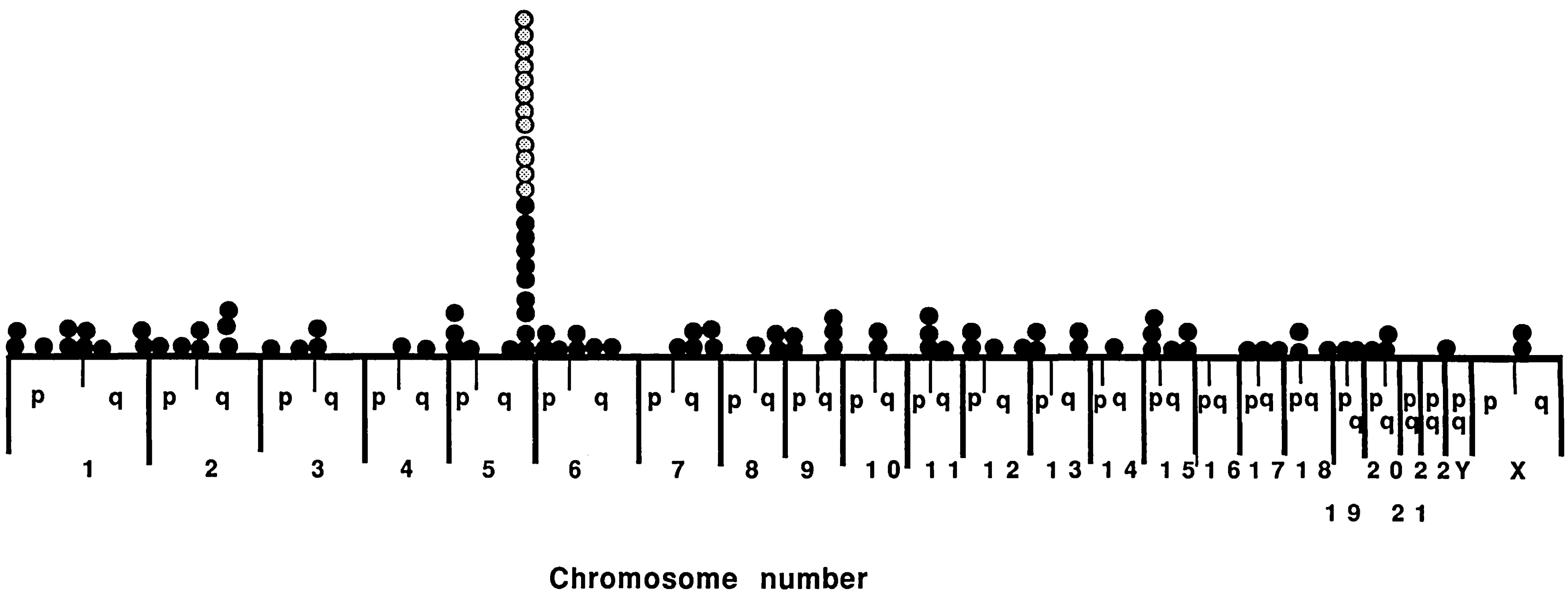
5.8.2 Localisation of the MYB proto-oncogene in patient 3

The breakpoint on chromosome 6 band q23 in the complex translocation t(6;9;22) occurred in the region of the cellular oncogene MYB (Harper et al, 1983). MYB, the normal human homologue of the transforming gene of the avian myeloblastosis virus, is localised to 6q22-q25. I localised the MYB oncogene in metaphases obtained from patient 2 to find out whether the chromosomal break at chromosome 6q23 involved the movement of MYB to another chromosomal site.

For localisation of the MYB oncogene in the metaphases of patient 3 I used a 2 kb cDNA Kpn I/ Xba I viral myb fragment (viral) as a probe. In 50 metaphases analysed with a total number of 120 grains, 12 (10%)

Table 19 **Localisation of the FMS proto-oncogene in metaphases from a CML patient with variant translocation t(5;17;22)(q34;21;q11)**

The grain localisation of the FMS proto-oncogene to chromosome 5q34 exclusively in 45 metaphases by in situ hybridisation. The stippled circles indicate hybridisation to the abnormal chromosome 5.



grains hybridised to the normal chromosome 6 and 11 (9.1%) grains to the abnormal chromosome 6q+. The clustering of grains on chromosomes 6 and der(6) was significant ($p = 0.029$ and $p = 0.059$). There was no other significant clustering of grains on any of the other chromosomes (Table 20).

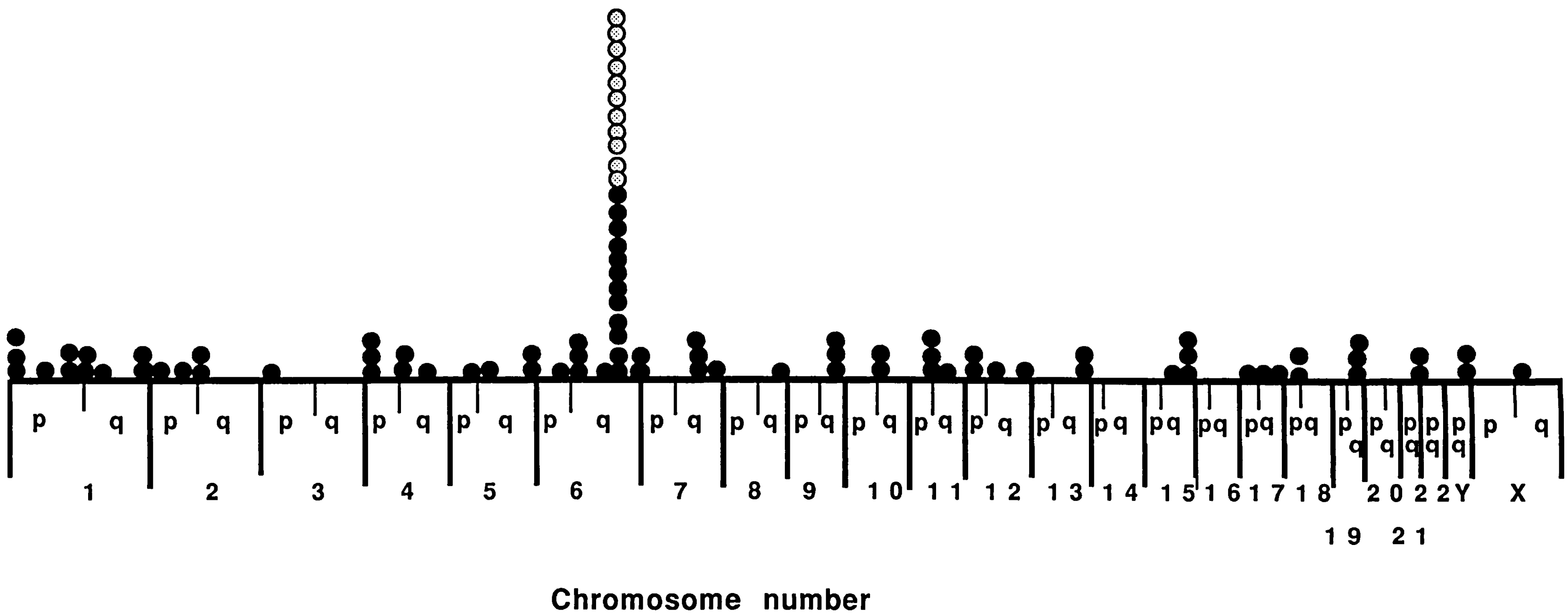
5.9 DISCUSSION

The clinical and haematological features of all four CML patients with variant Ph translocations were indistinguishable from CML with a classical Ph chromosome. Analysis of M-BCR by Southern blotting demonstrated rearrangements in all four patients.

The analysis of chromosomes from BrdU-synchronised cultures by cytogenetics and in situ hybridisation helped to interpret the complex pattern of chromosomal rearrangements that may occur in 'complex' Ph translocations. Localisation of the ABL oncogene in the metaphases of patient 2 showed that ABL sequences had been translocated to chromosome 22q-. This demonstrated that the apparently 'simple' translocation t(5;17;22) was in fact a 'complex' translocation involving the four chromosomes 5, 9, 17,and 22 [t(5;9;17;22)]. This confirmed the original report of Hagemeijer et al (1984) that 'simple' variants always involved the chromosomal region 9q34. Cytogenetic analysis of chromosomes from patient 3 suggested that the chromosomal rearrangements occurred in one direction and not reciprocally. It appeared that a translocation had occurred between chromosome 6q and 9q and subsequently between chromosome 6q and 22q. These cytogenetic findings seemed to

Table 20 Localisation of the MYB proto-oncogene in metaphases from a CML patient with variant translocation t(6;9;22)(q23;q34;q11)

The grain localisation of the MYB proto-oncogene to chromosome 6q23 exclusively in 60 metaphases by in situ hybridisation. The stippled circles indicate hybridisation to the abnormal chromosome 6.



corroborate reports of Sandberg et al (1980) and others that a reciprocal translocation between chromosome 9 and 22 was not necessary in the formation of 'complex translocations'. However the localisation ABL sequences on chromosome 22q- and the findings of M-BCR rearrangement suggested that a Ph translocation had occurred.

The localisation of the BCR gene and the SIS oncogene in the metaphases of patient 4 enabled me to interpret this complex pattern of chromosomal rearrangements. BCR gene sequences were localised to chromosome 9q+ and SIS oncogene sequences were localised to the p arm of chromosome 10. These results suggested that a classical Ph chromosome translocation had occurred resulting in the movement of the chromosome segment 22q11 22qter to the q arm of chromosome 9, followed by several other chromosome rearrangements. Ohyashiki et al (1987) also localised 3' BCR in the chromosomes of CML patients with complex Ph translocations. In one patient 3' BCR sequences were found on the chromosome 9q+ (as in patient 4). In a second patient these sequences were located on chromosome 9q22. A most plausible interpretation for this finding was an interstitial deletion of the region 9q22 9q34 prior to the translocation event between chromosome 9 and 22. In a third patient 3' BCR sequences were found on 22q11 and on chromosome 12p11 (a breakpoint in this complex translocation) suggesting that a Ph translocation had not occurred.

Studies of variant translocations of CML by in situ hybridisation suggest a formation by at least three routes. In the majority of cases there is a formation of a standard Ph translocation prior to other

chromosomal rearrangements; in other cases the Ph translocation occurs after one or more other chromosome changes and in still other cases there is no conventional Ph translocation. Furthermore in situ hybridisation studies of Hagemeijer et al (1987) suggest that a common feature of all Ph translocations (standard and 'complex') is the juxtaposition of ABL sequences with 5' BCR sequences. Therefore it seems that in 'complex' Ph translocations, as in standard Ph translocations, there is the formation of the chimeric BCR-ABL gene. This chimeric gene is formed mainly by means of a reciprocal translocation event but also by other mechanisms.

The phenomenon of the other cytogenetic changes in variant translocations has been of interest to investigators of CML. The significance of these chromosome alterations, given that these patients all had the same clinical and haematological features of CML patients with a standard Ph chromosome, has been doubted. Although a recent report by de Braekeleer (1987) demonstrated a non-random association between the breakpoints in variant translocations and the positions of oncogenes, fragile sites or consistent breakpoints in other malignancies, molecular analysis of these breakpoints has not been done.

The breakpoints 5q35 in patient 2 and 6q23 in patient 3 defined the positions of the cellular oncogenes FMS and MYB respectively. We localised these oncogenes in the metaphases of our patients and found that in patient 2 FMS remained in its normal position on chromosome 5 and in patient 3 MYB remained in its normal position on chromosome 6. These findings suggest two possible explanations. One possibility is

that in these patients the activity of the MYB and FMS oncogenes may be altered by a means other than its translocation into a transcriptionally active area of the genome, for example by point mutations. A perturbation of these oncogenes may be involved in some minor aspect of the disease process which is not immediately apparent. The more probable explanation is that these chromosomal breakpoints bear no direct relationship to disease features in CML patients with variant translocations. Sites within the genome, which by their structural nature are 'fragile', may predispose the cell to genetic rearrangement. These rearrangements may be 'productive' i.e. lead to an alteration of expression of genes at these breakpoints, or 'non-productive'. Yunis et al (1984) and Le Beau and Rowley (1984b) recognised the association of fragile sites and chromosome breakpoints in cancer. Several oncogenes have been mapped to chromosome sites which are also heritable fragile sites. For example the proto-oncogene ETS has been mapped to chromosome 11q23 which is the region of the breakpoint in t(9;11) in acute myelomonocytic leukemia (AMoL) and in the t(4;11) in acute lymphoblastic leukemia (ALL), as well as the position of a heritable fragile site (de Taisne et al, 1984). It can also be imagined that not all genetic rearrangements at sites of chromosomal fragility lead to an alteration of these oncogenes. It may be that in the 'complex' Ph translocations of CML the chromosome rearrangements other than those leading to the formation of the chimeric BCR/ABL gene are 'non-productive'.

The chromosome breakpoint 5q35 in patient 2, the position of the FMS oncogene, is (also) a fragile site (Seventh International Workshop on Chromosomes in Leukaemia, 1984). The chromosome breakpoint 6q23 in

patient 3, the position of the MYB oncogene, is not a fragile site and neither are the breakpoints in the other 'complex' translocations of patient 1 and 4. However several investigators have suggested that the actual number of fragile sites in the human genome may be much higher than reported. Glover et al (1984) observed breaks at 25 sites induced by aphidicolin, Yunis and Soreng (1984b) described fragility at 51 sites in cells deprived of thymidine and exposed to caffeine, and Barbi et al (1984) noted 11 folic-acid sensitive sites in cells exposed to methotrexate. However the localisation of these new fragile sites as well as the genetic analysis of the breakpoints in variant Ph translocation is necessary to understand the significance of these chromosomal rearrangements.

Nevertheless our findings are in line with the molecular analyses found in the standard Ph translocation and emphasise the fact that the juxtaposition of ABL sequences with 5' BCR sequences, a result of the Ph translocation in the majority of CMLs with a variant translocations, may be obscured by more complicated cytogenetic events. Other changes, though they may have a significance of their own, are of a somewhat secondary nature and do not participate in the basic causation of the leukaemia.

CHAPTER 6: FURTHER CYTOGENETIC AND GENETIC CHANGES IN BLAST CRISIS CML

6.1 BACKGROUND

The chronic phase of CML is characterised by the presence of the Ph chromosome in dividing cells of the myeloid lineage. The molecular basis of this marker chromosome has been studied extensively in recent years (see chapter 1). The relatively 'benign' chronic phase lasts typically for about 2 to 6 years but sooner or later evolves to an acute (and usually rapidly fatal) phase of blastic transformation, which may be predominantly myeloid or lymphoblastic. This advanced phase of disease is heralded or accompanied in 80% of cases by characteristic additional chromosomal changes: +Ph, +8, i(17q), and +19. Recently, attempts have been made to correlate the chromosomal abnormalities in blast crisis with the morphological, immunological, and cytochemical features of the emerging blasts. The number of cases investigated is still small, but the results of three studies indicate that the pattern of additional abnormalities correlates with the predominant lineage involved in the transformation process (O'Malley and Garson, 1985, Yoa, 1985, Parreira et al, 1986). In general, all the typical secondary aberrations of +8, i(17q), +19, and +Ph may be found in blast crises with myeloid differentiation. With the exception of an extra Ph chromosome so new abnormalities are found less frequently in lymphoid blast crisis. Although these secondary karyotypic changes have been extensively reviewed and have been of some practical value as diagnostic and prognostic indicators of transformation of CML, their origin and

molecular significance are still poorly understood.

The elucidation of the molecular changes in chronic phase and observations of the course of disease provide a basis for a hypothetical model of CML in which a number of steps (presumably synonymous with genetic changes) occur. The first genetic change might result in the clonal growth of myeloid stem cells without a proliferative advantage, a situation that would be clinically inapparent in most instances. The next step is a Ph translocation, resulting in the formation of the BCR/ABL chimeric gene; this would lead to expansion of the myeloid stem cell compartment and to clinically apparent chronic phase disease. Further genetic change(s) then result in loss of differentiation, maturation and progression to the acute phase of CML. It is possible that these genetic changes result in increased expression of BCR/ABL (which in the chronic phase is expressed at similar levels to that of normal genes) or to activation of other oncogenes leading to their inappropriate expression. In general oncogene activation may result from one of three types of genetic changes (Fig 38):

(a) Chromosomal translocations may place proto-oncogenes into transcriptionally active regions of DNA or create fused genes which transcribe and translate fused gene products (as in BCR/ABL gene formation).

(b) Oncogene amplification may result in increased amounts of oncogene-specific RNA and protein.

(c) Point mutations in oncogenes can lead to altered expression or formation.

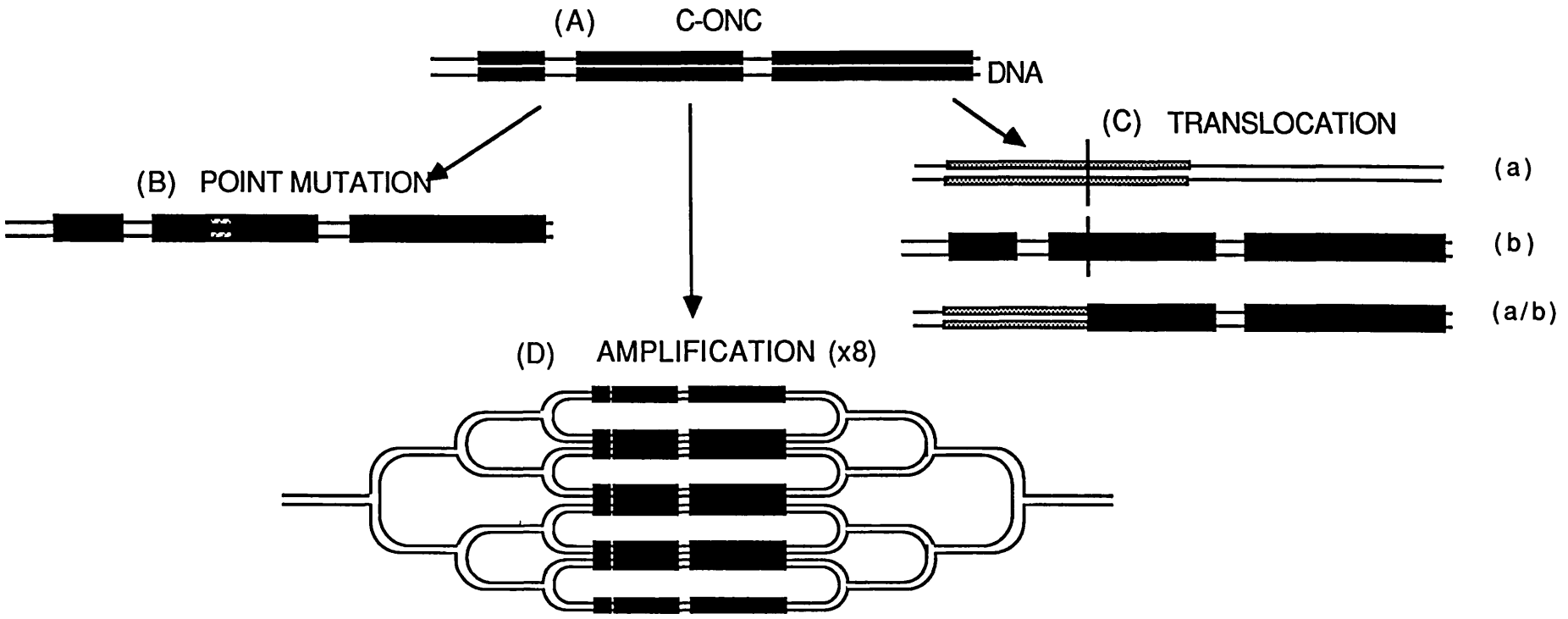


Fig 38 **Three mechanisms of activation of cellular oncogenes.**

(A) represents the haploid component of a proto-oncogene, composed of three exons (in black boxes) in a segment of DNA. The different activated forms of (A) are depicted in (B), (C) and (D).
(B) A point mutation, resulting in a base change (the hatched box) can occur at a particular site in the proto-oncogene and lead to its activation.
(C) Chromosome translocations may place proto-oncogenes into transcriptionally active regions of chromatin or create fused transcripts and proteins from the two genes.
(D) Unscheduled replication of a proto-oncogene, amplification, can lead to an increase in transcription. Amplified proto-oncogene is depicted schematically as superimposed replication 'bubbles' (modified from Alitalo and Shwab, 1986).

The molecular investigation of the blastic phase of CML is in its infancy. Nevertheless some investigators have attempted to demonstrate further genetic changes consistant with blastic transformation. Bartram et al (1986) and Andrews et al (1987) used Southern blotting analysis to study the breakpoints in M-BCR in the chronic phase and again in blast crisis of a number of CML patients. All but two patients showed the identical breakpoints in both phases of the disease. The different breakpoint in M-BCR found in these two exceptional cases have been interpreted as changes specific to blast crisis but unique to these particular patients.

Some groups have reported that the relatively early onset of blast crisis correlates with breakpoints in M-BCR 3' of the central Hind III restriction enzyme site (Fig 39). Schaefer-Rego et al (1987) reported that 8 out of 9 patients in blast crisis had 3' M-BCR breakpoints while all 17 chronic phase patients had 5' M-BCR breakpoints. Mills et al (1988) reported that 10 patients with a relatively short duration in chronic phase had 3' M-BCR breakpoints. However others have failed to confirm this result. Torelli et al (1987) found no correlation between the breakpoints within M-BCR and the duration of the chronic phase in 40 CML patients.

Collins and Groudine (1983) studied the cell line K562 derived from a CML patient in blast crisis, and found rearrangement and amplification (4-8 fold) of ABL. There also was a similar amplification of the C immunoglobulin gene. It was unclear whether the gene amplification seen in K562 was attributable to the disease process or to in vitro culture

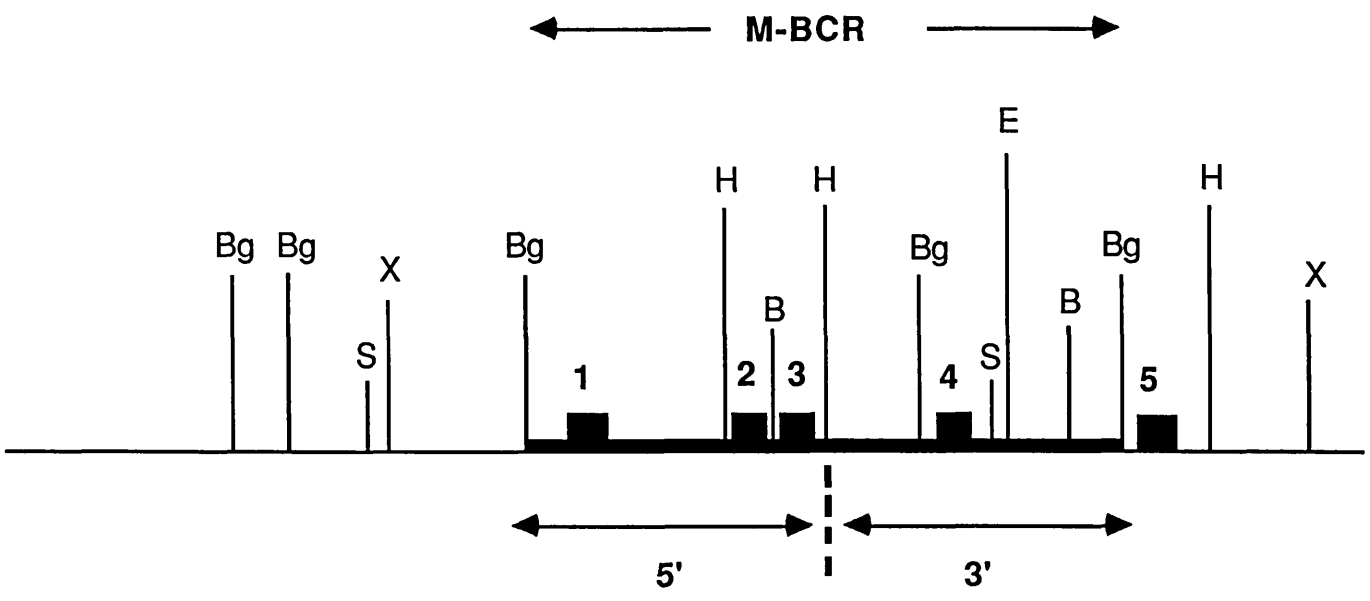


Fig 39 Breakpoints within M-BCR associated with survival in CML chronic phase.

Schematic diagram of M-BCR showing the five exons within the region represented by black boxes and the restriction endonuclease sites Bgl II (Bg), Hind III (H), Eco RI (E), Xba I (X) and Sal I (S).
Based on the report by Schaefer-Rego (1987), the arrow to the left of the vertical dashed line denote those patients with breakpoints 5' of the central Hind III site and who have a relatively longer survival in chronic phase. The arrow to the right of the Hind III site represents those patients with 3'M-BCR breakpoints and with a relatively shorter survival in chronic phase.

conditions necessary for establishment of the cell line. Seldon et al (1983), also working on K562, reported that the amplified C and ABL genes were present on the same marker chromosome. This result implied that gene amplification occurred after the formation of the BCR/ABL chimeric gene and that the amplification unit extended from ABL through to C . In 1987 Collins and Groudine reported similar findings in a CML patient who presented in lymphoid blast crisis. They showed that the rearranged ABL fragment was amplified to a similar degree in both the patient's blast cells and in his terminally differentiated granulocytes, although the expression of the BCR/ABL transcript was 8-16 fold higher in the blast crisis cells.

Eva et al (1983) also studied a cell line, IM9, derived from a CML patient in blast crisis. They used the techniques of DNA mediated gene transfer into NIH 3T3 cells and a sensitive in vivo assay of tumorigenesis. They identified the human DNA responsible for tumour formation in athymic nude mice as N-RAS. This oncogene is closely related to the transforming genes of the Harvey and Kirsten murine sarcoma virus. Pierce et al (1984) also demonstrated transforming genes presumed to be related to the RAS family in two CML patients. Recently Liu et al (1988), using the same assay for tumorigenicity used by Eva et al (1983), found that 7 out of 12 CML patients harboured transforming genes. Point mutations of codon 12 of the RAS oncogene were found in the leukaemic cells of 4 of these patients, one of whom was in chronic phase. The demonstration of RAS point mutations in the chronic phase as well as blast crisis of CML suggested that activation of RAS was not

the primary genetic event responsible for blast crisis but might play diverse roles in the pathogenesis of CML.

McCarthy et al (1984) reported rearrangement and amplification of MYC (the normal cellular homologue of the transforming gene of the avian myelocytomatosis virus) in the cells of a patient with promyelocytic transformation of CML. This genetic change was not evident in the chronic phase cells. At blast crisis analysis of DNA showed a 4-8 fold amplification of MYC and analysis of mRNA showed a two-fold increase in expression of MYC. The finding of MYC rearrangement and amplification in CML pointed to a strong causal association with the final stage of this disease but one that was unique to this particular patient.

Ahuja et al (personal communication) have recently studied the expression of the oncogene p53 in blast crisis CML of myeloid or intermediate phenotypes. This oncogene codes for a family of proteins ranging in molecular weights from 48-55 kd, expressed in high amounts in mammalian cells transformed by DNA and RNA tumour viruses and chemical carcinogens. They found that 8 out of 17 blast crisis CML patients had genomic rearrangement or deletion of p53. However, the p53 message uniformly expressed in chronic phase CML was expressed variably in blast crisis cells; no transcripts were detectable in four cases and high levels were present in a fifth case. One case without transcripts had no detectable gene rearrangement. However another group was unable to demonstrate this phenomenon and reported the lack of any rearrangement of the p53 gene in human haematological malignancies (Veneturelli et al, 1988).

I studied a group of blast crisis CML patients by cytogenetics, Southern blotting and in situ hybridisation and looked for differences in M-BCR breakpoints at presentation and blast crisis. I tried to identify, by means of in situ hybridisation, a chromosome marker found in one patient. Rearrangement of the p53 oncogene and amplification, by Southern blotting, of MYC and ABL that would that would normally be unidentifiable by standard cytogenetic means was looked for.

6.2 CLINICAL AND HAEMATOLOGICAL FEATURES

The clinical and haematological features, at presentation, of four CML patients studied in blast crisis are shown in Table 21.

6.3 CELL MARKER STUDIES

The results of cell marker studies at blast crisis are shown in Table 22. All 4 patients had blast cells of myeloid origin. In patients 1, 2 and 4 between 46 and 58% of cells stained positive with the monoclonal antibody CD13 (MCS2) which recognises early myeloid cells and is positive with the blast cells of 80% of AML patients. 34% and 51% of cells from patient 2 and 3 respectively stained positive for CD33 (MY9) which reacts with cells of the myeloid phenotype.

Table 21 Clinical and haematological features of four CML patients studied in blast crisis

Peripheral blood at diagnosis

Patient	Age/ sex	Spleen (cm)	Hb (g/dl)	WBC (x10/l)	Plat's (x10/l)	Differential (% of 300 cells)									NAP	Treatment & response	Survival (months)
						Bl	Pm	My & Met	Neut	Eos	Baso	Mono	Lymph	NRBCs			
1	F/42	6	8.0	166	255	45	15	8	14	4	2	4	0	0		BU,PR	28
2	F/23	15	10.4	299	201	2	44	18	2	0	0	0	3	0		HU,PR	40
3	F/29	12	10.8	212	208	0	0	24	4	0	2	0	0	2		HU,CR BMT	51
4	M/18	8	4.9	322	620	0	0	20	65	18	17	0	2	0		BU,HU,CR, BMT	17

HU = hydroxyurea BU = busulphan NT = not treated CR = complete haematological response

PR = poor or partial haematological response BMT = bone marrow transplantation

NAP = neutrophil alkaline phosphatase

Table 22 **Cell marker studies performed on four CML patients in blastic transformation**

		Percentage positive cells from patient no.			
		1	2	3	4
B- cell	CD19 (B4)	-	-	-	5
	CD10 (CALLA)	-	-	-	4
T-cell	CD7 (3A1)	-	-	4	10
	CD2 (E-rosettes)	4	2	-	3
Myeloid	CD13 (MCS2)	52	46	-	58
	CD33 (My-9)	-	34	51	1
	CD14 (FMC17)	-	-	5	-
Others	Tdt	-	-	-	-
	CD34 (3C5)	-	-	18	-
	HLA-DR (GRB-1)	60	25	10	25

Tdt = terminal deoxynucleotidyl transferase

Summary of studies performed by R Morilla

6.4 CYTOGENETICS

These patients were studied at presentation and at blast crisis in order to detect cytogenetic changes coincident with blastic transformation.

6.4.1 Patient 1

15 G-banded metaphase chromosomes from bone marrow at presentation were Ph positive [46XX, t(9;22)(q34;q11)]. The patient referred to us from another centre was already in the accelerated phase of CML. Analysis of 15 G-banded metaphases from bone marrow cultures (48 hours) at blast crisis showed that 9 metaphases were exclusively Ph-positive, while 6 metaphases had acquired an extra metacentric G group marker (Fig 40).

6.4.2 Patient 2

Cytogenetic analysis of 10 G-banded metaphases from bone marrow was done at presentation. All the metaphases were Ph-positive [46XY, t(9;22)(q34;q11)]. At blast crisis 15 G-banded bone marrow metaphases were analysed: 7 metaphases were exclusively Ph-positive while 8 metaphases had acquired an extra marker chromosome which we defined as an extra chromosome 19 with a translocation involving the terminal portion of the q arm of chromosome 17 [47XY, t(9;22)(q34;q11), + t(17;19) (q22;q13)] (Fig 41).

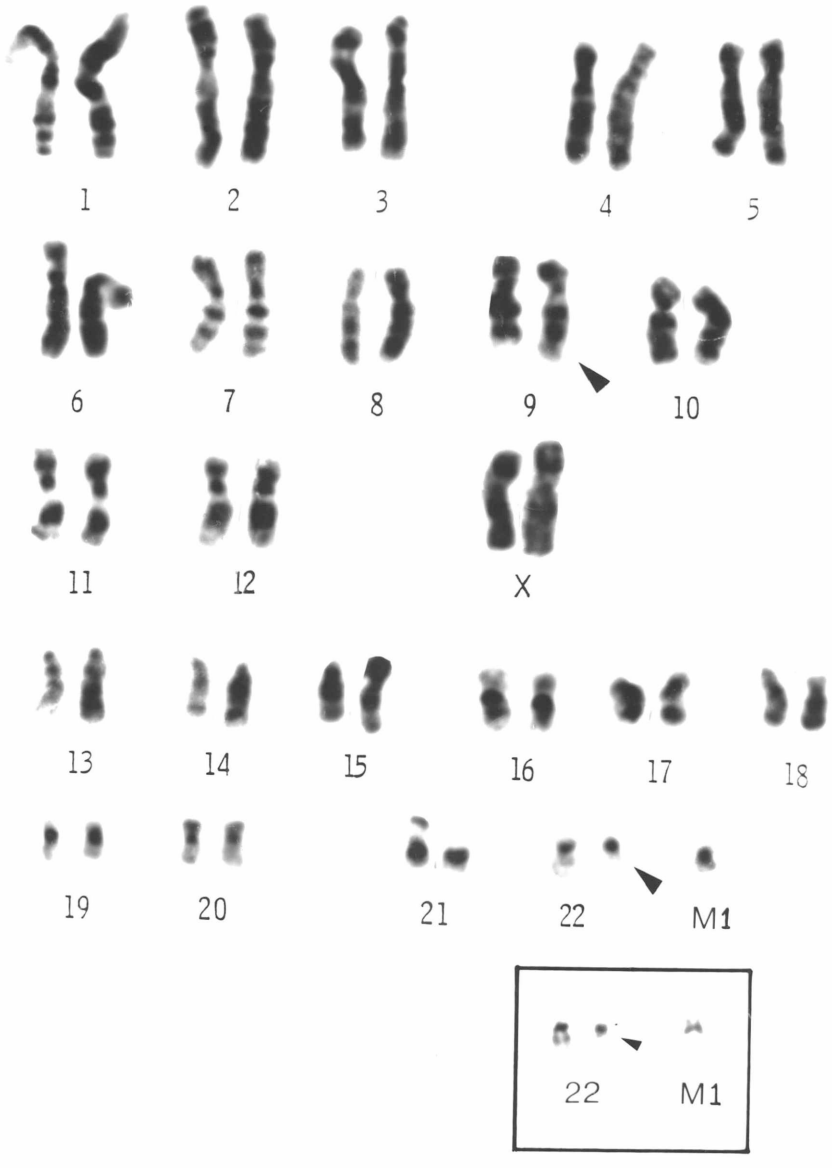


Fig 40 **Karyotype of G-banded metaphase from a patient with CML in blast crisis (no. 1) with a Ph chromosome and an extra marker chromosome.**

G-banded chromosomes showing the translocation between chromosoms 9 and 22, t(9;22), resulting in the Ph chromosome as well as an extra metacentric G-group marker chromosome (M). Inset: partial karyotype from another metaphase showing the normal chromosome 22, the Ph chromosome and the marker chromosome.

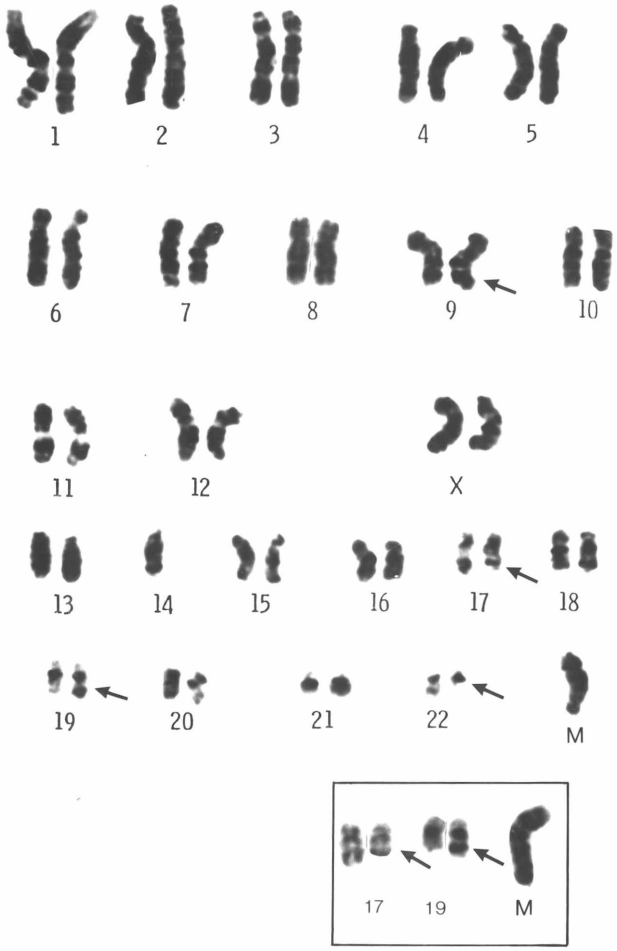


Fig 41 **Karyotype of a G-banded metaphase from a patient with CML in blast crisis (no. 2) blast crisis CML showing translocations, t(9;22)(q34;q11), t(17;19)(q22;q13) and an uncharacterised C group marker chromosome.**

Inset: partial karyotype of a second metaphase showing the abnormal chromosomes 17 and 19 and marker.

6.4.3 **Patient 3**

Cytogenetic analysis was performed on two occasions during chronic phase, once at presentation and one a year later. On both occasions the study of 15 and 10 G-banded bone marrow metaphases respectively analysed were Ph-positive [46XY, t(9;22)(q34;q11)]. At blast crisis all 15 G-banded bone marrow metaphases were also Ph-positive (Fig 42). No secondary karyotypic change had taken place.

6.4.4 **Patient 4**

At presentation 20 G-banded metaphases obtained from bone marrow cultures were all Ph-positive. At blast crisis 20 G-banded metaphases analysed from bone marrow cultures at 24 hours: 2 were Ph-negative, 12 were exclusively Ph-positive, 5 had acquired an extra chromosome 8 [47XY, t(9;22)(q34;q11), +8] (Fig 43) and 1 metaphase had acquired an extra A group marker and a D group marker [48XY, t(9;22)(q34;q11), + mar A, + mar D]

9.5 IDENTIFICATION OF A MARKER CHROMOSOME

The origin of the metacentric G-group marker chromosome found in patient 1 was unidentifiable by G-banding analysis. In some metaphase spreads it resembled an iso-Ph chromosome while in others the morphology was unclear. In situ hybridisation of an ABL probe to metaphase chromosomes was carried out to identify this marker.

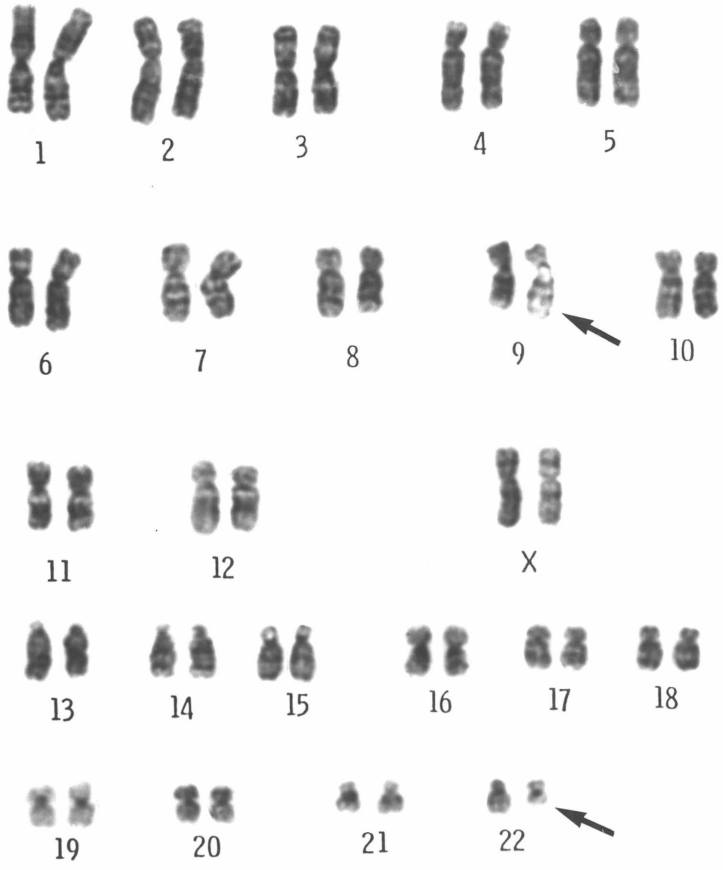


Fig 42 **Karyotype of a G-banded metaphase from blast crisis CML patient 3 showing a Ph translocation, t(9;22)(q34;q11) with no further change.**

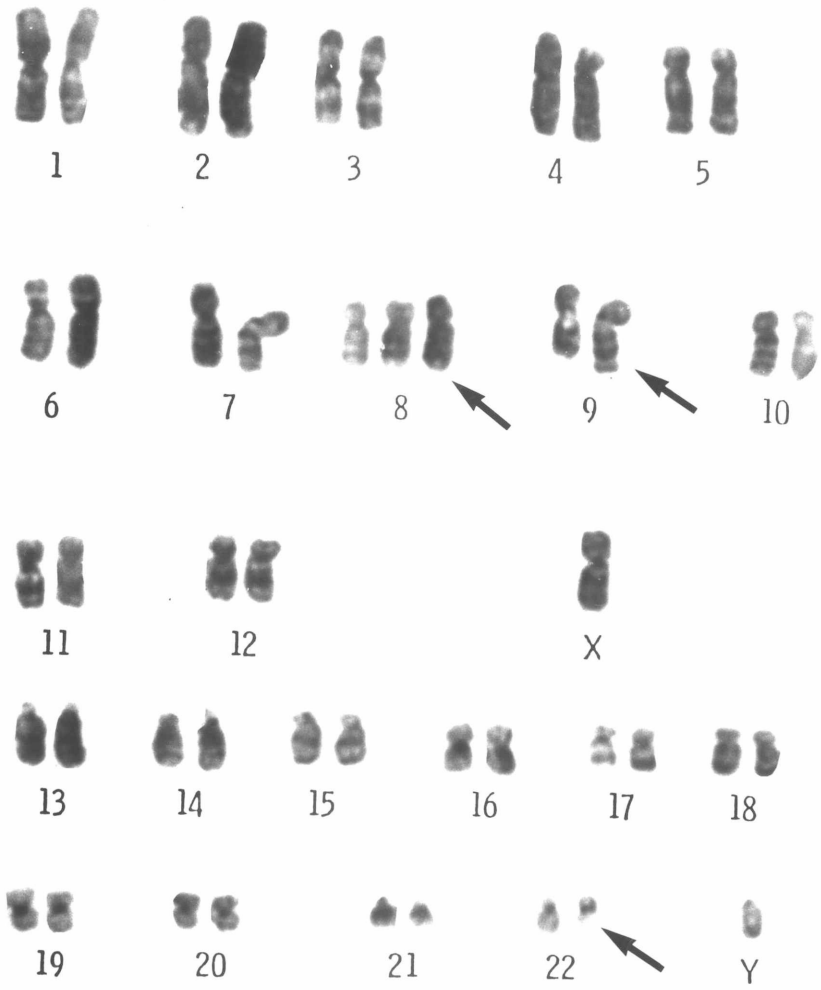


Fig 43 **Karyotype of a G-banded metaphase from blast crisis CML patient (no. 4) showing a Ph translocation and an extra chromosome 8, [47XY,t(9;22)(q34;q11), +8]**

The grain localisation of the ABL oncogene in 40 metaphases containing the marker showed that with a total of 102 grains 10 (9.8%) grains hybridised to chromosome 9, 9 (8.8%) grains to the Ph chromosome and 10 (9.8%) grains to the metacentric marker chromosome (Table 23). The clustering of grains on chromosomes 9, 22q- and M was significant ($p = 0.009$, $p < 0.0001$ and $p = 0.0008$ respectively). The ABL probe did not hybridise to both chromosome arms of the metacentric marker in any one metaphase.

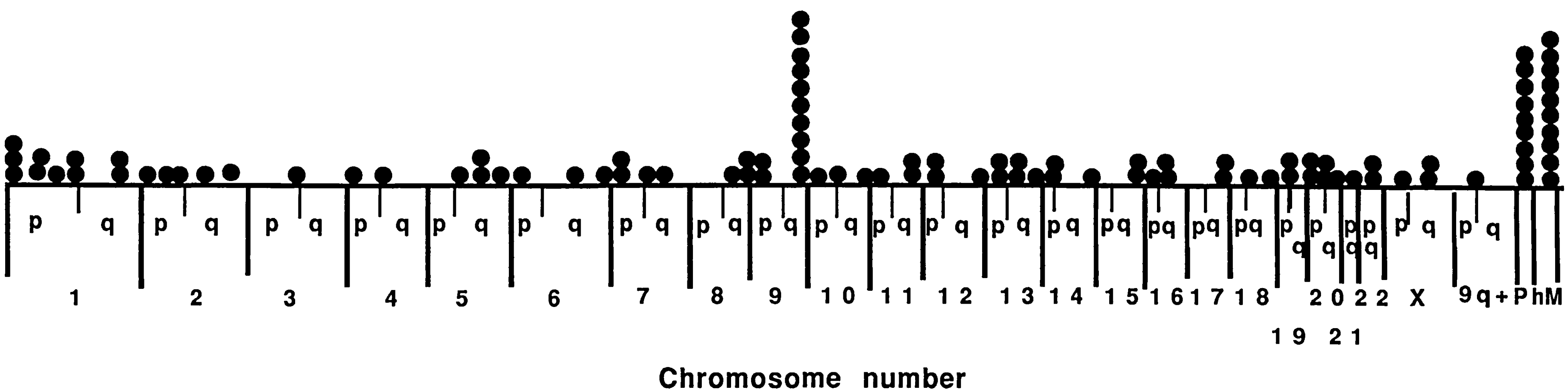
The localisation of ABL sequences on the marker chromosome and its size suggested that it had been formed in part by duplication of the Ph chromosome. This was confirmed subsequently by in situ hybridisation studies performed on the cell line derived from this patient using both ABL and BCR probes.

6.6 M-BCR REARRANGEMENT

High molecular weight DNA extracted from the leukaemic cells of patients at chronic phase and blast crisis was digested with Bam HI, Eco RI and Hind III and probed with the 0.6 kb probe for M-BCR (BCR-G). Southern analysis of M-BCR was carried out to look for differences in the breakpoint within M-BCR. The pattern of rearrangement was identical in all 4 patients at both stages of the disease (Fig 44). In 1, 2 and 4 the breakpoint within M-BCR occurred 3' of the central Hind III site.

Table 23 Localisation of the ABL proto-oncogene in metaphases from blast crisis CML patient 1

The grain localisation of the ABL proto-oncogene to chromosomes 9q34, 22q- and the metacentric G group marker chromosome (M) in 45 bone marrow metaphases by in situ hybridisation.



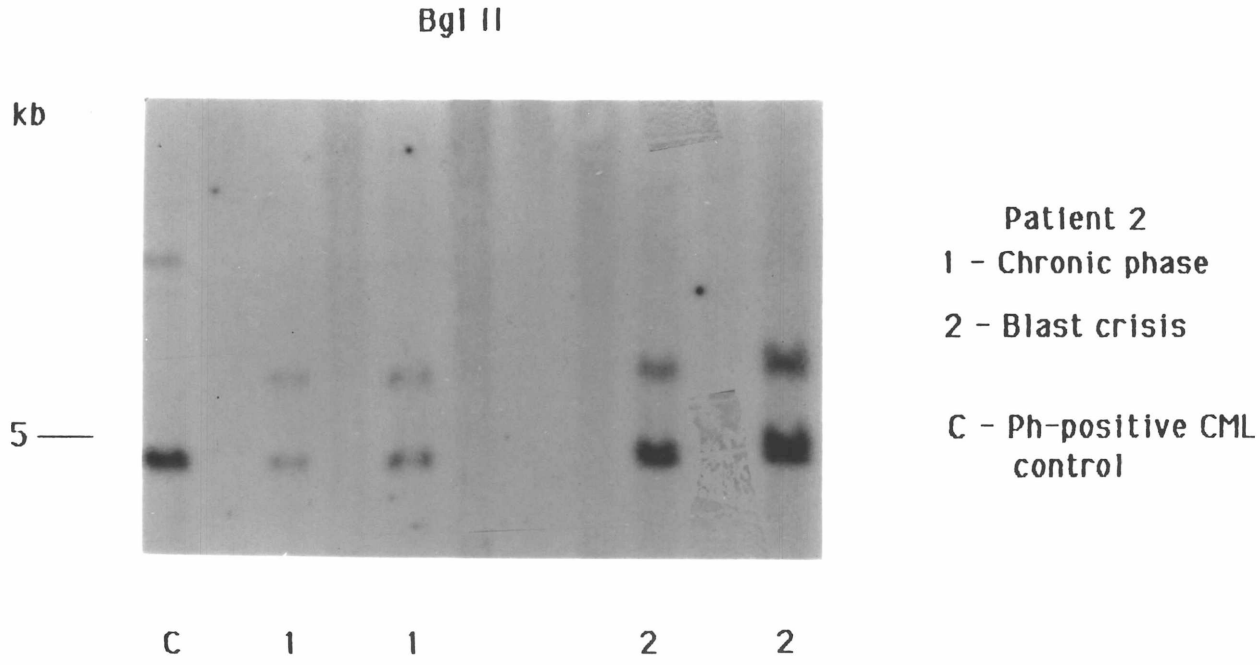


Fig 44 Southern blot analysis of DNA from blast crisis CML patient 2 using the 0.6 kb M-BCR probe.

High molecular weight DNA extracted from the leukaemic cells of patient 2 at chronic phase (1) and blast crisis (2) was digested with restriction endonuclease Bgl II and the blot hybridised with the M-BCR probe.
The same pattern of genomic rearrangement of M-BCR is seen in patient 2 at chronic phase (1) and blast crisis (2)

6.7 P53 REARRANGEMENT

Southern blots of high molecular weight DNA digested with Eco RI and Bam HI were hybridised with a 1.76 kb human p53 cDNA probe which contained all the known exons of the P53 gene. All four patients had germline configurations of the p53 gene (Fig 45).

6.8 SEARCH FOR GENE AMPLIFICATION

Amplifications of ABL (Collins and Groudine, 1985) and MYC (McCarthy et al, 1985) to the levels seen in K562 and HL60 (4-8 fold) respectively was looked for by Southern analysis using a 1.4 kb ABL probe corresponding to its tyrosine kinase domain (Ben-Neriah et al, 1986), a and a 1.5kb MYC probe corresponding to exon 2 (Rabbitts et al, 1985). DNA extracted from the K562 cell line was used as a positive control in the search for ABL amplification. DNA extracted from the HL60 cell line established from a patient with promyelocytic leukaemia was used as a positive control in a search for MYC amplification. A strain of HL60 was used which had a 4-8 fold amplification of MYC. To assess whether any amplification had occurred I compared autoradiographs of filters hybridised with ABL or MYC with autoradiographs of filters rehybridised with a probe for a single copy gene (pre-proenkephalin-1). Hybridisation signals were quantitated by transmission densitometry. The ratio of the area of hybridisation signal was calculated for ABL and MYC probes and compared with that of the control enkephalin probe for each sample. The relative gene dosage for MYC and ABL in each patient was established by comparing the MYC and ABL signal ratio to the mean signal ratio of all

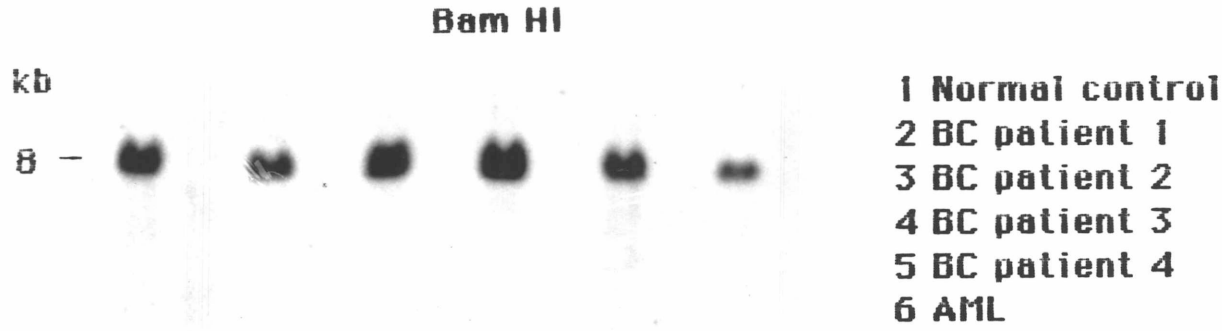


Fig 45 Southern analysis of the p53 oncogene in DNA from four blast crisis CML patients.

High molecular weight DNA was digested with Bam HI and hybridised with the 1.76 kb cDNA p53 probe. DNA from the four CML patients, the AML patient and the normal control showed a single band in the germline configuration at 8 kb.

control subjects on the same blot, with the latter value normalised to 1 (Podlisny et al, 1987).

The relative gene dosage for ABL and MYC in the four blast crisis CML patients studied is shown in Table 24. No amplification of ABL or MYC of the level seen in the control cell lines was found in the DNA from any of the 4 patients studied (Fig 46). Due to technical limitations it was not possible to measure an increase in band intensity equivalent to duplication of a gene. For example we were not able to show one extra copy of the ABL gene in the DNA from patient 1 even though in situ hybridisation studies demonstrated duplicated ABL proto-oncogene sequences on the marker chromosome. Also no clear duplication of the MYC proto-oncogene in the DNA was demonstrated from patient 4 with trisomy of chromosome 8 in a significant proportion of his cells.

6.9 DISCUSSION

A study of cell surface markers demonstrated blasts of myeloid lineage in four blast crisis CML patients. Cytogenetic analysis showed that in addition to the Ph chromosome one patient had acquired an extra chromosome 8, while the other three patients had karyotypes not typical of the cytogenetic changes seen in 70 % of blast crisis CMLs. One patient had an extra marker chromosome derived from a translocation between chromosome 17 and 19, a second had an extra marker unidentifiable by conventional G-banding analysis, and a third had no additional chromosomal abnormalities. The secondary karyotypic change of +19 is seen frequently in blast crisis CML but a marker with

Table 24 **Densitometric analysis of MYC and ABL proto-oncogene dosage in four CML patients in blastic transformation compared to normal controls and cell lines with known oncogene amplification**

Proto-oncogenes	Normal	HL60*	K562+	Patient nos. 1	2	3	4
MYC	1	5.3	NA	1.15	1.06	1.2	1.13
ABL	1	NA	10.2	1.04	1.12	1.17	1.21

* The HL60 cell line with a 5-fold amplification of the MYC proto-oncogene

\+ The K562 cell line with a 10-fold amplification of the ABL proto-oncogene

NA - not applicable

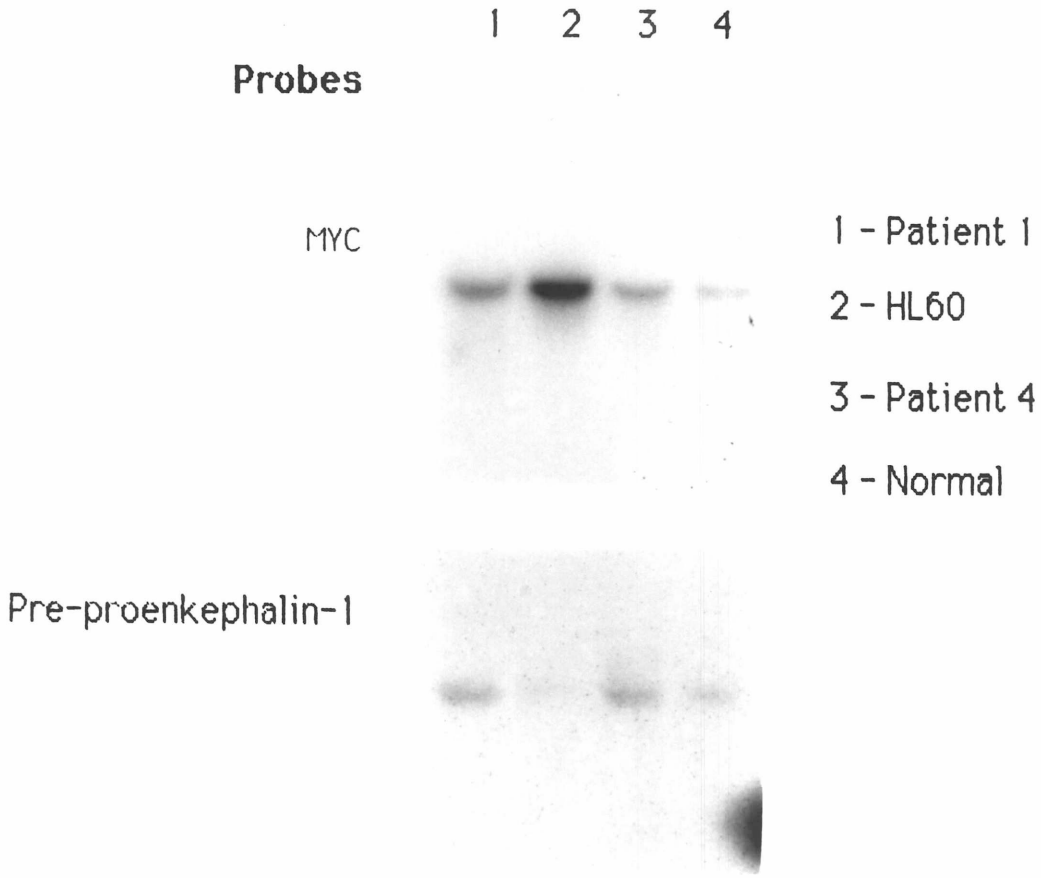


Fig 46 **Representative Southern blot analysis of the MYC proto-oncogene in DNA from two blast crisis CML patients.**

High molecular weight DNA was digested with Eco RI and probed with a 1.5 kb MYC probe and a control probe pre-proenkephalin-1.

Patients 1 and 4 (lanes 1 and 3 respectively) showed no amplification with respect to the normal control. The HL60 cell line (lane 2) showed amplification of the MYC proto-oncogene. The control blot shows that less DNA was loaded in lane 2, suggesting a higher level of MYC amplification.

involvement of chromosome 17q has not been reported before. Approximately 20% of CML patients have the Ph chromosome as the sole abnormality throughout their disease and additional cytogenetic aberrations do not always occur in the development of blast crisis (First International Workshop on Chromosomes in Leukemia, 1978). I identified the marker chromosome found in patient 1 by means of in situ hybridisation. ABL oncogene sequences hybridised to the normal chromosome 9, the Ph and the marker chromosome. This suggested that at least one chromosome arm of the metacentric marker contained a copy of ABL. Recent results obtained from the cell line established from this patient suggest that this marker was derived through duplication of the Ph. In situ hybridisation studies in the cell line demonstrated that both BCR and ABL were present on this marker chromosome.

M-BCR rearrangement studies demonstrated no difference in the breakpoint between presentation and blast crisis. Although three of the patients studied had breakpoints within M-BCR 3' of the central Hind III site this did not correlate with a shortness of duration of chronic phase. These results have been confirmed by a larger study in our laboratory in which 67 CML patients have showed no correlation between position of the M-BCR breakpoint and the period before relapse (Jaubert et al, 1989).

Southern analysis of p53 demonstrated no genomic rearrangement. Although p53 was not rearranged in these patients, this does not rule out alteration of the gene by other means. Ahuja et al (1988) reported a heterogeneous pattern of genetic alteration and expression of p53 in some blast crisis CML patients with a myeloid phenotype. It may be

that alterations of the p53 gene are merely an epiphenomenon. It is also likely that disruption of normal gene function is the common factor that unites this variant pattern of gene alterations and contributes to the development of blast crisis. It has been recently reported that murine p53 competes with alpha DNA polymerase for binding to large-T-antigen and in so doing can inhibit SV40 origin dependent DNA replication, suggesting a role for p53 in initiation and maintenance of replicative DNA synthesis (Gannon and Lane, 1987). It seems reasonable to suggest that loss of this protein could lead to a disruption of normal DNA replication and thereby contribute to carcinogenesis.

No amplification of ABL or MYC was detected in any of the 4 patients studied, although amplification of these oncogenes in CML blast crisis has been reported in isolated cases (Collins and Groudine, 1984, McCarthy et al, 1984). In neuroblastoma N-MYC amplification has been correlated with more aggressive tumour growth and poor prognosis (Brodeur et al, 1984), so it is possible that gene amplification represents one of the mechanisms, albeit rare, by which blast crisis is achieved.

A duplication of chromosomal material associated specifically with the blast crisis phenotype was demonstrated in three of the four patients studied. It may be that the duplication of particular oncogenes (located on these chromosomes) which result in perturbation of oncogene expression is sufficient to incline the cell towards acute transformation. The duplication of the BCR/ABL chimeric gene in patient 1 may be significant. Although Shtivelman et al (1987) suggested that

further changes of BCR/ABL were not important in the transition from the chronic to the acute phase in CML, qualitative and quantitative studies of ABL/BCR expression in blast crisis are yet sufficient to rule out this possibility. Likewise a duplication of MYC in patient 4 may be important in blast crisis. No oncogenes have yet been localised to chromosome 19 or the terminal end of chromosome 17. The translocation t(17;19) found in patient 2 may involve the activation of an as yet unidentified oncogene. It has been debated whether the important event in the formation of the isochromosome 17 in CML is the loss of genes from the short arm or the duplication of genes on the long arm. p53 is located on the p arm of chromosome 17 (van Tuinen et al,1986). The formation of iso(17q) results in the loss of one allele of p53. It may be that loss of the wildtype allele allows the expression of an abnormal p53. However the incidence of t(17;19) in patient 2 indicates that genes located on the terminal end of the 17q may also be important in blast crisis. No additional cytogenetic abnormalities were detected in patient 3.

Previous studies by us (see chapter 3) and other investigators indicate that genetic changes may occur in CML without any evidence of cytogenetic abnormalities (Morris et al, 1986, Bartram et al, 1986, Ganesan et al, 1986, Dreazen et al, 1987, Wiedemann et al, 1988). It is likely therefore that cytogenetically invisible genetic changes analogous to those presumably occurring in other blast crisis CMLs also occurred in patient 3.

The genetic changes involved in blast crisis CML may be many and varied. One or several oncogenes may be activated and operate synergistically to transform the cell from a 'benign' chronic phase to blast crisis. However, our understanding of these events remains poor. Greater insight may be gained by careful genetic analysis of the cytogenetic changes that occur in transformation.

CHAPTER 7: Ph-POSITIVE ACUTE LEUKAEMIA

7.1 BACKGROUND

Cytogenetically, the Ph translocation in the acute leukaemias is indistinguishable from the t(9;22) found in CML. In approximately 50% of acute leukaemias the Ph chromosome is the only chromosomal aberration whereas in other cases the Ph translocation occurs together with other chromosomal changes. The most common additional abnormalities are monosomy of chromosome 7 and structural rearrangements of one or both chromosomes 9. In acute leukaemia the incidence of variant translocations is higher (approximately 15%) than in CML (5%) and complex variants involving especially chromosome 14q32 have been reported by a number of investigators (Takeuchi et al, 1981, Sandberg et al, 1985, De Klein et al, 1986).

The molecular events resulting in the Ph translocation in CML have been studied in considerable detail and comparable studies are in progress for the acute leukaemias. Erikson et al (1986) and de Klein et al (1986) investigated the breakpoints at 22q11 in a total of 20 patients with Ph-positive acute leukaemia by Southern blot analysis using M-BCR probes. They found that in some cases M-BCR remained unaffected. In other cases the breakpoints occurred within M-BCR and were indistinguishable from the molecular findings in CML. Data from these and other studies suggested that about half of patients with Ph-positive acute leukaemia may have a disease closely related to CML whereas others

had a molecularly distinct disorder. These results also indicated that contrary to earlier suggestions both molecular subgroups occurred in the adult and childhood form of Ph-positive ALL.

Three studies have shed more light on the problem (Chan et al, 1987; Clark et al, 1987; and Kurzrock et al, 1987). These workers investigated the expression of ABL-related proteins in Ph-positive ALL patients without rearrangement of M-BCR by the immune precipitation kinase assay and detected a unique ABL-related tyrosine kinase of 185 or 190 kd. They also found that expression of this protein correlated with the expression of a novel 6.5 or 7.4 kb mRNA. The authors observed that these novel mRNAs and proteins must result from the fusion of the ABL oncogene with then undetermined sequences. They speculated that these sequences could be upstream of M-BCR (but within the BCR gene) or part of another gene. Hermans et al (1987) identified these sequences. They reported that genomic breaks occurred within the first intron of the BCR gene, 5' of M-BCR involved in CML. They proposed that this alternative BCR/ABL fusion gene was created when the 3' end of this first exon of the BCR gene alone was linked to the second ABL exon (see chapter 1, Fig 4). Using a panel of genomic probes, Chen et al (1988) showed the breakpoints in 6 out of 7 Ph-positive , BCR-negative ALL patients to occur within a 10.8 kb region of the first intron of the BCR gene. These authors have recently reported a second cluster of breakpoints within a 5 kb segment of the first intron, approximately 16 kb upstream of the previously described cluster (Chen et al, 1989)

Few molecular investigations of Ph-positive AML have been reported thus far. The results of investigating Ph-positive ALL seemed at the molecular level to suggest that M-BCR rearrangement and expression of the P210 might be pathogenetically associated with myeloid leukaemogenesis whereas the absence of M-BCR rearrangement and the expression of the P190 might be associated with lymphoid leukaemogenesis.

An initial report from Erikson et al (1986) of M-BCR rearrangement in one Ph-positive AML patient seemed to support this hypothesis. However Kurzrock et al (1987) reported that the genomic alteration and the gene product in Ph-positive AML could be similar to those in M-BCR-negative ALL, such that the breakpoint at 22q11 occurred outside the BCR region and resulted in expression of a P190 protein.

I studied two Ph-positive acute leukaemia patients with unique features at the cytogenetic and molecular levels. One was a Ph-positive ALL patient who at presentation showed both lymphoid and myeloid populations of cells and both Ph-positive and normal metaphases and the other was a case of de novo AML classified as erythroleukaemia (FAB-M6) with a complex karyotype including a 'masked' Ph chromosome. These studies are described in greater detail below.

7.2 Ph-POSITIVE ACUTE LYMPHOBLASTIC LEUKAEMIA

7.2.1 Case Report

A 20 year old male presented in May 1986 with purpura, lymphadenopathy, and splenomegaly. His white cell count was $120x10^9/l$; the majority of cells in his peripheral blood were blasts. Cell surface markers studied by R Morilla were positive for CD14 (FMC17, monocytoid) and CD19 (B4, early lymphoid) markers (Table 25). Staining with immunoperoxidase suggested the existence of two distinct populations of blast cells, one of monocytic and the other of B cell lineage (Fig 47). He did not respond to treatment with courses of daunorubicin, cytarabine and 6-thioguanine (DAT) and amsacrine, 5-azacytidine and etoposide (MAZE). He responded subsequently to treatment with the UKALL-X intensification regimen followed by maintenance treatment. The number of blasts in his bone marrow fell to 6% and remained low until January 1987 when he relapsed. At this stage his cell-surface markers showed a B-lymphoid phenotype (positive for terminal deoxynucleotidyl transferase and CD10 [B4+, CALLA+]). Treatment with mitoxantrone and cytarabine again reduced the percentage of blasts in the bone marrow to 6% but he relapsed again in March 1987. His cell surface markers were again consistent with the B lineage. He died in April 1987.

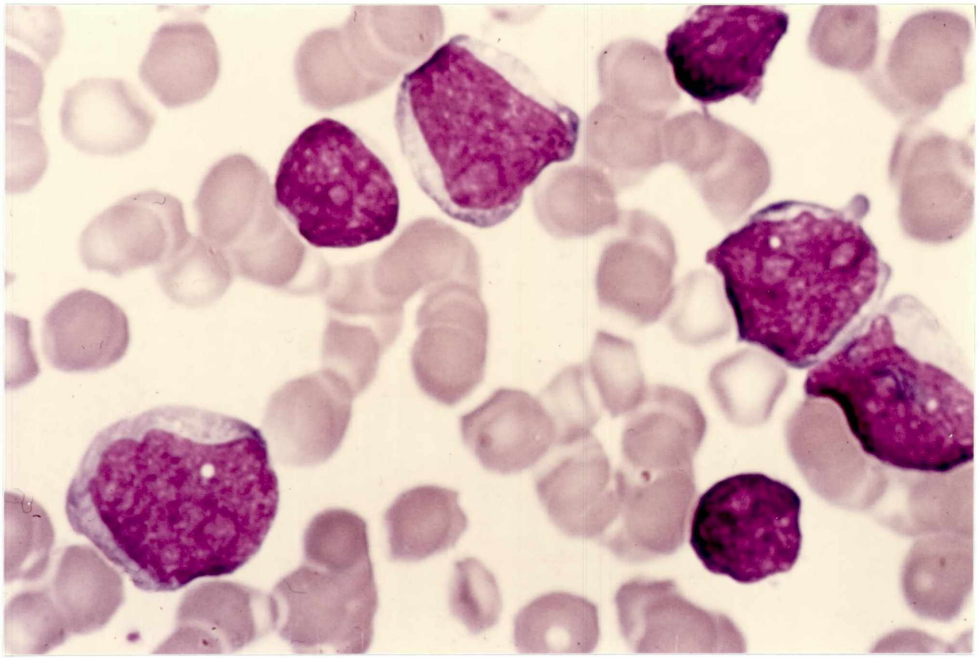
Table 25 Cell marker studies performed on patient's leukaemic cells at presentation and at relapse.

		April 1986	April 1987
Total leukocyte count (x10/l)		90.0 [a]	104
B-cell	CD19 (B4)	49%	64%
	CD10 (CALLA)	47%	83%
T-cell	CD7 (3A1)	neg	17%
	CD2 (E-rosettes)	neg	10%
Myeloid	CD13 (MCS2)	18%	neg
	CD33 (My-9)	15%	-
	CD14 (FMC17)	30%	-
Others	Tdt	48%	86%
	CD34 (3C5)	26%	neg
	HLA-DR (GRB-1)	71%	69%

Tdt = terminal deoxynucleotidyl transferase

Summary of studies performed by R Morilla working in Prof. Catovsky's laboratories at the RPMS, London.

A



B

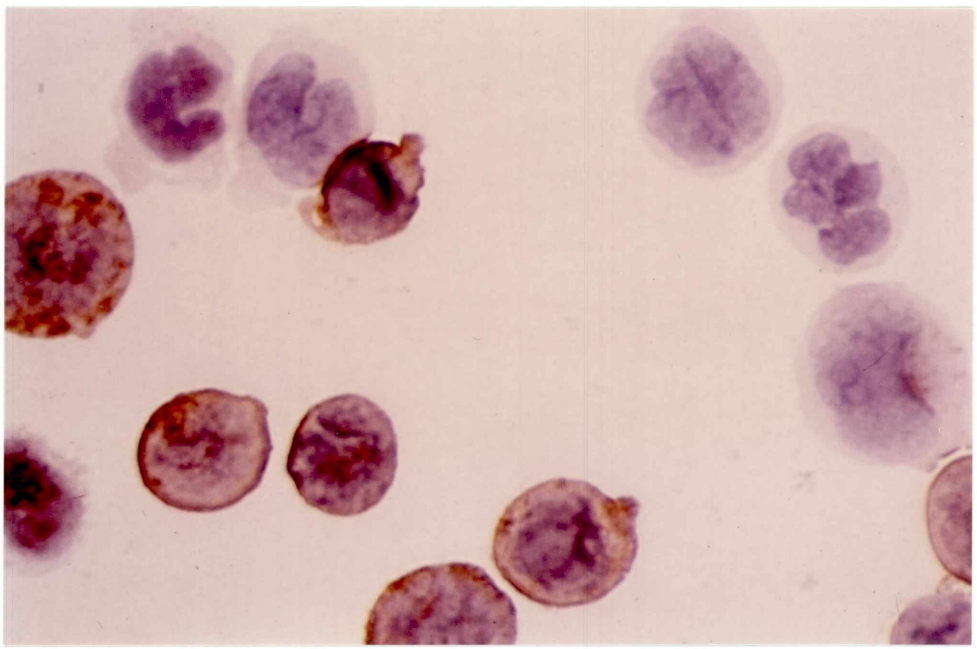


Fig 47 **Marker studies in a patient with Ph-positive ALL**

A May Grunwald Giemsa stain showing small and large blasts.

B Small blast cells showing CD10 positivity by the immunoperoxidase stain.
(This study was performed by R Morilla)

7.2.2 **Cytogenetics**

Cytogenetic studies performed on 20 marrow metaphases at presentation in May 1986 showed that 16 (80%) had the Ph translocation, t(9;22) (q34;q11) and 4 (20%) had a normal male karyotypes. Cytogenetic studies were performed again at relapse in March 1987 shortly before the patient's death. There were three different populations of cells: a predominantly Ph-positive population, 46,XY,t(9;22) (q34;q11) (n=15), normal metaphases with an extra chromosome 19, 47,XY, +19 (n=3) (Fig 48), and karyotypically normal cells, 46,XY (n=2).

7.2.3 **Southern analysis**

To determine whether there was genomic rearrangement of M-BCR Southern analysis of DNA was carried out by Dr L Wiedemann. DNA was prepared from the leukaemic cells of the patient at presentation and digested with restriction enzymes Bam HI, Eco RI, Sac I, Bgl II and Hind III and probed with a 4kb genomic M-BCR probe (see Chapter 2, Fig 8). Only germline bands were present in each lane (Fig 49) suggesting that there was no rearrangement of M-BCR. Rearrangement of Ig and T-cell receptor (TCR) genes are of interest in the study of differentiation since they may identify lineage affiliation prior to and independent of its expression at the protein level, and they are by their nature markers of clonality. Thus material from the patient was studied by Dr L Foroni using appropriate cloned DNA probes. DNA was digested with Hind III, Eco RI and BglII and hybridised with a probe for

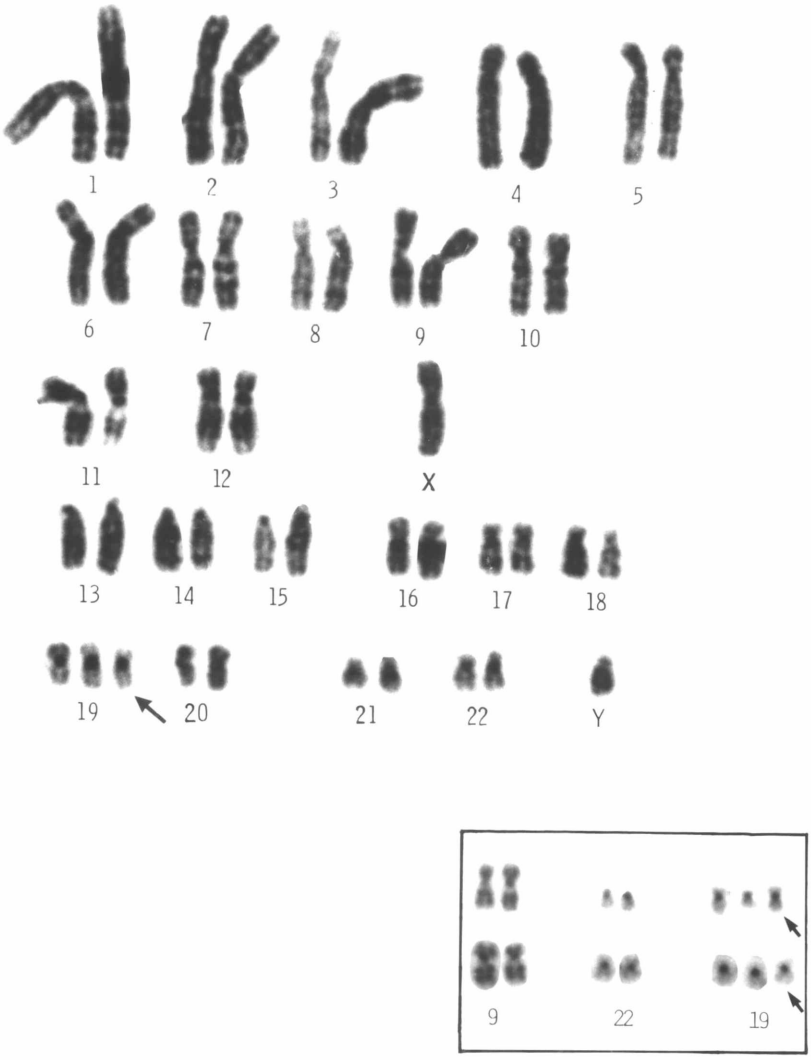


Fig 48 **Karyotype of a G-banded metaphase with normal chromosomes and an additional chromosome 19, 47, XY, +19.**

Inset: Partial karyotypes from two other metaphases showing also normal chromosomes 9 and 22 and trisomy 19.

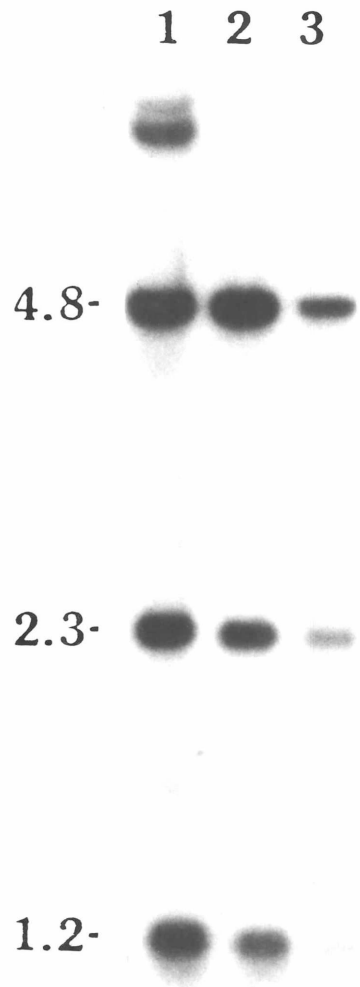


Fig 49 Southern blot analysis of DNA from Ph-positive ALL patient hybridised with the 4 kb M-BCR probe

Lane 1 represents a Ph-positive CML control, lane 2 represents DNA from the patient with Ph-positive ALL, lane 3 represents a normal control. There are three germline bands at 1.2 kb, 2.3 kb, 4.8 kb. In lane 1 there are three germline bands and one rearranged band. The DNA in lane 2 as with the normal control shows only the germline configuration of M-BCR.
(This Southern blot was performed by Dr L Wiedemann).

the J_H region of the immunoglobulin gene (for details see chapter 2). One band was present in the rearranged configuration but there was no band in the germline position. A study of the TCR chain gene showed a rearrangement of the locus with deletion of both CBI segments and rearrangement of one CB2 segment, the second CB2 being in the germline configuration. The same procedure was carried out using DNA extracted at relapse and identical rearrangements of the Ig and TCR genes were detected. Therefore the same clone of cells was present at presentation of the disease and at relapse.

7.2.4 **In situ hybridisation**

Because the patient's cells had a standard Ph chromosome but showed no evidence M-BCR rearrangement, I tried to localise the breakpoint at 22q11 by the technique of in situ hybridisation using probes that normally localise proximal or distal to BCR on chromosome 22. I used a probe for the ABL proto-oncogene, the SIS proto-oncogene, 5' BCR which mapped 5' of the K562 breakpoint (see Chapter 2, Fig 8), and the C Ig gene (see Chapter 2 for details). It was also interesting to know whether these probes were located in their normal positions in the cytogenetically normal population of cells.

ABL localisation

In 50 Ph positive metaphases analysed with a total number of 79 grains 12 (15%) grains mapped to the Ph chromosome and 11 (14 %) grains to the normal chromosome 9 at 9q34 (Fig 50). The clustering of grains on chromosomes 22q- and 9 was significant ($p < 0.0001$ and $p = 0.0003$



Fig 50 Localisation of the ABL proto-oncogene to chromosome 22q- in a metaphase from Ph-positive ALL patient

The black grain on the 22q-, indicated by the arrow, represents the localisation of the ABL proto-oncogene in situ.

respectively). In 45 Ph negative metaphases with a total number of 100 grains 10 (10%) mapped to chromosome 9q34 and none to chromosome 22. The clustering of grains to chromosomes 9q34 was significant ($p = 0.03$)

SIS localisation

In 40 Ph positive metaphases analysed with a total number of 78 grains 15 (19 %) grains mapped to the 9q+ and 14 (18 %) grains to chromosome 22q13. The clustering of grains on chromosomes 9q+ and 22 was significant ($p < 0.0001$). In 40 Ph negative metaphases analysed with a total number of 76 grains 16 (21 %) grains mapped to chromosome 22q12-13. The clustering of grains on chromosomes 22 was significant ($p < 0.0001$).

5' BCR localisation

In 35 Ph positive metaphases analysed with a total number of 80 grains 11 (14%) grains mapped to the 9q+ and 8 (10%) grains to the normal chromosome 22 at 22q11. The clustering of grains on chromosomes 9q+ and 22 was significant ($p = 0.0004$ and $p < 0.0001$ respectively).

C localisation

In 40 Ph positive metaphases analysed with a total number of 68 grains 8 (12%) grains mapped to the Ph chromosome and 6 (9%) grains to the normal chromosome 22 at 22q11. The clustering of grains on chromosomes 22q- and 22 was significant (p 0.0002).

A composite of histograms showing the grain localisation of the above mentioned probes in both Ph-positive and negative cells is shown in Fig 51.

7.2.4 mRNA PROTECTION ASSAY

Dr O Dreazen looked for presence of the BCR/ABL chimeric mRNA by the protection assay to confirm the Southern data. The details of this assay were described in detail in Chapter 3.

Using the b3a2 RNA (450 bp) probe, 172 and 287 b fragments were detected. These fragments could have resulted from the normal expression of BCR and ABL genes or possibly from other rearrangements of these genes distinct from those that characterise Ph-positive CML. Neither the 450 bp (b3a2) probe nor the 280 bp (b2a2) probe was protected in its entirety. These results excluded the presence of either of the chimeric mRNAs that characterise Ph-positive CML (data not shown).

7.2.5 Protein kinase assay

The analysis of ABL related PTK activity was performed by Dr L Wiedemann on cells during the first relapse. A phosphorylated protein band of Mr 190 kd was observed (Fig 52). Its precipitation was inhibited in the presence of the immunogen against which the antibody was raised.

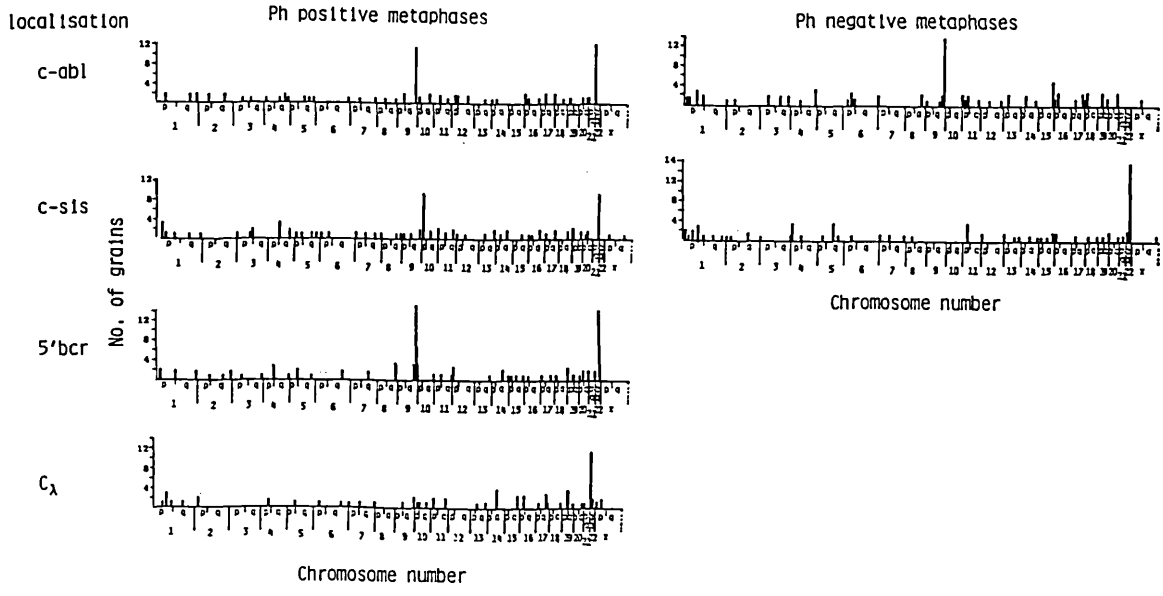


Fig 51 **Composite diagram showing grain distribution in 30-50 metaphases for each of the DNA probes: ABL, SIS, 5' BCR, and C in Ph-positive and -negative metaphases.**

The x axes represent the chromosomes in their size proportion; the y axes show the number of grains. There was obvious clustering of grains in one or two distinct regions in comparison with nonspecific 'background' grains found on all chromosomes.

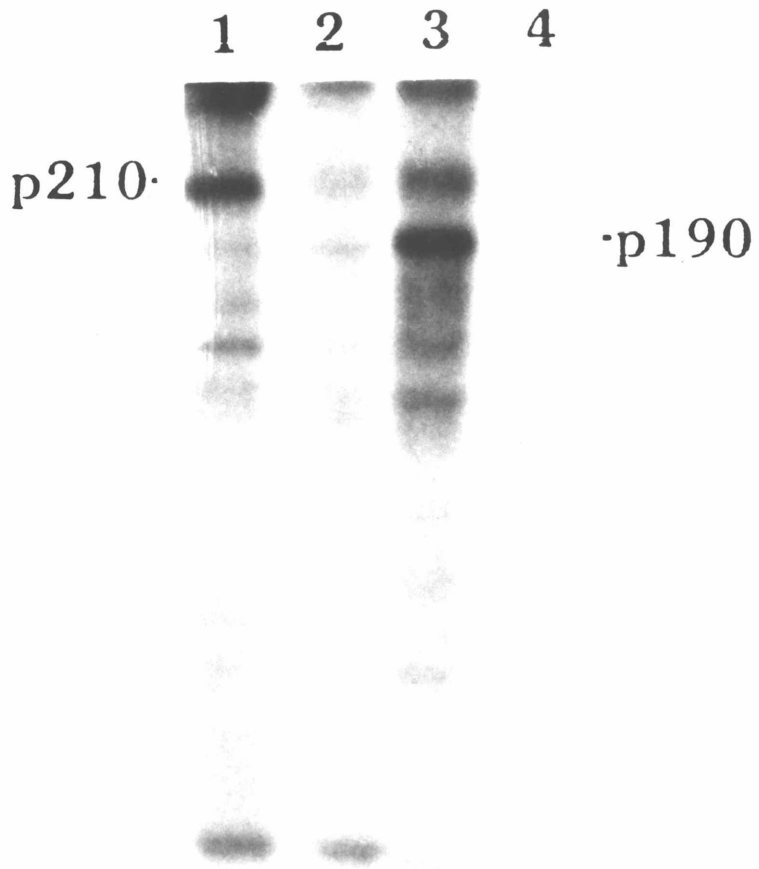


Fig 52 **In vitro assay for PTK in the leukaemic cells of Ph-positive ALL patient.**

The light density cell fraction was isolated on a Percoll discontinuous gradient (Martin et al, 1986) and analysed by the autophosphorylation assay. Lanes 1 and 2: K562; lanes 3 and 4; patient. In lanes 2 and 4 the immunogen peptides were present during the antibody-antigen complex formation step. The apparent band at 210 kd in lane 3 is an artefact. Such a diffuse band of similar size to P210 has also been seen in other studies and generally its precipitation was not prevented by the presence of the immunogen.
(This study was performed by Dr L Wiedeman)

7.3 Ph-POSITIVE ACUTE MYELOID LEUKAEMIA

7.3.1 Case Report

A 41 year old female presented with a short history of fever, cough and symptoms of anaemia in February 1986. There was no preceding history of haematologic illness. On examination she had marked hepatosplenomegaly. Her blood on admission showed a haemoglobin of 8.8 g/dl and platelets of 51 x 10^9/l. The total white cell count (WBC) was 26.5 x 10^9/l with 66% neutrophils, 10% lymphocytes, 2% monocytes, 2% metamyelocytes and 20% blasts. The bone marrow showed 85% infiltration by blasts with deeply basophilic cytoplasm; the nuclei had up to four nucleoli. Occasional blast cells exhibited vacuolation. Intermediate and late normoblasts were absent. Cytochemical staining with periodic acid Schiff and Sudan black were negative, but 100% of the blast cells reacted with a monoclonal antibody for glycophorin A (Fig 53). The blood and bone marrow findings were therefore consistent with AML, FAB-type M6 (erythroleukaemia).

Remission induction therapy was started with CCNU (lomustine) 200 mg/m^2 on day 1, daunorubicin 50 mg/m^2 on day 1 to 3, and cytosine arabinoside 100 mg/m^2 on days 1 to 7 inclusive. Examination of marrow on day 42 following start of therapy confirmed complete haematological remission; the peripheral WBC was 2.8x10^9/l without blasts.

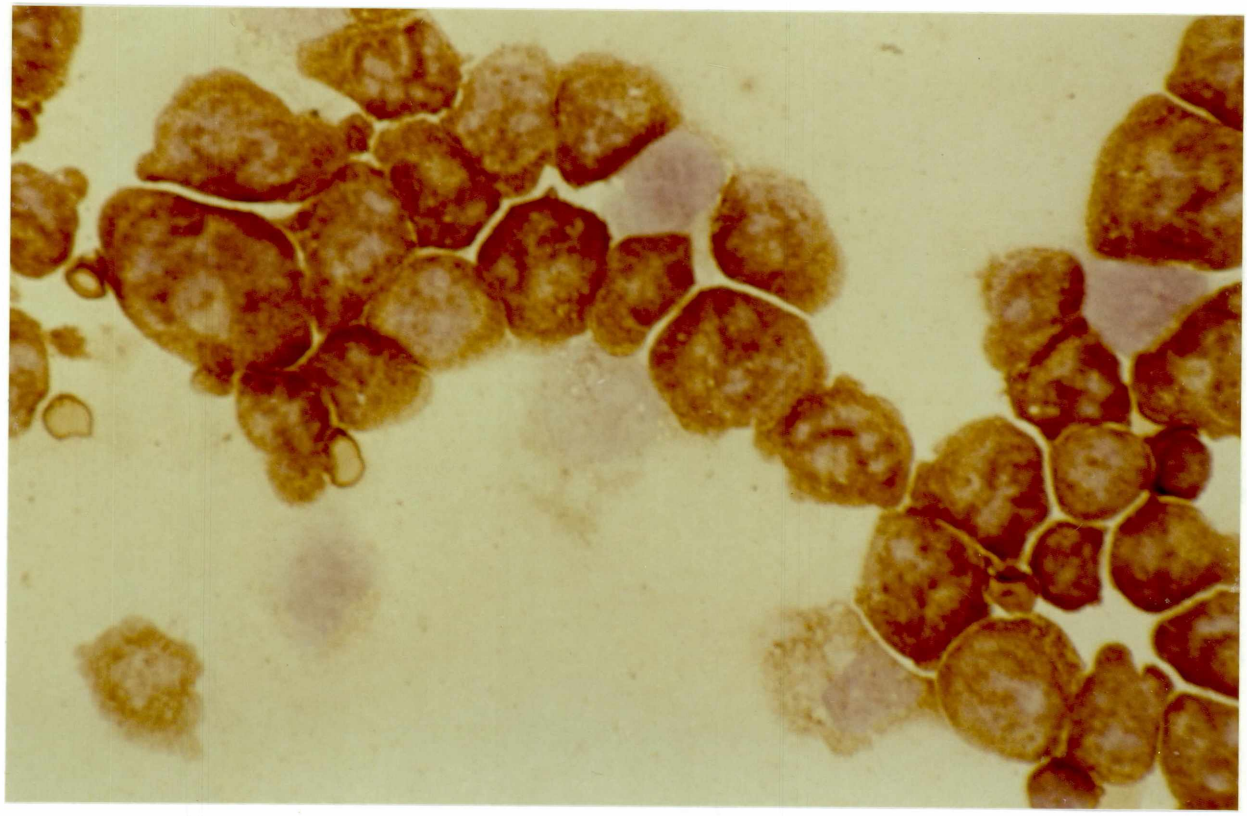


Fig 53 Marker studies in Ph-positive AML patient

Blast cells showing showing alpha-glycophorin positivity by the immunoperoxidase stain.
(This study was performed by R Morilla)

7.3.2 **Cytogenetics**

Cytogenetic analysis at presentation was done on metaphases obtained from direct bone marrow preparations and from peripheral blood at 48 hours (cultured without PHA). 20 metaphases were analysed from the peripheral blood and 10 metaphases from the bone marrow preparations. The patient showed a highly variable karyotype which ranged from 42,XX to 96,XXXX. Most of the metaphases were hyperdiploid with a predominant clone of 48,XX. No normal cells were present. Trisomy 11 and a common chromosome arrangement involving chromosome 1, 4, both chromosomes 9, and chromosome 22 was seen in all cells analysed. Some metaphases carried a duplicated marker chromosome (der 22) which indicated that evolution of the original clone had occurred. Non-clonal abnormalities were also observed and these included trisomy 12, 15 and 17. Many cells contained markers of unknown origin. The involvement of chromosomes 9q34, 22q11, 4q35, and 9p12 suggested that a complex variant translocation might have occurred producing a 'masked' Ph chromosome (Fig 54) (Cytogenetic analysis was done in collaboration with Dr C Price).

To confirm that this der 22 marker chromosome was in fact a 'masked' Ph chromosome I localised the ABL proto-oncogene in the metaphase chromosomes of this patient by in situ hybridisation. The ABL oncogene hybridised to the 9p- chromosome, in its normal position at 9q34 and to the der 22 (Fig 55). In 50 metaphases analysed 11 (11.3%) grains mapped to chromosome 9q34 and 17 (17.5%) grains to der22. The clustering of

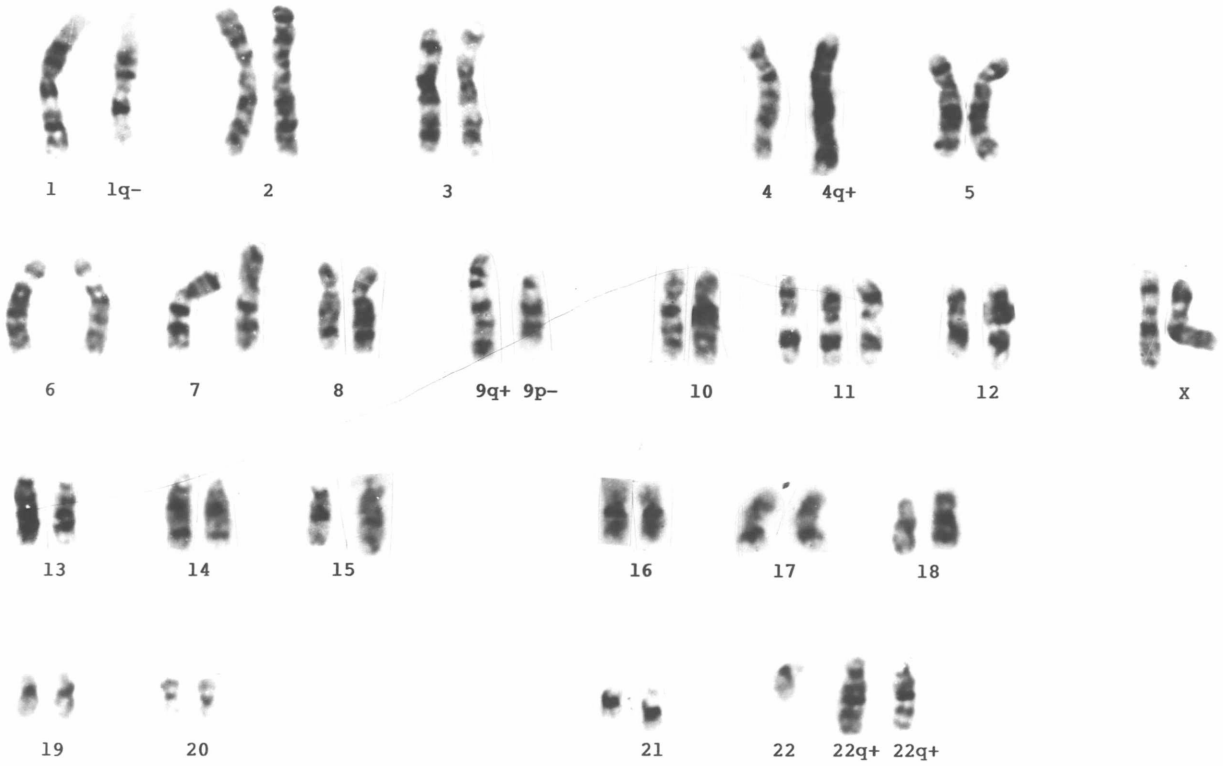


Fig 54 Representative G-banded karyotype showing the 'complex' translocation t(1;4;9;9;22) resulting in a 'masked' Philadelphia chromosome.

The G-banded karyotype of the predominant clone showing trisomy of chromosome 11 and a complex rearrangement involving chromosomes 1, 4, 22 both homologues of chromosome 9 and a duplication of chromosome 11 and the 'masked' Ph [der (22)], t(1;4;9;9;22) +11, + der(22).

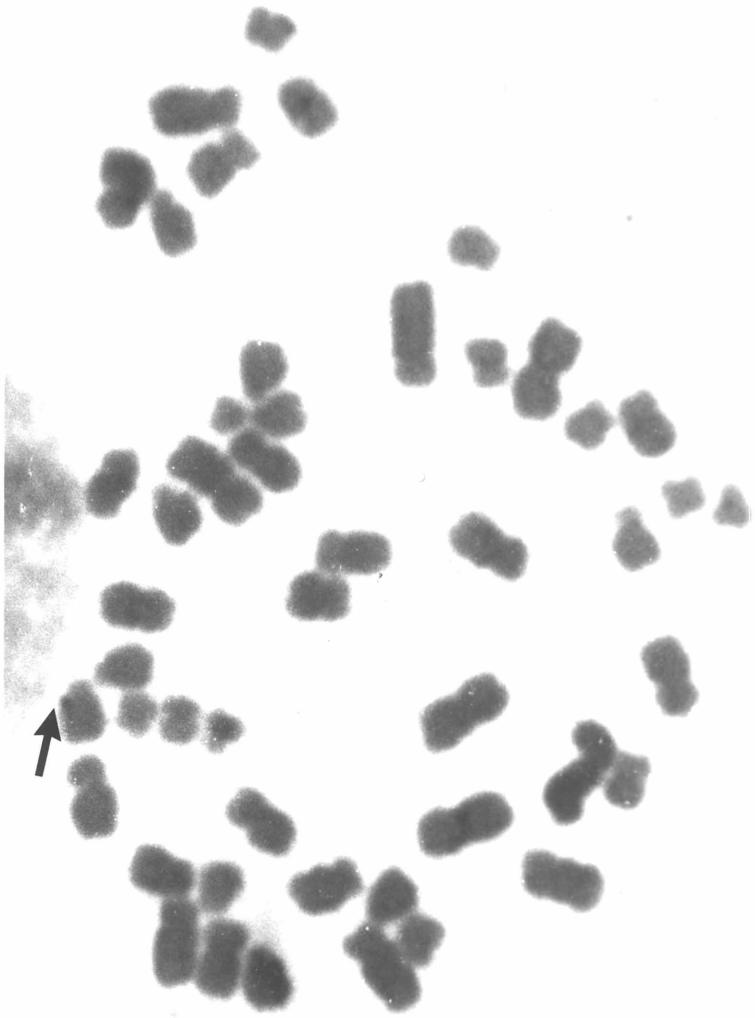


Fig 55 **Localisation of the ABL proto-oncogene to the 'masked' Ph chromosome in a metaphase from Ph-positive AML patient with translocation , t(1;4,9;9;22)**

The black grain on the der 22, indicated by the black arrow, represents the localisation of the ABL proto-oncogene in situ.

grains on chromosomes 9p- and der(22) was significant ($p = 0.008$ and $p < 0.0001$ repectively). This result suggested that a standard Ph translocation had occurred first and subsequently a translocation occurred between the Ph chromosome and chromosome 1 resulting in masking of the Ph chromosome.

Conventional cytogenetic analysis of the G-banded karyotype suggested that the additional material on the 9q+ was not derived from chromosome 22q11-qter. The additional material was dark staining (Giemsa-band positive) whereas the the material derived from chromosome 22, as a result of a standard Ph translocation, was Giemsa band negative. I therefore postulated that further rearrangement had taken place involving chromosome 9q34 and another chromosome. The deletion of most of the p arm of the other chromosome 9 and the aberrant staining at chromosome 4q31 suggested that either chromosome could be a candidate in this rearrangement. It was argued that if there had been a subsequent translocation involving another chromosome the location of the SIS oncogene (translocated to the 9q+ in the standard Ph translocation) would give us some insight into these karyotypic events.

In situ hybridisation of SIS showed that it was located on chromosome 22q23, in its normal position, and on the 4q+ chromosome. In 50 metaphases analysed 12 (12.2%) grains mapped to the chromosome 4q+ and 12 (12.2%) mapped to chromosome 22. The clustering of grains on chromosomes 4q+ and 22 was significant ($p = 0.02$ and $p = 0.0001$). A composite diagram of ABL and SIS proto-oncogene localisation is shown in Fig 56.

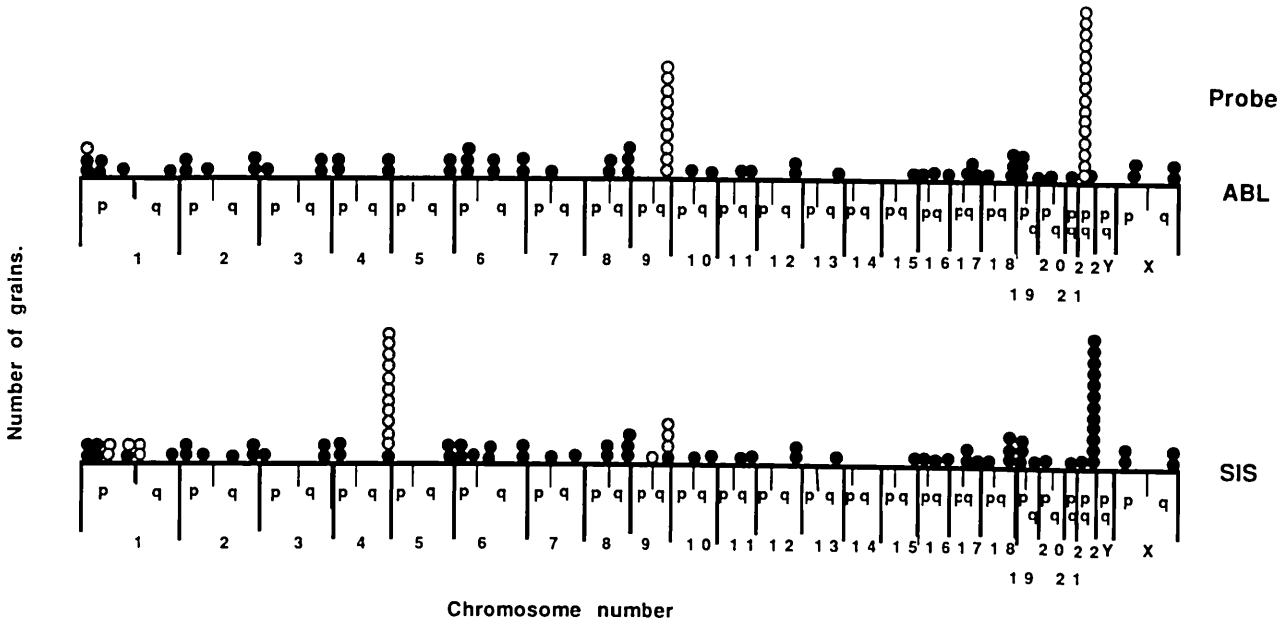


Fig 56 Composite diagram showing the grain distribution in 50 metaphases for each of the proto-oncogene probes: ABL and SIS from AML patient with translocation t(1;4;9;9;22).

The x axes represent the chromosomes in their size proportion; the y axes showthe number of grains. There was localisation of the ABL proto-oncogene tochromosomes 9p- and der(22). The SIS proto-oncogene localised to chromosomes 22q13 and 4p. The stippled circles indicate hybridisation to abnormal chromosomes 9p- and der(22) using the ABL probe and to 4p using the SIS probe.

These in situ hybridisation results made it possible to construct a theoretical sequence of chromosomal exchanges that gave rise to the predominant clone (Fig 57). This was as follows: First a Ph translocation occurred, (9pter 9q34::22q11 22qter; 22pter 22q11::9q34 9qter); secondly a translocation occurred between the Ph chromosome and the q arm of chromosome 1 to form a 'masked' Ph, 1pter 1q24::22q11; 22pter 22q11::1q24 1qter; thirdly a translocation occurred between the chromosome 9q+ (position q34) and the q arm of chromosome 4 (q31), (4pter 4q31::9q34 9qter; 9pter 9q34::4q31 4qter); and fourthly a translocation occurred between the normal chromosome 9 and the derived chromosome 9q+, 9qter 9p13::9ter; 9pter 9qter::9p13 9q34. A duplication of chomosome 11 and the 'masked' Ph also occurred. Trisomy 11 was seen in all the cells analysed, so it presumably occurred around the time of formation of the 'masked' Ph. The karyotype of the predominant clone was interpreted as 48XX, t(1;4;9;9;22), (q24;q31;q34;p13;q11) +11, +'masked' Ph; the duplication of the marker (der 22q-) resulted in a double 'masked' Ph chromosome.

Cytogenetic analysis was done at 2 and 5 months after remission induction therapy. The metaphases from unstimulated bone marrow preparations after 24 hours of culture showed exclusively a normal karyotype (46, XX).

7.3.4 DNA, RNA and protein studies

Southern analysis of DNA by Dr L Wiedemann using a 4.5 kb DNA probe spanning the M-BCR (see Chapter 2, Fig 8) demonstrated a rearranged band

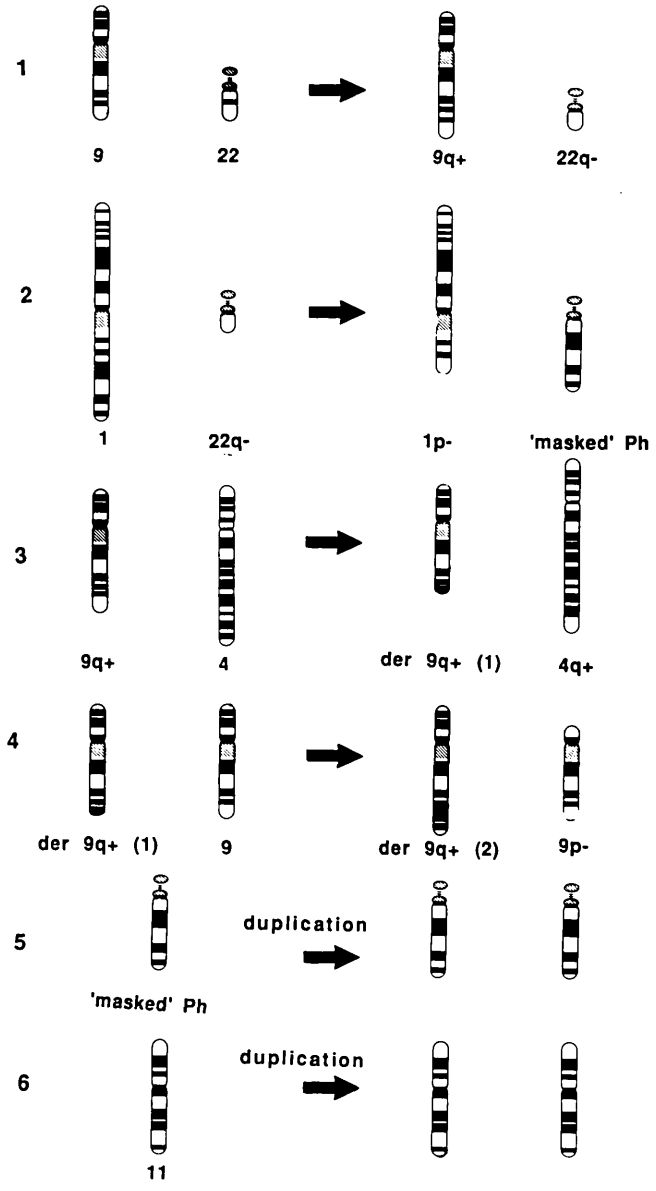


Fig 57 Proposed sequence of chromosomal exchanges in AML patient leading to translocation t(1;4;9;9;22)(q24;q31;q34;p13;q11) including a 'masked' Ph chromosome.

Stage 1 - the Ph translocation
Stage 2 - translocation between chromosomes 1q and 22q- resulting in a 'masked' Ph
Stage 3 - translocation between chromosomes 9q+ and 4
Stage 4 - translocation between chromosomes der 9q+ and normal 9
Stage 5 - duplication of the 'masked' Ph chromosome
Stage 6 - duplication of chromosome 11

in addition to the germline band in the XbaI digest (data not shown). This finding was consistent with rearrangement within the M-BCR region. The presence of an 'active' BCR/ABL chimeric gene was demonstrated by the results of the protein tyrosine kinase (PTK) assay which detected a P210 BCR/ABL fusion protein (data not shown). In order to confirm the presence of the hybrid gene coding for the 210 kd protein with ABL epitopes G Morgan isolated mRNA from this patient and subjected it to specific amplification of the putative junction region using the polymerase chain reaction. An amplified fragment of 394 bp bounded by the primers A/C from BCR and ABL gene sequences was observed, suggesting the presence of a b3a2 junction. The predicted band of 218 bp was observed in the reaction with primer pair B/C. In addition the 394 bp but not the 218 bp band hybridised to the primer (oligo D) spanning the predicted b3a2 junction sequence which was further confirmation of the hybrid identity of the amplified region. A schematic representation of the hybrid mRNA present in the patient's leukaemic cells at presentation is shown in Fig 58.

Analysis of both protein and DNA during haematological and cytogenetic remission showed no evidence of the aberrant ABL-protein or rearrangement of M-BCR (data not shown).

7.3.6 **Discussion**

Patients with Ph positive ALL show two different molecular patterns. Some patients show evidence of rearrangement at the genomic level within M-BCR and these patients have the chimeric BCR/ABL 8.0kb mRNA and P210,

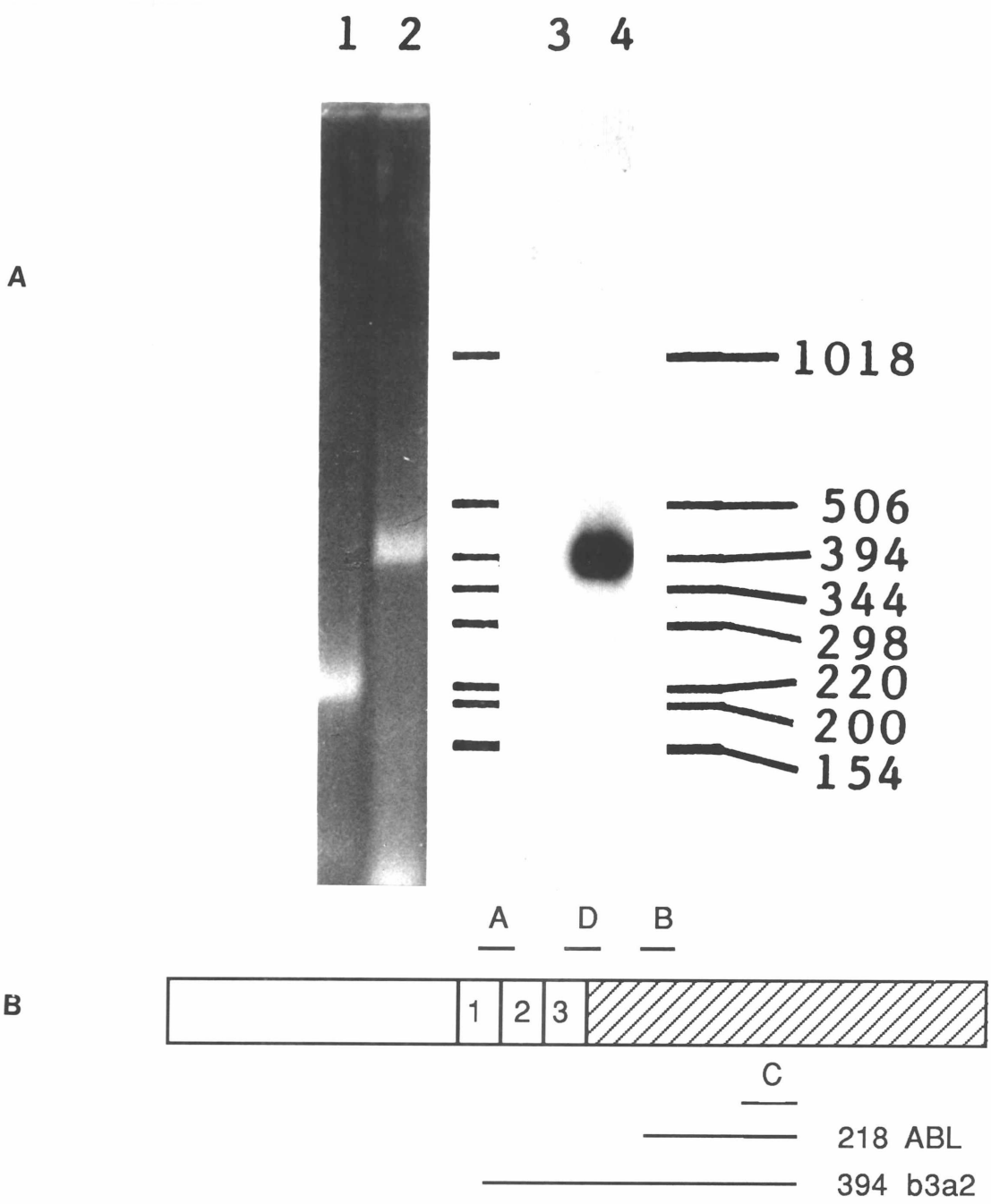


Fig 58 Analysis of m-RNA from the Ph-positive AML patient by the polymerase chain reaction.

(A) Amplified material was size fractionated through a 2% agarose gel and stained with 1 g/ml ethidium bromide and photographed with appropriate size markers (lanes 1 and 2) The material in the gel was then immobilised on Gene Screen Plus and hybridised to oligo D (lanes 3 and 4). This probe spans the b3a2 junction region of the RNA and will hybridise only to b3a2-amplified material under stringent conditions. Lanes 1 and 3 consisted of amplfied material using primer pair B/C and lanes 2 and 4 consisted of amplified material using primer pair A/C. The positions of the DNA mol-wt markers`are given in base pairs.
(B) Schematic of the hybrid mRNA present in the leukaemic cells showing the position of the oligomers used in this study. Sizes of the amplified units for primer pairs B/C (ABL) and A/C (b3a2) are also indicated. Hatched regions represent the ABL sequences; open region represents BCR sequences. Boundaries of the three exons of the BCR gene refer to exons b1, b2 and b3 present within M-BCR.

while other patients lacking M-BCR rearrangement express a P190 (or P185) that also has enhanced PTK activity. The DNA sequence on chromosome 22 that activates ABL appears to include the first exon of the BCR gene; in these patients the genomic breakpoint falls within the first intron of the gene.

The ALL patient reported here had leukaemic cells at diagnosis with phenotypic features of both the myeloid and lymphoid lineages. DNA analysis revealed rearrangement of both the J_H and the TCR chain genes. These changes have been observed in cells from patients with other forms of acute leukaemia and this pattern suggests features of a very early lymphoid precursor cell.

The results of studies with in situ hybridisation of gene probes to metaphase chromosomes made it possible to place the breakpoint on chromosome 22 proximal (5') to M-BCR and distal (3') to the C gene.

Because both the Ph chromosome and the gene rearrangements described above were present in the great majority of blast cells, it was inferred that both coexisted in the same leukaemic clone. Thus as far as I know, this patient is unique in having Ph-positive, BCR rearrangement negative, acute leukaemia with rearrangement of both the J_H and TCR chain genes.

The finding of both Ph positive and Ph negative metaphases in the bone marrow of a patient with acute leukaemia at diagnosis poses questions as to the timing of the acquisition of the Ph chromosome and its precise

role in the pathogenesis of the disease. It is not known if the Ph negative cells in this patient were or were not part of the malignant clone. It has been suggested, for example, that the acquisition of the Ph chromosome may not be the primary neoplastic event in the genesis of CML (Fialkow et al, 1981). These authors showed that some of the Ph negative B cell lines derived from a G6PD heterozygous female with CML had a variety of clonal abnormalities. They took this as evidence that in that patient a clonal expansion of leukaemic cells had preceded the acquisition of the Ph chromosome. This sequence of events may or may not apply to other cases of leukaemia including Ph positive acute leukaemia.

Cytogenetic analysis at the time of relapse showed that some of the Ph negative cells had acquired an extra chromosome 19. Trisomy 19 commonly occurs as a secondary karyotypic change in CML in blastic transformation and in some cases of acute leukaemia. This clonal change in the Ph negative cell population suggests the existence of a cytogenetically normal population of cells that may have been part of the malignant process and may represent a clonal population susceptible to the acquisition of further cytogenetic (or genetic) changes. Alternatively, it is possible that the same microenvironmental or other external factors acted on a normal population of haemopoietic cells to induce the Ph chromosome on the one hand and the trisomy 19 on the other.

One case of Ph positive AML with M-BCR rearrangement has been reported previously but detailed haematological and protein data were not provided (Erikson et al, 1986). I have studied a 'masked' Ph positive

AML which showed BCR rearrangement and expression of a P210.
It might be argued that this patient is not actually a Ph positive AML arising de novo but rather CML in erythroid transformation. However,the absence of features in the peripheral blood and bone marrow (eg, eosinophilia, basophilia,and/or thrombocytosis) that might have suggested an occult preceding CML, the presence of morphological abnormalities in the erythroid series and, most importantly, the ease with which both haematological and cytogenetic remission was achieved are consistent with de novo AML. It therefore is probable that the association of the P210 BCR/ABL with CML and the P190 BCR/ABL with the acute leukaemias is a loose one and that other secondary events must be responsible for determining the predominant malignant phenotype.

The molecular changes underlying the formation of the t(9;22) are conserved in masked Ph translocations despite extensive karyotypic changes (Alimena et al, 1987a). It could be speculated that one or other additional chromosome changes could activate another gene (or protooncogene) involved in specifying the lineage for malignant transformation. Although no specific chromosomal translocations have yet been correlated with erythroleukaemia, CML in erythroid transformation and AML M6 may both be associated with bizarre disruptions of the karyotype (Alimena et al, 1987b).

Recent reports have suggested that the position of breakpoints in complex translocations is non random (De Braekeleer, 1986, Huret et al, 1986, Maserati et al, 1986). In a recent review De Braekeleer et al (1986) reported that in complex translocations 28 chromosome bands were

rearranged more frequently than expected by chance. Twenty-seven bands were also associated with fragile sites, known oncogene locations or breakpoints common in other malignancies.

The complex translocation resulting in a 'masked' Ph chromosome in our patient was interpreted as 48,XX, t(1;4;9;9;22), (q24;q31;q34;p13;q11) +11, +der(22). The breakpoints other than 9q34 and 22q11 occurred at chromosomes 1q24, 4q31,and 9p13. 'Masked' Ph translocations involving a breakpoint at chromosome 1q24 have been reported in CML by Najfeld et al (1983). Brito-Babapulle et al (1985) reported the non random association of aberrations of chromosome 1 with human neoplasia. Translocations involving chromosome 4q31 have been reported in several cases of ALL (Mitelman et al, 1986) and chromosome 4q31 is the position of a fragile site (Tedeschi et al, 1987). These sites of fragility have been implicated in the predisposition to chromosomal rearrangements associated with malignancy (Yunis J, 1984, Le Beau, 1986). The deletion of the short arm of chromosome 9 (del[9p]) is frequently observed in malignant cells of patients with ALL and non-Hodgkin's lymphoma (Chilcote et al, 1985, Kowalczyk and Sandberg, 1983, Pollak and Hagemeijer, 1987). Recently Diaz et al (1988) reported the homozygous deletion of the interferon genes and the deficiency of 5'-methylthioadenosine phosphorylase (an enzyme of purine metabolism), and have suggested that these deletions may be associated with the loss of a tumour suppressor gene that is involved in the development of these neoplasia. The other clonal chromosomal abnormality in our patient was trisomy of chromosome 11. The proto oncogene H-RAS is located at chromosome 11p15 (Jhanwar et al, 1984).

Thus I speculate that one or other of the additional cytogenetic changes that occurred in this patient's leukaemic cells might have led to the activation of other oncogenes - events which might have dictated to some extent the phenotypic characteristics of this leukaemia.

CHAPTER 8: GENERAL CONCLUSIONS

8.1 THE JUSTIFICATION FOR THE NEW NOMENCLATURE

Various recent reports demonstrating that a small minority of patients with apparently typical Ph-positive CML have breakpoints outside the classical M-BCR (and incidentally outside the first intron of the BCR gene) suggest that there may be additional (minor) breakpoint cluster regions in the gene. For example Selleri et al (1987) demonstrated that in three Ph-positive CML patients the breakpoints on chromosome 22 were located neither within M-BCR nor immediately upstream of it. Northern blot analysis of the mRNA from two of these patients showed the absence of a detectable 8.5 kb chimeric mRNA. In the third patient a chimeric mRNA was detected by an ABL cDNA probe but failed to hybridise with the conventional BCR cDNA. Moreover Saglio et al (1988) reported seven Ph-positive CML patients whose breakpoint on chromosome 22 occurred outside BCR. In two of these cases the breakpoint was mapped between 9 and 12 kb proximal to M-BCR. In another case the breakpoint was located approximately 16 kb distal to M-BCR. In the remaining four cases, the precise position of the breakpoint could not be localised with the available BCR probes.

We have identified one patient with Ph-positive CML (out of a total of 67 studied at the DNA level) who appeared to have genomic rearrangements outside M-BCR. This patient has now been studied in some detail. High molecular weight DNA extracted from the leukaemic cells showed no rearrangement by Southern blotting using two different probes for M-BCR

but a rearranged band could be shown by pulse field gel electrophoresis (Gao Ling Min unpublished data). In situ hybridisation using a DNA probe for M-BCR localised BCR sequences on the normal chromosome 22 and the Ph chromosome, whereas the 'full length' BCR probe localised to the normal 22, the Ph chromosome and the chromosome 9q+. These results suggested that the breakpoint on chromosome 22 in this patient occurred distal to M-BCR but within the BCR gene (unpublished data). ABL sequences on the other hand were localised both to the normal chromosome 9 and the Ph chromosome. Taken together, these data suggest that in a minority of Ph-positive CML patients breakpoints may occur in at least two other regions within the BCR gene. These breakpoints may be the sites of formation of new chimeric BCR-ABL genes. Therefore using the nomenclature outlined in Chapter 1, the putative breakpoint cluster region which occurs 5' of M-BCR would be designated m-BCR-2 and the putative breakpoint cluster region which occurs 3' of M-BCR would be designated m-BCR-3.

The inability of Selleri et al (1987) and Saglio et al (1988) to demonstrate BCR rearrangement with available probes for the BCR gene in some of these Ph-positive, M-BCR rearrangement-negative CML patients has two possible explanations; either the breakpoints occur in the part of the BCR gene not yet completely characterised or the breakpoints occur in another gene altogether. Regions 5'and 3' of M-BCR have not been completely sequenced and cloned and we still do not know the precise number of exons contained in the BCR gene. These putative m-BCRs would result in the formation of new BCR-ABL chimeric proteins, shorter or longer than the P210 depending on which side of M-BCR these breakpoints

occurred. Selleri et al (1987) also were unable to detect a chimeric mRNA using ABL cDNA probes in two Ph-positive, M-BCR rearrangement-negative CML patients. Bartram et al (1985) on the other hand described a CML patient with rearrangement of M-BCR but without juxtaposition of the ABL proto-oncogene. These data suggest that the ABL oncogene is not involved in these particular genetic rearrangements and that another gene on chromosome 9q34 might play a role in the pathogenesis of this disease.

8.2 Ph-NEGATIVE CML

8.2.1 Significance of investigation

I studied a group of patients with Ph-negative CML whose disease resembled Ph-positive CML to a greater or lesser extent. Some patients were indistinguishable from Ph-positive CML on the basis of morphology, peripheral blood films and clinical details. These patients also had rearrangement of M-BCR, the ABL oncogene translocated to chromosome 22, and expression of the BCR-ABL chimeric message. These observations support the suggestion that Ph-negative, M-BCR-positive CML can be distinguished from Ph-negative, M-BCR negative CML by careful evaluation of clinical and haematological data in the absence of a Ph chromosome (Wiedemann et al, 1987, Morris et al, 1986, Shepherd et al, 1988).

However the same BCR/ABL chimeric gene was also found in patients who had some features not typical of Ph-positive CML. The biological implications of these studies are profound because they cast doubt on

the extent to which the haematological manifestations in CML are determined merely by the formation of the BCR/ABL gene. For these cases it is likely that the phenotypic diversity is generated by variations in other genes.

The localisation of ABL oncogene sequences on chromosome 22 without the concomitant movement of 3'BCR gene and SIS oncogene sequences suggested that the chimeric BCR/ABL gene in these patients was formed by a mechanism of interstitial insertion. The localisation of two RFLP probes that recognise different polymorphic regions distal to ABL on chromosome 9 in three Ph-negative patients gave us more information as to the position of the 'distal' breakpoint on chromosome 9. In one patient the breakpoint was within 6 cM of the ABL oncogene, while in two patients the breakpoint was at least 17 cM downstream of the ABL oncogene. Therefore it seems that a variable amount of ABL-containing chromosome material is inserted into chromosome 22q11 to make a functional BCR/ABL chimeric gene in Ph-negative CML. This chromosomal material could not be identified by conventional cytogenetic methods.

One patient with features not typical of the Ph-positive CML, did not have M-BCR rearrangement, or translocation of ABL into M-BCR on chromosome 22. These preliminary studies suggest that the formation of the BCR/ABL chimeric gene does not play a significant role in the development of this disease and that other genetic aberrations are responsible.

8.2.2 Future investigations

The next step in the investigation of the mechanism by which the ABL oncogene is inserted into into M-BCR is to clone and sequence the 'distal' breakpoint on chromosome 9 (9 22). The fact that the 3' sequences of the BCR gene remain in their normal position on chromosome 22 and ABL-containing sequences are inserted into M-BCR should enable us to clone the 'distal' breakpoint with relative ease. DNA probes for the BCR gene 3' of M-BCR can be used to clone sequences upstream on chromosome 22q11. Chromosome 9 sequences and therefore the 'distal' breakpoint could be identified by a comparison of these cloned nucleotide sequences with published BCR nucleotide sequences. A knowledge of these sequences may elucidate the amount of ABL-containing genetic material that is interstitially inserted. There may be 'signal' sequences on chromosome 9q34 distal to ABL homologous to sequences flanking the BCR gene and translocation of ABL may involve a mechanism of homologous recombination (Borzillo et al, 1987).

Some Ph-negative, M-BCR negative CML patients have the ABL proto-oncogene only in its normal position on chromosome 9 and the leukaemia must therefore have a genetic basis that does not involve formation of the chimeric BCR/ABL gene. A study to investigate the genetic changes that occur in this disease is necessary. Because of the random nature of the search for genetic alteration in these patients it is necessary initially to exclude involvement of the BCR gene. There may be formation of a new chimeric gene consisting of BCR sequences juxtaposed

with a gene other than ABL. A number of probes corresponding to the regions 3' and 5' of M-BCR are now available so genomic rearrangement of these areas should be easy to establish. If in fact there is rearrangement of the BCR gene in these patients then cloning of these breakpoints would be the next step to establish the presence or absence of any 'alien' sequences.

If however there is no involvement of the BCR gene in this disease then another approach would be to search for activated oncogenes by the technique of DNA-mediated transfection. Between 15 and 50% of human tumours and tumour cell lines analysed by this technique have been found to contain genes which are able to transform NIH/3T3 cells (review by Cooper, 1982). If however the oncogeneic potential of this unknown gene resembles that of the BCR/ABL chimeric gene then the gene product will not readily transform NIH 3T3 cells (Daley et al, 1987). Therefore another approach to the study of other 'activated' genes in CML must be sought.

Yet another approach to an investigation of Ph-negative, M-BCR-negative patients would be to look for genetic alteration of candidate oncogenes. The expression of these genes may be altered by various means including gene rearrangement, amplification, point mutation and insertion of promotor or enhancer elements (review by Bishop M, 1983).
Candidate oncogenes could be chosen on the basis of two criteria. Firstly, oncogenes expressed abnormally in other haematologic malignancies may also be 'activated' in this disease. For example the MYC proto-oncogene transcriptionally activated in Burkitt's lymphoma may

also be expressed inappropriately in this disease (Croce et al, 1984). Secondly other oncogenes that have a similar site of action and kinase activity to ABL may be involved. The ABL proto-oncogene like approximately one third of the known oncogenes, encodes a protein that phoshorylates tyrosine residues on other proteins. Other oncogenes with tyrosine specific protein activity include SRC (the normal cellular homologue of the Rous avian sarcoma virus), YES (the normal cellular homologue of the Yamaguchi avian sarcoma virus) and FES (the normal homologue of the feline sarcoma virus). Daley et al (1987) suggested that the substrate of the oncogenic ABL protein may be localised to the cell membrane. Other oncogene products which also bind to the plasma membrane include SRC and RAS (the normal cellular homologue of the Harvey and Kirsten murine sarcoma virus).

8.3 CML WITH VARIANT Ph TRANSLOCATIONS

8.3.1 Significance of investigation

The study of variant translocations of CML by the technique of in situ hybridisation served to elucidate the many and complex chromosome changes that had occurred, some of which could not be interpreted by standard cytogenetic analysis. These investigations primarily confirmed the involvement of both chromosome 9 and 22 and the rearrangement of M-BCR. These data are in line with the view that the clinical, haematological and molecular features of CML patients with variant translocations do not differ significantly from those with a classical Ph chromosome.

8.3.2 Future investigations

The significance of the correlation between fragile sites, the position of oncogenes and the breakpoints in variant Ph-translocations needs to be established. It would be important to know whether the somatic cells of these patients have inherent sites of chromosomal fragility which coincide with the chromosomal breakpoints seen in the leukaemic cells. To this end skin fibroblasts can be grown in folate deficient media and examined for fragile sites (Le Beau, 1986). It may then be determined whether the chromosomes of these patients are predisposed to breakage and hence rearrangement in these regions.

The fact that the nature and course of disease between CML patients with standard and variant translocations does not differ significantly implies that the other genetic changes, if they do indeed occur, are secondary and do not affect the phenotype in an obvious way. A study of the mRNA and protein expressed by the oncogenes located at these breakpoints may show differences between CMLs with a standard Ph chromosome and those with a variant Ph translocation.

8.4 BLAST CRISIS OF CML

8.4.1 Significance of investigation

I found no amplification of ABL and MYC proto-oncogenes in blast crisis CML compared to the levels seen in the K562 and HL60 cell lines. However in situ hybridisation of an ABL probe to the metaphases of a blast crisis patient with a Ph translocation and an extra marker chromosome demonstrated ABL sequences on the Ph chromosome and also on the marker chromosome. Although a second Ph chromosome apparently identical to the original Ph chromosome may be seen in transformation, our data indicate that duplication or indeed triplication of a gene may be achieved by other mechanisms. This marker chromosome containing both BCR and ABL sequences was also demonstrated in the cell line derived from this patient. Similar amplification not clearly related to the Ph chromosome is seen in K562 cells, but whether such amplification was present in the fresh cells is not known. In K562 the unit of amplification encompasses virtually a complete cytogenetic sub-band, extending as far up stream as the C immunoglobulin gene locus. This may also be the case in patient 1. I conclude that amplification of BCR/ABL or an unknown gene on the marker chromosome in vivo may contribute in some cases to the transformation of CML.

8.4.2 Future directions

The effect of amplification of the BCR/ABL chimeric gene on the levels of mRNA and protein produced is still unclear and a comprehensive study of BCR/ABL message and protein expression in CML chronic phase and blast crisis is necessary. In studies of mRNA expression in CML patients some investigators have found no difference in the levels of BCR/ABL mRNA transcription between chronic phase and blast crisis (Shtivelman et al, 1987). Other investigators have found a 16-fold increase in expression of BCR/ABL mRNA in the blast cells of a CML patient (Collins et al, 1987). It is not known whether there is an increase in expression of the BCR/ABL protein in blast crisis CML. There may be further enhancement of ABL tyrosine kinase activity in blast crisis CML which could alter the transforming activity of the protein from that of weakly transforming to more acutely transforming. Injecting the complete BCR/ABL cDNA contained in murine retroviruses into nude mice (BALB/c) Young and Witte (1988) showed that there was a clonal outgrowth of B lymphoid cells expressing the P210 BCR/ABL protein but that these cells had a limited transformation potential (Young and Witte, 1988).

8.5 **Ph-POSITIVE ACUTE LEUKAEMIA**

8.5.1 Significance of investigation

The study of two unique Ph-positive acute leukaemia patients helped elucidate the genetic changes that occur in these patients and also provided some insight into the biology of the disease.

Southern analysis of DNA from the Ph-positive ALL patient at diagnosis showed no rearrangement of M-BCR. However the cells expressed an ABL-related protein of Mwt 190 kd with enhanced tyrosine kinase activity. In situ hybridisation to Ph-positive metaphase chromosomes demonstrated that the ABL proto-oncogene mapped to chromosome 22q11, 5' BCR mapped to the chromosome 9q+ and the C immunoglobulin mapped to chromosome 22q11. Thus the genomic breakpoint in this patient lay upstream of M-BCR. In situ hybridisation in the Ph-negative metaphases demonstrated that the ABL, 5'BCR and C genes were in their normal positions and had not moved. However cytogenetic analysis at relapse showed an extra chromosome 19 in the Ph-negative population of cells. These cells may represent a leukaemic proliferation susceptible to specific chromosomal changes.

The study of a patient with AML (FAB M6) arising de novo by cytogenetic and in situ hybridisation demonstrated a masked Ph chromosome. The leukaemic cells initially showed a rearrangement of M-BCR, the presence of a hybrid mRNA, and expression of the P210 BCR/ABL. These changes were absent in remission. These results and the finding of a P190 BCR/ABL in Ph-positive AML (Kurzrock et al, 1987) support the concept that the BCR/ABL chimeric gene plays a crucial role in leukaemogenesis but suggest that factors other than the position of the breakpoint in the BCR gene may determine the lineage of the target cell for malignant transformation. Therefore the specificity of lineage and type (chronic or acute) of Ph-positive leukaemia may be dependent on other tumorigenic events superimposed on BCR/ABL rearrangements.

8.5.2 Future directions

In patients with an initial diagnosis of Ph-positive acute leukaemia and no history of CML, the cell origin and extent of lineage involvement of the disease is often unclear. The cytogenetic studies of Kalousek et al (1988) on granulocyte-macrophage colonies support the concept that patients presenting with Ph-positive ALL comprise a heterogeneous group. In some cases the disease may arise in a restricted lymphopoietic cell not capable of myeloid differentiation and in others involvement of myeloid cells suggest these may be variant forms of CML. Furthermore Turhan et al (1988) found genomic rearrangement in the purified granulocytes in two out of three Ph-positive ALL patients. The patient who exhibited no M-BCR rearrangement in the granulocytes, however showed rearrangement in blast cells. These findings demonstrate the origin of the disease in the first two patients in a cell with myelopoietic as well as lymphopoietic potential and confirm a restricted lymphoid cell origin of the neoplastic clone in the third patient. It also indicates that different target cells for transformation within the haematopoietic system may be affected by very similar BCR (both M-BCR and m-BCR) rearrangements. Preliminary investigation of Ph-positive AML suggests that in these patients the Ph chromosome originated in a multipotent stem cell. Chen et al (1988) found the blasts positive for both lymphoid and myeloid antigenic markers in all Ph-positive AML patients studied. They also showed the presence of both breakpoints within M-BCR and within exon one of the BCR gene in these patients.

Further definition of the lineages involved in both Ph-positive AML and ALL is necessary .

References

Alimena G, Dallapiccola B, Gastaldi R et al. Chromosomal, morphological and clinical correlations in the blastic crisis of chronic myeloid leukemia. A study of 69 cases. Scand. J. Haem. 1982; 28: 103-117.

Alimena G, de Cuia MR, Diverio D et al. The karyotype of blastic crisis. Cancer Genet. Cytogenet. 1987a; 26: 39-50.

Alimena G., Hagemeijer A, Bakhius J et al. Cytogenetic and molecular characterisation of a masked Philadelphia chromosome in chronic myelocytic leukemia. Cancer Genet. Cytogenet. 1987b; 27: 21-26.

Andrews DF, Collins SJ. Heterogeneity in expression of the bcr-abl fusion transcript in CML blast crisis. Leukemia 1987; 1: 718-724.

Arthur DC, Bloomfield CD. Partial deletion of the long arm of chromosome 16 and bone marrow eosinophilia in acute non-lymphocytic leukemia: A new association. Blood 1983; 61: 994-998.

Baikie AG, Court Brown WM, Buckton KE et al. A possible specific chromosome abnormality in human chronic myeloid leukaemia. Nature 1960; 188: 1165-1166.

Barbi G, Steinbach P, Vogel W. Non random distribution of methotrexate-induced abberations on human chromosomes. Detection of further folic acid sensitive sites. Hum. Genet. 1984; 68: 290-294.

Barnes D and Sato G. Serum-free cell culture: a unifying approach. Cell 1980; 22: 649-655

Bartram CR, de Klein A, Hagemeijer A et al. Localization of the c-sis oncogene in Ph-positive and Ph-negative chronic myelocytic leukemia by in situ hybridization. Blood 1981; 63: 223-225.

Bartram CR, de Klein A, Hagemeijer A et al. Translocation of c-abl oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. Nature 1983; 306: 277-280.

Bartram CR, Kleihauer C, de Klein A et al. c-abl and bcr are rearranged in a Ph'-negative CML patient. EMBO J. 1985a; 4: 683-686.

Bartram CR. bcr rearrangement without juxtaposition of c-abl in chronic myelocytic leukemia. J. Exp. Med. 1985b; 162: 2175-2179.

Bartram CR, Carbonell F. bcr rearrangement in Ph'-negative CML. Cancer Genet. Cytogenet. 1986; 21: 183-184.

Bartram CR, de Klein A, Hagemeijer A et al. Additional c-abl-bcr rearrangements in a CML patient exhibiting two Ph chromosomes during blast crisis. Leuk. Res. 1986; 10: 221-225.

Le Beau MM, Larson RA, Bitter MA et al. Association of an inversion of chromosome 16 with abnormal eosinophils in acute myelomoncytic leukemia: a unique cytogenetic clinicopathological association. New Eng. J Med. 1983; 309: 630.

Le Beau MM, Westbrook CA, Diaz M, Rowley JD. Evidence for two distinct c-src loci on human chromosomes 1 and 20. Nature 1984; 312: 70-71.

Le Beau MM, Rowley JD. Heritable fragile sites in cancer. Nature 1984; 308: 607-608.

Le Beau M.M, Diaz M.O., Karin M., Rowley J.D. Metallothionein gene cluster is split by chromosome 16 rearrangements in myelomonocytic leukaemia. Nature 1985a; 313:709-711.

Le Beau MM, Westbrook CA, Diaz MD, Rowley JD. c-src is consistently conserved in the chromosomal deletion 20q observed in myeloid disorders. Proc. Natl. Sci. USA 1985b; 82: 6692-6696.

Le Beau MM. Chromosomal fragile sites and cancer specific breakpoints. Blood 1986; 67: 849-859.

Ben-neriah Y, Daley GQ, Mes-messon AM, Witte ON, Baltimore D. The chronic myelogenous leukemia specific P210 protein is the product of the bcr/abl hybrid gene. Science 1986; 233: 212-219.

Bernstein R, Morcom G, Pinto MR et al. Cytogenetic findings in chronic myeloid leukemia (CML): Evaluation of karyotype, blast morphology and survival in the acute phase. Cancer Genet. Cytogenet. 1980; 2: 23-27.

Bishop MJ. Cellular oncogenes and retroviruses. Ann. Rev. Biochem. 1983; 52: 301-354.

Bloomfield CD, Lindquist LL, Brunning RD, Yunis JJ, Coccia PF. The Philadelphia chromosome in acute leukemia. Virchows Archiv Biol. 1978; 29: 81-91.

Bloomfield CD, Goldman AI, Alimena G. Chromosomal abnormalities identify high-risk and low-risk patients with acute lymphoblastic leukemia. Blood 1986; 67: 415-420.

Borzillo GV, Cooper MD, Kabazawa H et al. Isotype switching in Human B lymphocyte malignancies occurs by DNA deletion: Evidence for non-specific switch recombination. J. Immunol. 139: 1326-1335.

Boyd Y, Buckle V, Choo K, Migeon B, Craig I. Assignment of the haemophilia B (factor 9) locus to q26-qter region of the X chromosome. Ann. Hum. Genet. 1984; 48: 145-152.

Brodeur GM, Seeger RC, Schwab M et al. Amplification of N-myc in untreated neuroblastomas correlates with advanced disease stage. Science1984; 224: 1121-1124.

Brito-Babapulle V, Atkin WB. Breakpoints in chromosome 1. Abnormalities of 218 human neoplasms. Cancer Genet. Cytogenet. 1981; 4: 215-225.

Cannani E, Gale RP, Steiner-Saltz D et al. Altered transcription of an oncogene in chronic myelogenous leukaemia. Lancet 1984; i: 593-595.

Caspersson T, Zech L, Johansson C, and Modest EJ. Identification of human chromosomes by DNA-binding fluorescent agents. Chromosoma 1970a; 30: 215-227.

Caspersson T, Gahrton G, Lindsten J, Zech L. Identification of the Philadelphia chromosome as a number 22 by quinacrine mustard fluorescence analysis. Exp. Cell Res. 1970b; 63: 238-240.

Catovsky D. Ph'-positive acute leukaemia and acute granulocytic leukaemia: one or two diseases? Brit. J. Haematol. 1979; 42: 492-498.

Cervantes F, Ballesta F, Mila M, Rozman C. Cytogenetic studies in blast crisis of Ph-positive chronic granulocytic leukemia: Results and prognostic evaluation in 52 patients. Cancer Genet. Cytogenet. 1986; 21: 239-246.

Chan LC, Karhi K, Rayter S et al. A novel abl protein expressed in Philadelphia chromosome positive acute lymphoblastic leukemia. Nature 1987; 325: 635-637.

Chen SJ, Chen Z, Grausz D et al. Molecular cloning of a 5' segment of the genomic phl gene defines a new breakpoint cluster region (bcr2) in Philadelphia-positive acute leukemias. Leukaemia 1988; 2: 261-273.

Chen SJ, Chen Z, Hillion J et al. Ph1-positive, bcr-negative acute leukemias: Clustering of breakpoints on chromosome 22 in the 3' end of the BCR gene first intron. Blood. 1989; 73: 1312-1315.

Chikkappa G, Borner G, Burlington H et al. Periodic oscillation of blood leukocytes and reticulocytes in a patient with chronic myelocytic leukemia. Blood 1976; 47: 1023-1030.

Chilcote RR, Brown E, Rowley JD. Lymphoblastic leukemia with lymphomatous features associated with abnormalities of the short arm of chromosome 9. N. Engl. J. Med. 1985; 313: 286-291.

Clark S, Mc Laughlin J, Christ WM, Champlin R, Witte ON. Unique forms of the abl oncogene tyrosine kinase distinguish Ph'-positive CML from Ph'-positive ALL. Science 1987; 235: 85-88.

Clein JP, Flemens RJ. Involvement of the erythroid series in blastic crisis of chronic myeloid leukaemia. Further evidence for the presence of the Philadelphia chromosome in erythroblasts. Brit. J. Haematol. 1966; 12: 754-758.

Collins SJ, Groudine MT. Rearrangement and amplification of c-abl gene sequences in human chronic myelogenous leukemia cell-line K562. Proc. Natl. Acad. Sci. USA 1983; 80: 4813-4817.

Collins SJ, Groudine MT. Chronic myelogenous leukemia: Amplification of a rearranged c-abl oncogene in both chronic phase and blast crisis. Blood 1987; 69: 893-898.

Cooper GM. Cellular transforming genes. Science; 1982; 217: 801-806.

Croce CM, Erikson J, ar-Rushidi A et al. Translocated c-myc oncogene of Burkitt lymphoma is transcribed in plasma cells and repressed in lymphoblastoid cells. Proc. Natl. Acad. Sci. USA 1984; 81:3170-3174.

Daley GQ, McLaughlin J, Witte ON. The CML-specific P210 bcr/abl protein unlike v-abl does not transform NIH 3T3 fibroblasts. Science 1987; 237: 532-535.

Dalla-Favera R, Gallo RC, Giallong A, Croce CM. Chromosomal localization of the human homologue (c-sis) of the simian sarcoma virus onc gene. Science 1982; 218: 686-688.

de Braekeleer M. Variant Philadelphia translocations in chronic myeloid leukemia. Cytogenet. Cell Genet. 1987; 44: 215-222.

de Klein A, van Kessel AG, Grosveld G et al. A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelogenous leukemia. Nature 1982; 300: 765-767.

de Klein A, Hagemeijer A. Cytogenetic and molecular analysis of the Ph translocation in chronic myeloid leukemia. Cancer Surveys 1984; 3: 515-529.

de Klein A, Hagemeijer A, Bartram CR et al. bcr rearrangement and translocation of c-abl oncogene in Philadelphia positive acute lymphoblastic leukemia. Blood 1986; 68: 1369-1375.

de Taisne G, Gegonne A, Stehelin D et al. Chromosomal localisation of the human proto-oncogene c-ets. Nature 1984; 310: 581-585.

Diaz MO, Ziemans L, Le Beau MM. Homozygous deletion of the alpha and beta 1-interferon genes in human leukemia and derived cell lines. Proc. Natl. Acad. Sci. USA 1988; 85: 5259-5263.

Donis-Keller H, Green P, Helms C. et al. A genetic linkage map of the human genome. Cell 1987; 51: 319-337.

Dreazen O, Klisak I, Rassool F et al. Do oncogenes determine clinical features in chronic myeloid leukaemia? Lancet 1987a; i: 1402-1405.

Dreazen O, Klisak I, Jones G et al. Multiple molecular abnormalities in Ph-positive acute lymphoblastic leukaemia. Brit. J. Haematol. 1987b; 67: 319-324.

Dube ID, Eaves CJ, Kalousek DK et al. A method for obtaining high quality chromosome preparations from single hemopoietic colonies on a routine basis. Cancer Genet. Cytogenet. 1981; 4: 157-168.

Dutrillaux B, Viegas-Pequignot E. High resolution R and G banding on the same preparation. Hum. Genet. 1981; 57: 93-95.

Erikson J, Griffen C.A, ar- Rushdi A et al. Heterogeneity of chromosome 22 breakpoint in Philadelphia positive (Ph+) acute lymphocytic leukemia. Proc. Natl. Acad. Sci. USA 1986; 83: 1807-1811.

Eva A, Tronick SR, Gol RA, Pierce JH, Aaronson SA. Transforming genes of human hematopoietic tumors: Frequent detection of ras-related oncogenes whose activity appears to be independent of tumor phenotype. Proc. Natl. Acad. Sci. USA 1983; 80: 4926-4930.

Fauser AA, Messner HA. Granulo-erythropoietic colonies on human bone marrow, peripheral blood and cord blood. Blood 1978; 52: 1243-1247.

Fauser AA, Lohr GW. Cloning of colonies positive for T cell associated antigens derived from mixed hemopoietic colonies. Proc. Soc. Exp. Biol. Med. 1982; 170: 220-224.

Fauser AA, Kanz L, Bross KJ, Lohr GW. T cells and probably B cells arise from the malignant clone in chronic myelogenous leukemia. J Clin Invest 1985; 75: 1080-1082.

Fialkow PJ, Gartler SM, Yoshida A. Clonal origin of chronic myelocytic leukemia in man. Proc. Natl. Acad. Sci. USA 1967; 58: 1468-1471.

Fialkow PJ, Denman AM, Jacobson RJ, Lowenthal MN. The origin of some lymphocytes from leukemic stem cells. J. clin. Invest. 1978; 62: 815-823.

Fialkow PJ, Martin PJ, Najfeld V et al. Evidence for a multistep origin of chronic myelogenous leukemia. Blood 1981; 58: 158-163.

First International Workshop on Chromosomes in Leukaemia. Chromosomes in Ph-positive chronic granulocytic leukaemia. Brit. J. Haematol. 1978; 39: 305-309.

Flanagan JG, Rabbitts TH. The sequence of the human immunoglobulin epsilon heavy chain constant region gene, and evidence for three non-allelic genes. EMBO J.; 1982; 1: 655-660.

Fleishman EW, Prigogina EL, Volkova MA et al. Correlation between the clinical course characteristics of blast cells and karyotype patterns in chronic myeloid leukemia. Hum. Genet. 1981; 58: 285-293.

Foch J. Cultivation, characterisation, and identification of human tumor cells with emphasis on kidney, testes, and bladder tumors. Natl. Cancer Inst. Monogr. 1978; 49: 5-11

Foroni L, Catovsky D, Rabbitts TH, Luzzatto L. DNA rearrangements of immunoglobulin genes correlate with phenotypic markers in B-cell malignancies. Mol. Biol. Med. 1984; 2: 63-79.

Gabrilove J, Welte K, Harris P et al. Pluripoietin-alpha: a second human hematopoietic colony-stimulating factor produced by the human bladder carcinoma cell line 5637. Proc. Natl. Acad. Sci. USA. 1986; 83: 2478-2482.

Gale RP, Cannani EA. Novel 8kb abl RNA transcript in chronic myelogenous leukemia. Proc. Natl. Acad. Sci. USA 1984; 81: 5648-5652.

Gale RP, Goldman JM. Rapid progress in chronic myelogenous leukemia. Leukemia 1988; 2: 321-323.

Ganesan TS, Rassool F, Guo AP et al. Rearrangement of the bcr gene in Philadelphia chromosome negative chronic myeloid leukemia. Blood 1986; 68: 957-960.

Gannon JV, Lane DP. p53 and DNA polymerase alpha compete for binding to SV40 T antigen. Nature 1987; 329: 456-458.

Glover TW, Berger C, Coyle J et al. DNA polmerase alpha inhibition by aphidicolin induces gaps and breaks at common fragile sites in human chromosomes. Hum. Genet. 1984; 67: 136-146.

Golde DW, Brugaleta C, Sparkes RS, Cline MJ . The Philadelphia chromosome in human macrophages. Blood 1977; 49: 367-370.

Groffen J, Heisterkamp NY, Stephenson JR et al. c-sis is translocated from chromosome 22 to chromosome 9 in chronic myelocytic leukemia. J. Exp. Med. 1983; 158: 9-15.

Groffen J, Heisterkamp N, Spurr N et al. Chromosomal localization of the human c-fms oncogene. Nuc. Acid Res. 1984a; 11: 6331-6339.

Groffen J, Stephenson JR, Heisterkamp N, et al. Philadelphia chromosome breakpoints are clustered within a limited region, bcr, on chromosome 22. Cell 1984b; 36: 93-99.

Groffen J, Heisterkamp N. The BCR/ABL hybrid gene. Baillieres Clinical Haematology. 1987; 1: 983-999.

Grosveld G, Verwoerd T, van Agtenhoven T et al. The chronic myelocytic leukemia cell line K562 contains a breakpoint in bcr and produces a chimeric bcr/c-abl transcript. Mol. Cell. Biol. 1986; 6: 607-616.

Hagemeijer A, Smitt EME, Bootsma D. Improved identification of chromosomes of leukemic cells in methotrexate-treated cultures. Cytogenet. Cell Genet. 1979; 23: 208-212.

Hagemeijer A, Bartram CR, Smit EME, van Agtenhoven AJ, Bootsma D. Is chromosomal region 9q34 always involved in variants of the Ph' translocation? Cancer Genet. Cytogenet. 1984; 13: 1-16.

Hagemeijer A, de Klein A, Godde-Saltz E et al. Translocation of c-abl to "masked" Ph in chronic myeloid leukemia. Cancer Genet. Cytogenet. 1985a; 18: 95-104

Hampe A, Marcelle G, Sherr CJ, Galibert F. Nucleotide sequence of the feline retroviral oncogene v-fms shows unexpected homology with oncogenes coding tyrosine-specific protein kinases. Proc. Natl. Sci. USA; 1984; 81: 85-88.

Hariharan IK, Adams JM. cDNA sequence for human bcr, the gene that translocates to the abl oncogene in chronic myeloid leukemia. EMBO J. 1987; 6: 115-119.

Harper ME, Saunders GF. Localisation of single copy DNA sequences on G banded human chromosomes by in situ hybridisation. Chromosoma 1981; 83: 431-439.

Harper M, Franchini G, Love J et al. Chromosomal sub localisation of human c-myb and c-fes cellular onc genes. Nature 1983; 304: 169-171.

Hayati I, Kakati S, Sandberg AA. A new translocation related to the Philadelphia chromosome. Lancet 1973; ii: 1385.

Hecht F, Morgan R, Schrier SL, Adams J, Sandberg AA. The Philadelphia chromosome in leukemia: A new mechanism due to interstitial deletion and insertion in chronic myelocytic leukemia. Canc. Genet. Cytogenet. 1985; 14: 3-10.

Heim S, Billstrom R, Kristoffersson U et al. Variant Ph translocations in chronic myeloid leukemia. Cancer Genet. Cytogenet. 1985; 18: 215-227.

Heim S, Mitelman F. Cancer Cytogenetics. 1987, New York, Liss.

Heisterkamp NY, Groffen J, Stephenson JR et al. Chromosomal localisation of human cellular homologues of two viral oncogenes. Nature 1982; 299: 747-750.

Heisterkamp NY, Stephenson JR, GroffenJ et al. Localisation of c-abl oncogene adjacent to a translocation breakpoint in chronic myelocytic leukaemia. Nature 1983; 306: 239-240.

Heisterkamp N, Stam K, Groffen J et al. Structural organization of the bcr gene and its role in the Philadelphia translocation. Nature 1985; 315: 758-761.

Hermans A, Gow J, Selleri L et al. BCR-ABL oncogene activation in Philadelphia chromosome positive acute lymphoblastic leukemia. Leukemia 1988; 2: 626-633.

Hermans AA, Heisterkamp N, von Lindern M et al. Unique fusion of bcr and c-abl genes in Philadelphia chromosome positive acute lymphoblastic leukemia. Cell 1987; 51: 33-40.

Hill RS, Carrington V, Park D, Fox JG, Goldman JM. A new controlled-rate apparatus for freezing hematopoietic cells for storage at -196 C. Cryobiology 1973; 10: 1-8.

Howard RO, Bregg WR, Albert DM, Lesser RL. Retinoblastoma and chromosome abnormality. Arch. Ophthalmol. 1974; 92: 490-493.

Hulten MA, Palmer RW, Laurie DA. Chiasma derived genetic maps and recombination fractions: chromosome 1. Ann. Hum. Genet. 1982; 46:167-175.

Human Gene Mapping 7: Seventh International Workshop on Human Gene Mapping. Cytogenet. Cell Genet. 1984; 37: 274.

Huret JL, Tanzer J, Henry-Amar M et al. Aberrant breakpoints in chronic myelogenous leukemia: oncogenes and fragile sites. Hum. Genet. 1986; 74: 447-448.

ISCN: An international system for human cytogenetic nomenclature. Cytogenet. Cell Genet. 1978; 21: 309-404.

ISCN: An International System for Human Cytogenetic nomenclature. Birth Defects: Original Article Series; 1985; 21; ISSN National Foundation - March of Dimes, New York.

Ishihara T, Minamihimatsu M. The Philadelphia chromosome. Considerations based on studies of variant Ph translocations. Cancer Genet. Cytogenet. 1988; 32: 75-92.

Jaubert J, Martiat P, Dowding C et al. The position of the M-BCR breakpoint does not predict the duration of chronic phase or survival in chronic myeloid leukaemia. (submitted for publication).

Jhanwar SC, Neel BG, Hayward WS et al. Localisation of c-ras oncogene family on human germ-line chromosomes. Proc Natl. Acad. Sci. USA. 1984; 80: 4794-4797.

Josephs SF, Ratner L, Clarke MF et al. Transforming potential of human c-sis nucleotide sequences encoding platelet derived growth factor. Science 1984; 225: 636-639.

Kalousek DK, Dube ID, Eaves CJ et al. Cytogenetic studies of haemopoietic colonies from patients with an initial diagnosis of acute lymphoblastic leukaemia. Brit. J. Haematol. 1988; 70: 5-11.

Kan YD, Dozy AM. Polymorphism of DNA sequence adjacent to human B-globin structural gene: Relationship to sickle mutation. Proc. Natl. Acad. Sci. USA 1978; 75: 5631-5635.

Kaneko Y, Rowley JD, Variakoji SD et al. Correlation of karyotype with clinical features in acute lymphoblastic leukemia. Cancer Res. 1982; 42: 2918-2929.

Kardinal CG, Bateman JR, Werner J. Chronic granulocytic leukemia: a review of 536 cases. Arch. Intern. Med. 1976; 61: 625-635.

Kearney L, Orchard KH, Hibbin J, Goldman JM: T-cell cytogenetics in chronic granulocytic leukaemia. Lancet 1982; 1: 858-859.

Kloetzer W, Kurzrock R, Smith L, et al. The human cellular abl gene product in chronic myelogenous leukemia cell-line K562 has an associated tyrosine protein kinase activity. Virology 1985; 140: 230-238.

Knudson AG, Meadows AT, Nichols WW, Hill R. Chromosome deletion and retinoblastoma. N. Engl. J. Med. 1976; 295: 1120-1123.

Kobori JA, Staines E, Minard K, Hood L. Molecular analysis of the hotspot of recombination in the murine major histocompatibility complex. Science 1986; 234: 173-186.

Konopka JB, Watanabe SM, Witte O. An alteration of the human c-abl protein in K562 leukemia cells unmasks associated tyrosine kinase activity. Cell 1984; 37: 1035-1042.

Konopka JB, Watanabe SM, Singer JW, Collins SJ, Witte O. Cell-lines and clinical isolates derived from Ph-positive chronic myelogenous leukemia patients express c-abl proteins with a common structural alteration. Proc. Natl. Acad. Sci. USA 1985a; 82: 1810-1814.

Konopka JB, Witte O. Detection of c-abl tyrosine kinase activity permits direct comparison of normal and altered abl gene products. Mol. Cell. Biol. 1985b; 5: 3116-3123.

Kowalczyk J, Sandberg AA. A possible subgroup of ALL with 9p-. Cancer. Genet. Cytogenet. 1983; 9: 383-385.

Kruh GD, King R, Kraus MH. A novel human gene closely related to the abl oncogene. Science 1986; 234: 1545-1548.

Kurzrock R, Blick MB, Talpaz M, et al. Rearrangement in the breakpoint cluster region and the clinical course in Philadelphia-negative chronic myelogenous leukemia. Ann. Int. Med. 1986b; 105: 673-679.

Kurzrock R, Shtalrid M, Talpaz M, Kloetzer WS, Gutterman JV. Expression of c-abl in Philadelphia positive acute myelogenous leukemia. Blood 1987a; 70: 1584-1588.

Kurzrock R, Stalrid M, Romero P et al. A novel c-abl product in Philadelphia-positive acute lymphoblastic leukaemia. Nature 1987b; 321: 631-633.

Lamb P, Crawford L. Characterization of the human p53 gene. Mol. Cell. Biol. 1986; 6: 1379-1385

Larson RA, Williams SF, Le Beau MM et al. Acute myelomonocytic leukemia with abnormal eosinophils and inv(16) or t(16;16) has a favorable prognosis. Blood 1986; 68: 1242-1249.

Lawler SD. Significance of chromosomal abnormalities in leukemia. Semin. Hematol. 1982; 19: 257-272.

Leibowitz D, Shaefer-Rego K, Popenoe DW, Mears JG, Bank A. Variable breakpoints on the Philadelphia chromosome in chronic myelogenous leukemia. Blood 1985; 66: 243-245.

Lifshitz B, Fainstein E, Shtivelman E et al. bcr related genes in the human genome. Recent advances in leukemia and lymphoma 1987a: 83-84; ISSN Alan Liss.

Lifshitz B, Fainstein E, Shtivelman E et al. bcr related genes in the human genome. Oncogene Res. 1987b: 113-117.

Lisker R, Casas L, Muschinick O, Lopez-Ariza B, Labardini J. Patient with chronic myelogenous leukemia and late appearing Ph' chromosome. Cancer Genet. Cytogenet. 1982; 6: 275-277.

Liu E, Hjelle B, Bishop M. Transforming genes in chronic myelogenous leukemia. Proc. Natl. Acad. Sci. USA 1988; 85: 1952-1956.

Louwagie A, Criel A, Verfailie CM. Philadelphia positive T acute lymphoblastic leukemia. Cancer Genet. Cytogenet. 1985; 16: 297-300.

Maddox AM, Keating MJ, Trujillo J. Philadelphia chromosome positive adult acute leukemia with monosomy of chromosome number seven: a subgroup of poor response to therapy. Leuk. Res. 1981; 4: 509-522.

Martin H, Hibbin JA, Dowding C, Matutes E, Tindle R, Goldman JM. Purification of haemopoietic progenitor cells from patients with chronic granulocytic leukaemia using percoll density gradients and elutriation. Brit J. Haematol. 1986; 63: 187-198.

Maserati E, Pasquali F, Peretti D. Different breakpoints in Philadelphia chromosome variant translocations and in constitutional and sporadic translocations. Ann. Hum. Genet. 1986; 50: 153-162.

McCarthy DM, Rassool FV, Goldman JM, Graham SV, Birnie GD. Genomic alteration involving the c-myc protooncogene locus during the evolution of chronic granulocytic leukaemia. Lancet 1984; i: 1362-1365.

McLaughlin J, Chianese E, Witte ON. In vitro transformation of immature hematopoietic cells by the P210 BCR/ABL oncogene product of the Ph chromosome. Proc. Natl. Acad. Sci. USA 1987; 84: 6558-6562.

Mendelsohn ML, Mayall BH, Bogart E, Moore DH, Perry BH. DNA content and DNA-based centromeric index of the 24 human chromosomes. Science 1973; 179: 1126-1129.

Mes-masson A.M., Mclaughlin J., Daley G.Q., et al. Overlapping cDNA clones defined the complete coding region for the P210 c-abl gene product associated with chronic myelogenous leukemia cells containing the Philadelphia chromosome. Proc. Natl. Acad. Sci. USA 1985; 83: 9768-9772.

Mills KI, McKenzie ED, Birnie GD. The site of the breakpoint within the bcr is a prognostic factor in Philadelphia-positive CML patients. Blood 1988; 72: 1237-1241.

Minden MD, Mak TW. Review: the structure of the T cell antigen receptor genes in normal and malignant T cells. Blood 1986; 68: 327-336.

Mitelman F. Comparative cytogenetic studies of bone marrow and extramedullary tissues in chronic myeloid leukemia. Ser. Haematol. 1975; 8: 113-117.

Mitelman F, Levan G, Nilsson PG, Brandt L. Non-random karyotypic evolution in chronic myeloid leukemia. Int. J. Cancer; 1976; 18: 24-30.

Mitelman F. Catalogue of chromosome aberrations in cancer. Cytogenet. Cell Genet. 1983; 36: 1-516.

Morris C, Reeve A, Fitzgerald P et al. Genomic diversity correlates with clinical variation in Ph-negative chronic myeloid leukaemia. Nature 1986; 320: 281-283.

Morris CM, Fitzgerald PH. Complexity of an apparently simple variant Ph translocation in chronic myeloid leukemia. Leuk. Res. 1987; 2: 163-169.

Myers CD, Katz FE, Joshi G, Millar JL. A cell line secreting stimulating factors for CFU-GEMM culture. Blood 1984; 64:152-155.

Nowell PC, Hungerford DA. Chromosome studies in normal and leukemic human leucocytes. J. Natl. Canc. Inst. 1960a; 25: 85-110.

Nowell PC, Hungerford DA. A minute chromosome in human chronic granulocytic leukemia. Science 1960b; 132: 1497.

O'Malley FM, Garson OM. Chronic granulocytic leukemia: correlation of blastic transformation type with karyotypic evolution. Am. J. Hematol. 1985; 20: 313-323.

O' Riordan ML, Robinson JA, Buckton KE, Evans H. J. Distinguishing between the chromosomes involved in Downs syndrome (trisomy 21) and chronic myeloid leukemia (Ph') by fluorescence. Nature 1971; 230: 167-168.

Ohyashiki K, Ohyashiki JH, Otaki K et al. Four cases with complex Philadelphia translocations including one with appearance de novo of a 'masked' Ph. Cancer Genet. Cytogenet. 1987; 24: 281-294.

Ohyashiki JH, Ohyashiki K, Ito H, Toyama K. Molecular and clinical investigations in Philadelphia chromosome-negative chronic myelogenous leukemia. Cancer Genet. Cytogenet. 1988a; 33: 119-126.

Ohyashiki K, Ohyashiki JH, Kinniburgh AJ et al. Transposition of breakpoint cluster region (3' bcr) in CML cells with variant Philadelphia translocations. Cancer Genet. Cytogenet. 1988b; 26: 105-115.

Oshimuru M, Ohyashiki K, Terada H et al. Variant Ph' translocations in CML and their incidence, including two cases with sequential lymphoid and myeloid crises. Cancer Genet. Cytogenet. 1982; 5: 187-201.

Paris Conference, standardisation in Human cytogenetics. Birth defects: Original Article Series 1971, Vol 3:1, The National Foundation-March of Dimes, New York.

Parreira L, Kearney L, Rassool F et al. Correlation between chromosomal abnormalities and blast phenotype in blast crisis of Ph-positive CGL. Cancer Genet. Cytogenet. 1986; 22: 29-34.

Pasquali F, Casalone R, Francesconi D et al. Transposition of 9q34 and 22(q11-qter) regions has a specific role in chronic myelocytic leukemia. Hum. Genet. 1979; 52: 55-67.

Pederson B. Co existence of cells with unmasked and masked Ph in a case of chronic myeloid leukemia in blastic phase. Cancer Genet. Cytogenet. 1981; 12: 129-137.

Perry P, Wolf S. New Giemsa method for the differential staining of sister chromatids. Nature 1974; 251: 156-158.

Peterson LC, Bloomfield CD, Brunning RD. Philadelphia chromosome positive acute leukemia : morphology, clinical features and survival of nine patients. J Laboratory Investigation 1976; 34: 346-351.

Pierce J, Eva A, Aaronson SA. Hematopoietic cell targets for the transformation by retroviral oncogenes. New Trends in Experimental Hematology 1984; Seron Symposia, Rome: 1-18.

Podlisny MB, Lee G, Selkoe DJ: Gene dosage of the amyloid beta precursor protein in Alzheimer's disease. Science 1987; 238: 669-671.

Pollak J, Hagemeijer A. Abnormalities of the short arm of chromosome 9 with partial loss of material in hematologic disorders. Leukemia 1987; 1: 541-548.

Potter AM, Watmore AE, Cooke P, Lilleyman JS, Sokol RJ. Significance of non-standard Philadelphia chromosomes in chronic granulocytic leukaemia. Brit. J. Haematol. 1981; 44: 51-54.

Price CM, Rassool F, Mahmud KS et al. Rearrangement of the breakpoint cluster region and expression of the P210 bcr-abl in a "masked" Philadelphia chromosome-positive acute myeloid leukemia. Blood 1988; 72: 1829-1832.

Prigogina EL, Fleischman EW, Volkova MA, Flenkel M.A. Chromosomal abnormalities and clinical and morphologic manifestations of chronic myeloid leukemia. Hum. Genet. 1978; 41: 143-156.

Proposed changes in cytogenetic records. Chicago Conference, 1966.

Pugh WC, Pearson M, Vardiman JW et al. Philadelphia chromosome negative chronic myelogenous leukaemia: A morphological reassessment. Brit. J. Haematol. 1985; 60: 457-467.

Rabbitts TH, Watson JV, Lamond A et al. Metabolism of c-myc gene products: c-myc mRNA and protein expression in cell cycle. EMBO J 1985; 4: 2009-2015.

Rastrick JH. A method for positive identification of erythropoietic cells in preparations of bone marrow. Brit. J. Haematol. 1969; 16: 185-191.

Ribeiro RC, Abramowitch M, Raimondi SC et al. Clinical and biological hallmarks of the Philadelphia chromosome in childhood acute lymphoblastic leukemia. Blood 1987; 70: 948-953.

Rodenhuis S, Smetss LA, Slater RM et al. Distinguishing the Philadelphia chromosome of acute lymphoblastic leukemia from its counterpart in chronic myelogenous leukemia. N. Engl. J. Med. 1985; 313: 51-52.

Roozendaal KJ, van den Reijden HJ, Geraedts JP. Philadelphia chromosome positive acute lymphoblastic leukemia with T-cell characteristics. Brit. J. Haematol. 1981; 47: 145-147.

Rosenthal S, Canellos GP, Wang-Peng J, Gralnick HR. Blast crisis of chronic granulocytic leukemia. Morphologic variants and therapeutic implications. Am. J. Med. 1977; 63: 542-547.

Rovigatti U, Mirro J, Kitchingman G, et al. Heavy chain immunoglobulin gene rearrangement in acute non-lymphocytic leukemia. Blood 1984; 63: 1023-1027.

Rowley JD. A new consistent abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature 1973; 243: 290-293.

Rowley JD. Identification of the constant chromosome regions involved in human hematologic malignancy disease. Science 1982; 216: 749-751.

Saglio G, Guerrasio A, Tassinari C et al. Variability of the molecular defects corresponding to the presence of a Philadelphia chromosome in human hematologic malignancies. Blood 1988; 72: 1203-1208.

Sandberg AA, Ishihara T, Miwa T, Hauschka TS. The in vivo constitution of marrow from 34 human leukemias and 60 non-leukemic controls. Cancer Res. 1961; 21: 678-689.

Sandberg A, Kohno S, Wake N, Minowada J. Chromosomes and causation of human cancer and leukemia XLII. Ph-positive ALL: An entity within myeloproliferative disorders? Cancer Genet. Cytogenet. 1980; 2: 145-174.

Sandberg AA, Kaiser-McCaw B, Hecht F. Nomenclature: The Philadelphia chromosome or Ph without the superscript. Cancer Genet. Cytogenet. 1985a; 14: 1.

Sandberg AA, Morgan R, Kips TJ et al. The Philadelphia (Ph') chromosome in leukemia II. Variant Ph' translocations in acute lymphoblastic leukemias. Cancer Genet. Cytogenet. 1985b; 14: 11-21.

Schaefer-Rego C, Dudek H, Popenoe D et al. CML patients in blast crisis have breakpoints localised to a specific region of bcr. Blood 1987; 70: 448-454.

Seabright M. A rapid banding technique for human chromosomes . Lancet 1971; ii: 971-972.

Seldon JR, Emanuel BS, Wang E et al. Amplified C and c-abl genes are on the same marker chromosome in K562 leukemia cells. Proc. Natl. Acad. Sci. USA 1985; 80: 7289-7292.

Selleri L, Narni F, Emilio G et al. Philadelphia-positive chronic myeloid leukemia with a chromosome 22 breakpoint outside the breakpoint cluster region. Blood 1987; 70: 1659-1664.

Shepherd S, Ganesan TS, Galton DA. Haematological classification of the chronic myeloid leukaemias. Baillieres Clin. Haematol 1987; 1: 887-906.

Shtivelman E., Lifshitz B, Gale RP, Cannani E. Fused transcripts of abl and bcr genes in chronic myelogenous leukaemia. Nature 1985; 315: 550-554.

Shtivelman E, Lifshitz B, Gale RP, Roe BA, Cannani E. Alternative splicing of RNAs transcribed from human abl gene and from bcr/abl fused gene. Cell; 1986; 47: 277-284.

Shtivelman E, Gale RP, Dreazen et al. bcr-abl RNA in patients with chronic myelogenous leukemia. Blood 1987; 69: 971-973.

Sims JA, Tunnacliffe A, Smith WJ, Rabbitts TH. Complexity of human T cell antigen receptor B chain constant and variable region genes. Nature 1984; 312: 541-545.

Singh S, Wass J, Vincent PC, Young GAR, Ginz FW. Significance of secondary cytogenetic changes in patients with Ph-positive chronic granulocytic leukemia in the acute phase. Cancer Genet. Cytogenet. 1986; 21: 209-220.

Spiers AD. The clinical features of chronic granulocytic leukaemia. Clinics in Haematology 1977; 6: 77-95.

Sumner AT, Evans HJ, Buckland RA. New technique for distinguishing between human chromosomes. Nature 1971; 232: 31-33.

Swan DC, McBride OW, Robbins KC, Keithley DA Reddy EP, Aaronson SA. Chromosomal mapping of the simian sarcoma onc gene analogue in human cells. Proc. Natl. Sci. USA 1982; 79: 4691-4695.

Swolin B, Weinfeld A, Westin J et al. Karyotypic evolution in Ph-positive chronic myeloid leukemia in relation to management and disease progression. Cancer Genet. Cytogenet. 1985; 18: 65-79.

Takeuchi J, Ohshima T, Amaki I. Cytogenetic studies in adult acute leukemias. Cancer Genet Cytogenet; 4: 293-303.

Tanzer J, Najean Y, Frocram C, Bernheim A. Chronic myelocytic leukemia with a masked Ph chromosome. N. Engl. J. Med. 1977; 296: 571-572.

Tedeschi B, Porfirio B, Vernole P et al. Common fragile sites: their prevalence in subjects with constitutional and acquired chromosomal instability. Am. J. Med. 1987; 2: 471-482.

Torelli G, Selleri L, Emilia G et al. Molecular study of the Philadelphia translocation in chronic myelogenous leukemia in different stages of the disease. Haematologica 1987; 72: 201-208.

Tough IM, Court-Brown WM, Baikie AG et al. Cytogenetic studies in chronic myeloid leukaemia and acute leukaemia associated with mongolism. Lancet 1961; 1: 411-417.

Travis LP, Pierre RV, De Wald GW. Ph-negative chronic granulocytic leukemia: a nonentity. Am. J. Clin. Path. 1986; 85: 186-193.

Turhan GA, Eaves CJ, Kalousek DK et al. Molecular analysis of clonality and bcr rearrangements in Philadelphia chromosome-positive acute lymphoblastic leukemia. Blood 1988; 71: 1495-1498.

van Tuinen P, Dobyns WB, Rich DC et al. Molecular detection of microscopic and submicroscopic deletions associated with Miller-Dieker syndrome. Am. J. Hum. Genet. 1986; 43: 587-596.

Veneturelli D, Ku DH, Narni F, Gatti C, Calabretta B. Lack of rearrangement of P53 tumor antigen gene locus in human hematological malignancies. Haematologica 1988; 73: 256-264.

Viegas-Pequignot E, Dutrillaux B. Une methode pour simple obtenir des prophases et des prometaphases. Annls. Genet. 1981; 21: 134-137.

Webber L.M., Garson M. Fluorodeoxyuridine synchronisation of bone marrow cultures. Cancer Genet. Cytogenet. 1983; 8: 123-125.

Westin EH, Wong-Staal, Gelmann EP et al. Expression of cellular homologues of retroviral onc genes in human hematopoietic cells. Proc. Natl. Acad. Sci USA 1982; 79: 2490-2494.

Whang J, Frei E, Tjio JH, Carbone PP and Brecher G. The distribution of the Philadelphia chromosome in patients with chronic myelogenous leukemia. Blood 1963; 22: 664-673.

White R, Lalouel JM. Genetic linkage in human families. Adv. Hum. Genet. 1987; 16:121-228.

Whitlock CA, Ziegler SF, Witte ON. Progression of the transformed phenotype in clonal lines of Abelson-virus infected lymphocytes. Mol. Cell Biol. 1983; 3: 596-604.

Wiedemann L, Karhi K, Shivji M et al. The correlation of the breakpoint cluster region and p210 phl/abl expression with morphological analysis of Ph-negative chronic myeloid leukemia and other myeloproliferative diseases. Blood 1988; 71: 349-355.

Williams JG, Mason PJ. Hybridisation in RNA analysis (Chapter 6). Nucleic acid hybridisation. A practical approach; 1985: Ed. Haines B.D. and Higgins S.J. ISSN IRL Press.

Wilson MG, Ebbin AJ, Towner JW, Spencer WH. Chromosomal abnormalities in patients with retinoblastoma. Clin. genet.; 1977; 12: 1-8.

Yao EI. Chromosomal abnormalities abnormalities in lymphoid crisis of chronic myelogenous leukemia. Cancer Genet. Cytogenet. 1985; 16: 235-243.

Yunis JJ. New chromosome techniques in the study of human neoplasia. Hum. Pathol. 1981; 12: 540-549.

Yunis JJ. Fragile sites in cancer and predisposition to leukemia and lymphoma. Cancer Genet. Cytogenet. 1984a; 12: 85-88.

Yunis JJ, Soreng AL. Constitutive fragile sites and cancer. Science 1984b; 226: 1129-1131.

APPENDIX Ia: SOLUTIONS

The solutions described in CHAPTER 2 (Materials and methods) are listed in alphabetical order below.

Carrier DNA

Herring sperm DNA (Boehringer) was made up in glass distilled water (GDW) at a concentration of 10 mg/ml. The solution was boiled for 10 mins and passed through a 19 gauge needle several times and stored at -20°C.

Chloroform

Mix with isopropanol 24:1 (v/v).

Deionised formamide

Formamide was deionised by stirring on a magnetic stirrer with Dowex XG8 mixed-bed resin (10 g/200 mls) for 1 hour and filtering twice through Whatman 1MM paper. Deionised formamide was stored in small aliquots at -20°C.

Denaturation solution

87.5 g of NaCl and 20 g of NaOH were dissolved in 800 ml of GDW. The volume was adjusted to 1 l with GDW.

Denhardt's solution

A X100 stock solution was made up. 10 g of 2% Ficoll, Bovine serum albumin and Polyvinyl pyrolidine was made up to 500 ml with GDW. This solution was filtered and stored at -20°C.

Dextran sulphate

A 50% (w/v) stock solution was made by weighing out 25 g of dextran sulphate into a 50 ml Falcon tube. 20 mls of GDW was added and left at 65°C to dissolve. The solution was mixed frequently by inverting the tube. The volume was adjusted to 50 mls with GDW and the solution stored at -20°C.

Elution buffer

99% (v/v) deionised formamide, 10 mM Tris. HCl (pH 7.8)

Ethidium bromide

A stock solution of 10 mg/ml was made up. This solution was kept in the dark at 4°C.

Hybridisation mix (for in situ hubridisation)

For 1 ml of hybridisation mix: 200 l 50X Denhardt's solution, 800 l 25% dextran sulphate in 12.5X sodium chloride sodium citrate phosphorus EDTA (SSPE) (pH 7.2), 40 l herring sperm DNA (10 mg/ml).

Hybridisation solution

Stock solution	Volume (ml) added	Final concentration
20xSSPE	25	5x SSPE
20%SDS	0.5	0.1% SDS
10mg/ml carrier DNA	1	100 g/ml
100x Denhardt's	2	2x Denhardt's
50x dextran sulphate 10		5% dextran sulphate
GDW	61.5	

L-B medium

For 1 liter of LB medium 10 g Bacto-tryptone, 5 g Bacto-yeast extract and 10 g NaCl was dissolved in 800 mls. The pH was adjusted to 7.5 with NaOH and the volume made up to 1 l with GDW.

L-B medium containing agar or agarose

Make up medium as above. Just before autoclaving, add one of the following was added (per l):

Bacto-agar	15 g (for plates)
Bacto-agar	7 g (for top agar)
Agarose	15 g (for plates)
Agarose	7 g (for top agarose)

When preparing plates, media containing agar or agarose should be sterilised by autoclaving and allowed to cool to 55°C before thermolabile substances are added (eg antibiotics).

Ligation buffer

A 10X stock solution was made. 0.5 M Tris (pH 7.4), 0.1 M $MgCl_2$, 0.1 M dithiothreitol, 10 mM spermidine, 10 mM ATP, 1 mg/ml bovine serum albumen (BSA).

Lysis buffer

A x10 stock solution was made. NaCl 175.2 g/l (3M), EDTA 37.2 g/l (100mM), Tris-HCl (pH 7.5) (100 mM) were mixed together and diluted in 7 vols of GDW. Urea (BDH) was added to this solution at 42g/100 ml.

Neutralising solution

Dissolve 60.5 g Trisma-base and 87.5 g in 700 mls GDW. Adjust the pH to 7.5 with concentrated HCl and add more GDW to make 1 l.

Prehybridisatiion solution

Stock solution	Amount of stock added (mls)	Final conc.20X
SSPE	25	5X SSPE
20% SDS	0.5	0.1% SDS
100X Denhardt's solution	5	5X Denhardt's
10 mg/ml carrier sperm DNA	1	100 g/ml
GDW	68.5	-

Phenol

250 mls of 1 M Tris.HCl (pH 9) was added to each 500 mls of water-saturated phenol. This solution was mixed well and left to stand until the layers separated. The top layer was removed and the process was repeated. Then 250 mls 0.1 M tris.HCl (pH 8) was added to the phenol and the process was repeated as above. Finally 250 mls TE (1X) was added to the phenol and the process repeated as above.

Restriction endonuclease buffers

Restriction endonucleases worked best at specific salt concentrations.

1 **Low-salt buffer**

10 mM Tris.Cl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol.

2 **Medium-salt buffer**

50 mM NaCl, 10 mM Tris.HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol.

3 **High-salt**

100 mM NaCl, 50 mM Tris.Cl (pH 7.5), 10 mM Mg Cl_2, 1 mM dithiothreitol.

SOB medium

For 1 l of SOB, 20 g Bactotryptone, 5 g yeast extract, and 0.5 g NaCl was dissolved in 800 mls GDW. The pH was adjusted to 7.5 and the volume was made up to 1 l with GDW.

20X sodium chloride / sodium citrate (SSC)

175.3 g NaCl and 88.2 g sodium citrate was dissolved in 800 mls of GDW. The pH was adjusted to 7 with concentrated NaOH and the volume adjusted to 1 l.

20X SSPE

174 g NaCl, 27.6 g NaH_2PO_4. H_2O and 7.4 g of EDTA was dissolved in 800 mls of GDW. The pH was adjusted to 7.4 with NaOH and the volume adjusted to 1 l with GDW.

sodium chloride Tris EDTA (STE)

10 mM Tris.HCl (pH 8), 100 mM NaCl, 1 mM EDTA (pH 8).

Transformation buffer

100 mM RbCl (rubidium chloride), 45 mM $MnCl_2$ (manganese chloride), 10 mM $CaCl_2$ (calcium chloride), 5 mM $MgCl_2$ (magnesium chloride), 0.5 MmM LiCl (lithium chloride), 35 mM KOAc and 15% sucrose.

Tris.HCl

For a 1 M solution of Tris.HCl 121 g Tris-base was dissolved in 800 mls GDW. This was adjusted to the desired pH by adding concentrated HCl and the volume was adjusted to 1 l with GDW.

Tris-borate

For a 10X stock solution 102 g Trisma-base, 6 g EDTA, 32 g boric acid was dissolved in 800 mls of GDW. The pH was adjusted to 8 and the volume made up to 1 l.

Tris-EDTA

For a 10X stock solution 12.1 g/l Trisma-base, 6 g/l and 32 g/l was made and the pH adjusted to pH 8.

APPENDIX Ib: DNA MARKERS

DNA size markers were as follows: Phage DNA digested with the restriction endonucleases Hind III and Eco RI and X174 DNA digested with the restriction endonuclease Hae III. size markers in DNA analysis on agarose and polyacrylamide gels. The sizes of the different restriction fragments of each digestion separated by gel electrophoresis are listed below:

Hae III digestion of Bacteriophage Ø X 174

base pairs (bp)
1353
1078
872
603
310
281
271
234
194
118
72

Hin DIII digestion of phage DNA

base pairs (bp)
2330
950
640
420
220
180
53

Eco RI digestion of phage

base pairs (bp)
21800
7550
5940
5530
4800
3380