ONCOGENE MOBILITY STUDIES IN PHILADELPHIA-NEGATIVE CHRONIC MYELOID LEUKEMIA AND OTHER LEUKEMIAS

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by

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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To my fiancé, David Scammell

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ABSTRACT

The Philadelphia chromosome (Ph') is the hallmark of chronic myeloid leukemia (CML). It may also be found in the blood cells of patients with chronic granulocytic leukemia (CGL), acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), and in pre-leukemic disorders. The Ph' chromosome results from the reciprocal chromosomal translocation, t(9;22)(q34;q11). This translocation relocates the proto-oncogene, abl, normally found on chromosome 9q34, to the breakpoint cluster region (bcr) on chromosome 22q11. At the same time, the proto-oncogene, sis, located at 22q13, is relocated to the terminus of chromosome 9. Approximately 10% of adult patients diagnosed with CML lack the Ph' chromosome.

The eleven untreated leukemia patients used in this investigation are characterized by the absence of a standard microscopic Ph' translocation. These Ph'-negative patients can be divided into two groups based on the movement of the abl proto-oncogene in the karyotype. The first group is distinguished by the movement of the abl proto-oncogene from chromosome 9 to chromosome 22. Five of the nine patients in this group have been diagnosed with CML. Their karyotypes are normal. In these patients, the movement of abl was not accompanied by the movement of sis as in Ph'-positive CML. This pattern of mobility was confirmed by the presence of two hybridization peaks in the distribution obtained from one CML patient after simultaneous hybridization with abl and sis. The genomic rearrangement of the abl

proto-oncogene was also demonstrated in Ph'-negative CGL and Ph'-negative ALL. The reciprocal mobility of both abl and sis was also demonstrated in one patient with a pre-leukemic disorder. The two remaining patients were not characterized by mobility of the oncogenes abl and sis. One patient was diagnosed with CML and the other patient with ALL.

The mobility of the abl proto-oncogene in Ph'-negative leukemia can aid in the development of a subclassification system for leukemias based on clinical, morphologic, and molecular characteristics.

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LIST OF ABBREVIATIONS

ALL	acute lymphoid leukemia
AMMOL	acute myelomonocytic leukemia
AML	acute myeloid leukemia
ANLL	acute nonlymphocytic leukemia
AT	adenine, thymine
BUdR	bromodeoxyuridine
bcr	breakpoint cluster region
c	cellular
°C	degrees Celsius
CGL	chronic granulocytic leukemia
Ci	Curie
CML	chronic myeloid leukemia
cpm	counts per minute
cs	centromeric stretch
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythmidine triphosphate
dL	decilitre, 10^{-1}
DNA	deoxyribonucleic acid
DNa	deoxyribonuclease
FAB	French-American-British
g	gram
h	hour
^{3}H	a radioactive isotope of hydrogen
ISCN	International System of Cytogenetic Nomenclature
kb	kilobase
KCL	potassium chloride
l	litre
LAP	leukocyte alkaline phosphatase
u	micro, 10^{-6}
m	milli-, 10^{-3}
mm^3	millimeter cubed
M	molar
min	minute
mol	mole
mRNA	messenger ribonucleic acid
n	nano-, 10^{-9}
p	protein
PDGF	platelet derived growth factor
Ph	Philadelphia
PHA	phytohemagglutinin
s	second
SDS	sodium dodecyl sulphate
U	units
UV	ultraviolet
v	viral
vol	volume
WBC	white blood cell

1.0 INTRODUCTION

Cytogenetic analysis of human cancer cells indicates that more than 90% of human malignancies carry clonal cytogenetic changes (Yunis et al., 1982). The discovery of the Philadelphia chromosome in CML (Nowell and Hungerford, 1960) and the subsequent findings that the great majority of leukemias carry specific chromosomal rearrangements (Yunis, 1983; Rowley, 1983) have implicated these rearrangements in the pathogenesis of human malignancies. Recent developments in the analyses of genes involved in chromosomal rearrangements observed in human leukemias indicate that such rearrangements are the critical steps in the pathogenesis of most leukemias (Croce, 1986). Fragile sites have been proposed to explain the predisposition for such rearrangements in cancer cells (Yunis, 1984a). In this thesis, the rearrangement of oncogenes in the karyotype has been investigated by *in situ* hybridization in Philadelphia-negative CML and other leukemias.

1.1 Literature Review

1.1.1 The Acute and Chronic Leukemias

The leukemias are a heterogeneous group of diseases characterized by infiltration of the bone marrow and peripheral blood by malignant cells of the hematopoietic system. Traditionally, the leukemias have been designated as acute or chronic. This classification is based on the untreated clinical course of the disease and on the morphologic appearance of the predominant malignant cell, namely, myeloid (nonlymphocytic) or lymphoid. Both the acute leukemias, acute lymphocytic leukemia (ALL) and acute myeloid leukemia (AML), and the

chronic leukemias, chronic granulocytic leukemia (CGL) and chronic myeloid leukemia (CML) will be discussed.

Acute leukemia can be categorized into two biologically distinct groups, that is, ALL and AML (for review see Burns et al., 1981). Differences in epidemiology, clinical behavior, and response to therapy vaguely justify the separation of ALL from AML. More definitive classification and subclassification within each of these two major categories is possible through the French-American-British (FAB) classification scheme (for review see Dick et al., 1982). This scheme is based on morphologic and cytochemical criteria (Appendix).

ALL

The three French-American-British (FAB) categories (Appendix) of ALL are designated ALL-L1, ALL-L2, and ALL-L3 (for review see Bennett et al., 1981). Various cytochemical techniques may be employed to facilitate morphological classification based on the FAB system into one of the three categories of ALL (for review see Catovsky et al., 1981). A large number of surface-markers have been described in ALL subtypes although few have been applied clinically. Particular physical signs and laboratory data complete the diagnosis of ALL (for review see Burns et al., 1981; Peterson and Bloomfield, 1982).

AML

There are six FAB subcategories (Appendix) of AML. This classification scheme designates the subcategories as M1 to M6 (for review see Bennett and Begg, 1981; McKenna et al., 1982). The cytochemical features (Appendix) and surface-markers of the subtypes of AML have been reviewed by Bennett and Begg (1981) and Bloomfield et

al. (1985), respectively. Certain physical signs and laboratory data complete the diagnosis of AML (for review see Burns et al., 1981).

CGL

The diagnosis of CGL is hematological and does not depend on clinical features (for review see Shaw, 1982). The two hematological features are a leucocytosis and a characteristic differential leucocyte count. The leucocyte count in new patients exceeds 100 x 10^9/l in 74% of cases (Spiers et al., 1977). Leucocytosis of this degree is uncommon in other leukemias.

A variant form of CGL is Ph'-negative CGL. It has been speculated from the similarity in the clinical coarse of Ph'-negative CGL and Ph'-positive CML that the genetic lesion in the CGL cells is the same as in the Ph'-positive cases (Shaw, 1982).

CML

CML is the best characterized human leukemia (for review see Bloomfield et al., 1985). It is manifested clinically by a marked proliferation of the granulocyte series. Evidence that CML is a clonal disorder arising at the level of the pluripotential bone marrow stem cell comes from cytogenetic, cell marker and isoenzyme studies (for review see Bloomfield et al., 1985). The diagnosis of CML is based mainly on laboratory features (for review see Gomez et al., 1981; Koeffler and Golde, 1981; Spiers et al., 1977) and also on clinical findings (for review see Bloomfield et al., 1985).

1.1.2 Philadelphia-Chromosome-Positive and -Negative Chronic Myeloid Leukemia

The two types of CML can be distinguished by the presence or absence of the Ph'-chromosome. This chromosome results from a translocation between chromosomes 9 and 22 (see section 1.1.3). Philadelphia-negative CML has traditionally represented a clinically and morphologically heterogeneous group of diseases (for review see Koeffler and Golde, 1981; Pugh et al., 1985). This group is a variant of CML possessing several distinctive features: the patients are older, show a striking male predominance, display unusual and difficult to classify hematologic profiles, and, in general, respond poorly to therapy (Krauss et al., 1964). While these features are most prevalent in Ph'-negative CML, they are not confined to it alone.

A few laboratory features characterize this group. The leukocytosis in these patients was of moderate degree rather than striking, as in Ph'-positive CML (Krauss et al., 1964). The shift toward more immature forms in blood and marrow, observed as the disease progressed, produced a picture like that described in acute leukemia in patients over the age of 50 (Gunz and Hough, 1956). Thrombocytopenia, decrease in megakaryocytes, and only moderate elevation of the myeloid:erythroid ratio were additional findings in the Ph'-negative CML, at variance with untreated Ph'-positive CML (Koeffler and Golde, 1981; Mintz et al., 1979). The demonstration of extramedullary hematopoiesis by splenic aspiration was of limited diagnostic usefulness because this was found in both Ph'-negative and Ph'-positive CML (Koeffler and Golde, 1981). Leukocyte alkaline phosphatase activity was very low to absent in CML regardless of the Ph'-chromosome (Mintz et

al., 1979).

The appearance of the Ph'-chromosome in CML has a significant prognostic implication. Patients with Ph'-positive CML live almost four times as long as patients with Ph'-negative CML (Ezdinli et al., 1970). However, evidence presented by Ezdinli et al. (1970) suggests that Ph'-negative CML patients may include two separate populations because of the bimodal survival curve of the Ph'-negative patients. For instance, most of the Ph'-negative CML patients died within the first year but the survival pattern of the remainder was similar to that among Ph'-positive patients. The issue of whether a subgroup Ph'-negative CML actually belongs to Ph'-positive CML remains unresolved (Bartram and Carbonell, 1986; Morris et al., 1986).

1.1.3 Chromosomal Anomalies in Leukemia

Certain chromosomal defects are consistently associated with some types of human cancer (review see Sandberg, 1980). The following human leukemias will be discussed with respect to characteristic chromosomal anomalies: ALL, AML, CGL and CML.

ALL

Patients with ALL are classified according to the chromosomal characteristics of the major proportion of their leukemic cells into five categories. These categories are hyperdiploid (more than 46 chromosomes), pseudodiploid (46 chromosomes including marker chromosomes), diploid (46 chromosomes), hypodiploid (less than 46 chromosomes) and mixed (consisting of any two of the preceding categories)(Secker-Walker et al., 1978). Achievement of complete remission was highly different in the hyperdiploid category versus the

pseudodiploid category (Williams et al., 1982). The modal number of chromosomes was revealed to be an independent prognostic factor (Hossfeld and Weh, 1984).

In addition to modal numbers, specific anomalies characterize ALL. For example, the translocation, t(9;22), occurs in 15% - 20% of cases of adult ALL and in 5% of cases of childhood ALL (Third International Workshop, 1981). The median survival was 11 months for adults and 15 months for children (Hossfeld et al., 1981). The Third International Workshop confirmed the association of t(8;14) and ALL-L3 morphology. Of these patients, 80% were males (Berger et al., 1979). Although a complete remission was obtained in 60% of the t(8;14) patients, the median survival was only 5 months (Mitelman et al., 1979). The translocation, t(4;11), is the most typical feature of patients with a very high tumor load, as evidenced by a increased WBC count (median 183 x 10^9/l), splenohepatomegaly, and lymphadenopathy (Third International Workshop, 1981).

AML

Patients with de novo AML have 17 types of chromosomal defects (Yunis, 1986). Nine of these categories represent single recurrent defects in AML subtypes (Appendix).

AML-M1 and AML-M3 are specifically characterized by the translocations, t(9;22) and t(15;17) respectively (Fourth International Workshop, 1984; Rowley, 1980a). Approximately 61% of AML-M3 cases reported by the Fourth International Workshop (1984) have the translocation, t(15;17). Remission rates and survival appear to be somewhat more favorable in patients with karyotypically normal AML-M3

than in those with the translocations, t(9;22) and t(15;17) (Second International Workshop, 1980).

The translocations, t(8;21) and t(6;9), are closely correlated with AML-M2 (Fourth International Workshop, 1984). The translocation, t(8;21), is also present in a few cases of AML-M1 and AML-M4 (Berger et al., 1982; Brodeur et al., 1983). The overall incidence of t(8;21) in AML-M2 was reported as 15% at the Fourth International Workshop (1984). Patients with t(8;21) are considered to have a favorable prognosis (Trujillo et al., 1979).

The deletion 11q is the most recent discovery of an anomaly specifically associated with AML-M5. The breakpoint was band q23 in 80% of cases (Fourth International Workshop, 1984). The deleted material can be translocated to the short arm of chromosome 9, resulting in t(9;11) (Hagemeijer et al., 1982; Dewald et al., 1983). A few cases of AML-M2, AML-M4 and AML-M5 also have t(9;11) (Hagemeijer et al., 1982; Dewald et al., 1983).

Certain chromosomal anomalies are consistently present in a range of AML subtypes. For example, three anomalies are seen in AML-M2, AML-M4, AML-M5 and AML-M6. These are a deletion in the long arm of chromosome 5 (Kerkhofs et al., 1982) and 7 (Fourth International Workshop, 1984) and an extra chromosome 8 (Fourth International Workshop, 1984). Response to chemotherapy in such patients is very poor and a complete remission is exceptional. Also, an inversion in chromosome 16 (Arthur and Bloomfield, 1983; Le Beau et al., 1983) is specifically seen in AML-M2 and AML-M4.

AML has an unusually high degree of complexity for a given type of

cancer. This explains the difficulties in identifying useful prognostic indicators in AML.

CGL

CGL is characterized by the presence of a translocation, t(9;22). Approximately 10 to 15% of CGL patients do not have this translocation (Canellos et al., 1976; Ishihara et al., 1974; Kohno et al., 1979). Clinical differences between the two types of CGL were recognized. Patients without a translocation, t(9;22), usually present at a higher median age and have lower median leukocyte and platelet counts than do those with the translocation (Canellos et al., 1976). Their response to chemotherapy is usually poor, and they have a consistently lower median survival time than patients with the translocation (Ezdinli et al., 1970; Gomez et al., 1981; Krauss et al., 1964; Whang-Peng et al., 1968). No consistent karyotypic abnormality has been associated with CGL in patients without a translocation, t(9;22). In most cases, only normal metaphases have been found at the time of diagnosis (Sandberg, 1980).

CML

The hallmark of CML is the Philadelphia (Ph') chromosome which was first described by Nowell and Hungerford in 1960. The nature of the aberration was determined to be the reciprocal translocation, t(9;22)(q34;q11), by Rowley in 1973a and 1973b. The breakpoints in this translocation are the same as the breakpoints evident in the translocation, t(9;22), in AML, ALL, and CGL (de la Chapelle and Berger, 1984; Shaw et al., 1982). The Ph' chromosome is found in the myeloid leukemic cells of the marrow, erythrocytic and megakaryocytic cell precursors (Sandberg, 1980a). The stem cell affected in this disease

seems to be a primitive one (Fialkow et al., 1980). This may help explain the equivocal usefulness of present treatments (Wintrobe, 1981).

A t(9;22) is observed in 90% of adults who are clinically diagnosed as having CML (Whang-Peng et al., 1968; Rowley, 1980a). These patients have a median survival time of 42 months. The presence of the t(9;22) in CML has been used as a prognostically favorable indicator because the remaining 10% of CML patients who do not have such a defect survive an average of only 15 months (Sandberg, 1980). The t(9;22) is also found occasionally in patients with a myeloproliferative syndrome that evolves into CML within several months (Sandberg, 1980).

In addition to the typical t(9;22), about 3% to 8% of the Ph' positive CML patients are known to have a variant Ph' translocation (First International Workshop, 1978; Pasquali et al., 1979; Lessard and Le Prise, 1982; Oshimura et al., 1982; Ishihara et al., 1983). Two major types of variant Ph' are the two-chromosome or "simple" translocations, involving chromosome 22 and another chromosome other than 9, and the three-or four-way "complex" translocations (Sandberg, 1980; Borgstrom, 1981). These complex translocations involve chromosome 9, 22 and at least one other chromosome. A third, very rare type of variant Ph' is called the "masked Ph'", where the Ph' chromosome does not show its usual morphology and is the recipient of part of another chromosome (Engel et al., 1974; Tanzer et al., 1977; Lessard et al., 1981). Masked Ph' chromosomes are complex translocations, very often involving chromosome 9 (Sessarego et al., 1983; Oshimura et al., 1981; Hagemeijer et al., 1984). The survival of patients with all types of

variant translocations is the same as those with the standard translocation (Sonta and Sandberg, 1977).

1.1.4 Clonal Evolution in Ph'-Positive

The t(9;22) is found throughout the evolution of the CML disease process. When patients with CML enter the terminal acute phase, blast crisis, about 20% appear to retain the 46, Ph'-positive cell line unchanged, whereas 80% of patients show karyotypic evolution (Rowley, 1980). That is, new chromosomal abnormalities in very distinct patterns are present in addition to the Ph' chromosome. In many cases, the change in karyotype precedes the clinical signs of blast crisis by 2-4 months (Rowley, 1980a). Thus, a change in karyotype is considered to be a grave prognostic sign. The median survival from the time of change until death was found by Whang-Peng et al. (1968) to be 2-5 months.

The most common changes, gain of chromosome 8 or 19 or a second Ph' and i(17q), frequently occur in combination to produce modal chromosome numbers of 47 to 50 (Rowley and Testa, 1982). When patients had only a single new chromosome change, this most commonly involved the gain of a second Ph', an i(17q), or a +8, in descending order of frequency (Mitelman and Levan, 1978). Chromosome loss occurs only rarely; that most often seen was -7, which occurred in only 3% of patients (Rowley and Testa, 1982). The relatively limited number of recurring chromosome abnormalities in the acute phase implies that the chromosomes involved carry genes that provide a proliferative advantage to the Ph'-positive cell that has an extra copy of one or a combination of these chromosomes (Le Beau and Rowley, 1986).

1.1.5 Clonal Evolution in Ph'-Negative CML

Mintz et al. (1979) found that 3 of the 10 Ph'-negative patients with CML showed chromosome abnormalities which are usually associated with blast crisis of Ph'-positive CML. For example, patients who showed karyotypic evolution associated with a change in modal chromosome number also had an extra chromosome 8. This observation is supported by the work of Vallejos et al. (1974) and Canellos et al. (1976). This abnormality is one of the most common in Ph'-positive CML during blast transformation and is the most common change seen in the progression of ANLL. Mintz also found that the clinical course of Ph'-negative patients with characteristic Ph'-positive clonal evolution was similar to the clinical course of Ph'-positive CML.

1.1.6 Molecular Basis of Philadelphia-Positive CML and ALL

The observation that proto-oncogenes are activated in specific nonrandom chromosomal rearrangements associated with certain cancers has provided insight into the potential role played by oncogenes in neoplasia (Varmus, 1984). Philadelphia-positive CML, which is characterized by the translocation, t(9;22), represents one such human cancer. In this chromosomal aberration, the abl oncogene is translocated from its normal position on 9q34 to the Ph'-chromosome (Heisterkamp et al., 1982; de Klein et al., 1982; Heisterkamp et al., 1983; Bartram et al., 1983) while the sis oncogene is translocated from 22q13 to chromosome 9 (Groffen et al., 1983; de Klein et al., 1982). Similar oncogene mobility data is unavailable for ALL.

Molecular studies have revealed that the breakpoints on chromosome

22 occur in a restricted region of about 5.8 kilobases, designated the breakpoint cluster region or bcr (Groffen et al., 1984). The result of the Philadelphia translocation is the shift of the abl gene from chromosome 9 into either of two small introns at the centre of the bcr gene (Bartram et al., 1983) which is located at 22q11 (de Klein et al., 1982; Groffen et al., 1984; Heisterkamp et al., 1985). The consequence of this molecular rearrangement is the production of aberrant 8 kb abl mRNA (Gale and Canaani, 1984) and an abnormal fusion protein, bcr-abl p210, with enhanced protein kinase activity (Konopka et al., 1984) compared with the normal p145 abl protein. The fused protein is expressed during both the chronic and acute phase of the disease (Shtivelman et al., 1987; Konopka et al., 1985; Maxwell et al., 1987).

In contrast to CML, the central region of bcr is not always involved in the translocation present in Philadelphia-positive ALL patients (de Klein et al., 1986; Erikson et al., 1986). In one such case, the breakpoint in 22q11 was distal (3') to the immunoglobulin gene for the constant region of the λ light chain, and proximal (5') to bcr (Erikson et al., 1986). In addition, these patients contain a new 190K abl-encoded protein kinase (Kurzrock et al., 1987; Clark et al., 1987; Chan et al., 1987), rather than the 210K protein typical of CML. The appearance of the p190 protein correlates with the expression of unusual abl mRNA (Kurzrock et al., 1987; Clark et al., 1987; Chan et al., 1987). It has been recently found that in some patients with Philadelphia-positive ALL the second abl exon is fused to the first exon of bcr (Fainstein et al., 1987) and the expression of the fused transcript results in the 190K protein kinase (Fainstein et al., 1987).

In addition to the original bcr gene (bcr1) associated with Philadelphia-positive CML and ALL, the human genome contains three bcr-related genes containing the 3' region of bcr, namely, bcr2, bcr3 and bcr4 (Croce et al., 1987). In both CML and ALL, the breakpoint is proximal to the bcr1 3' region, but distal to the λ light chain gene and bcr2 and bcr4 (Croce et al., 1987). Thus, the order of the loci on chromosome 22 is centromere -> bcr2, bcr4, and λ light chain gene -> bcr1 -> bcr3 -> sis (Croce et al., 1987). The precise loci involved as targets for juxtaposition to the abl gene is unknown at the present time.

1.1.7 Molecular Basis of Philadelphia-Negative CML

Philadelphia-negative CML is characterized by a bimodal distribution of survival in patients (Ezdinli et al., 1970). As a result, the disease is believed to include a small subgroup of patients whose clinical features and course parallels those of patients with Ph'-positive CML (Fialkow et al., 1980; Kurzrock et al., 1986).

Experimental evidence supporting the molecular translocation of the abl and sis oncogenes in Ph'-negative CML is inconclusive as compared to Ph'-positive CML. Mobility of the abl oncogene to chromosome 22 in the absence of the Ph'-chromosome has been shown by some investigators (Morris et al., 1986) and refuted by others (Bartram et al., 1983). Furthermore, abl has been shown to move to a variety of recipient chromosomes in complex translocations involving t(9;22) and other chromosomes (Hagemeijer et al., 1984) without the generation of the Ph'-chromosome. Similarly, in situ hybridization on a Ph'-negative CML patient with the translocation, t(9;12)(q34;q21) demonstrated mobility

of both abl and bcr to the derivative chromosome 12 (Bartram et al., 1985b). In contrast to the apparent mobility of the abl onogene, sis and bcr do not move to chromosome 9 in CML patients without a Ph'-chromosome (Morris et al., 1986).

Evidence supporting the molecular rearrangement of bcr is present in both Ph'-positive and Ph'-negative CML. Investigators unanimously support bcr rearrangement in Ph'-negative CML (Ganeson et al., 1986; Bartram and Carbonell, 1986; Benn et al., 1987; Kurzrock et al., 1986).

1.1.8 Fragile Sites

A fragile site is a region of a chromosome, present on both chromatids, that fails to become solid stained (Le Beau and Rowley, 1984). There are 104 established fragile sites according to the Ninth Human Gene Mapping Workshop. The established fragile sites belong to four classes, according to their mode of induction: folate sensitive, distamycin-A-inducible, BrdU requiring and aphidicolin-inducible fragile sites. The latter class of fragile sites is also referred to as the common fragile sites. Although the clinical significance of most fragile sites is unknown, fragile site Xq27.3 is associated with one form of X-linked mental retardation (Chudley and Hagerman, 1987).

While the precise structure of fragile sites is unknown, they may represent chromosomal segments which do not undergo normal compaction during mitosis (Le Beau and Rowley, 1984). The majority of fragile sites appear to be located either at the junction of Giemsa-negative and Giemsa-positive bands or in Giemsa-negative bands close to the junction (Yunis and Soreng, 1984). The bulk of the structural genes in humans are localized in Giemsa-negative bands. The Giemsa-positive bands are

enriched in middle-repetitive AT-rich DNA (Sanchez and Yunis, 1984). It is possible that most fragile sites represent an evolutionarily conserved class of T-rich sequences that flank proto-oncogenes and are particularly sensitive to thymidine deprivation (Yunis and Soreng, 1984). More current views represent fragile sites as reiterated DNA sequences of variable length (Warren et al., 1987).

The established autosomal fragile sites tend to occur in chromosomal bands where breaks leading to rearrangements in cancer have been identified (Yunis, 1983; Yunis, 1984a; Hecht and Sutherland, 1984; de la Chapelle and Berger, 1984). The locations of these sites (Hecht and Sutherland, 1984) were analyzed with respect to 50 cancer chromosome breakpoints accepted by the Seventh Human Gene Mapping Workshop (de la Chapelle and Berger, 1984). A significant statistical relationship was found ($p < 0.001$) consistent with the concept that established fragile sites may predispose to chromosomal rearrangements seen in some cases of neoplasia.

Although no direct evidence for this concept exists, a number of coincidences have been observed. For example, the t(8;21)(q22;22) is frequently seen in acute myeloblastic leukemia (Rowley, 1983). Correspondingly, a fragile site has been identified at 8q22. A fragile site at p21 of chromosome 9 is also the breakpoint in the t(9;11)(p21;q23) most commonly seen in acute monoblastic leukemia (Hagemeijer et al., 1982). There are fragile sites at q13 and q23 of chromosome 11, each of which can be the breakpoint in various translocations or deletions in both acute myeloid and lymphoid leukemias (Rowley and Fukuhara, 1980; Hagemeijer et al., 1982; Third International

Workshop on Chromosomes in Leukemia, 1981). The bone marrows of patients with acute myelomonocytic leukemia may have a rearrangement at 16q22. Such rearrangements include inv(16)(p13q22) (Le Beau et al., 1983; Fourth International Workshop, 1984), del(16)(q22) (Fourth International Workshop, 1984; Arthur and Bloomfield, 1983), t(16;16)(p13;q22) (Testa et al., 1984), and t(5;16)(q33;q22) (Bhambhani et al., 1986). A strong association of fragile site 16q22 in normal cells with the occurrence of a rearrangement involving band 16q22 in neoplastic cells from bone marrow has been reported in patients with acute myelomonocytic leukemia or AMMOL (Yunis, 1984a; Le Beau and Rowley, 1984; Le Beau and Rowley, 1986; Arthur et al., 1985; Glover et al., 1986). The coincidence of the relationship between chromosomal rearrangements of malignancy and fragile sites is substantiated by the number of cancer patients with chromosomal rearrangements that are themselves carriers of fragile sites (Le Beau and Rowley, 1984).

The association of common fragile sites and cancer chromosome breakpoints is less substantial than with the established fragile sites (Hecht and Sutherland, 1984). Eight of 25 aphidicolin-induced common fragile sites have been correlated with cancer breakpoints (Hecht and Hecht, 1984). Yunis and Soreng (1984) compared common fragile sites with cancer breakpoints. These fragile sites accounted for 70-80% of cancer breakpoints observed in these patients. The common fragile site at 3q27 has been found to be associated with the breakpoints in ins(3)(q27;q21q27) found in patients with myeloproliferative disorders (Norrby et al., 1982). Common fragile sites were also found to be located in both bands leading to deletion 3p in small cell carcinoma of

the lung (Whang-Peng et al., 1982a; Whang-Peng et al., 1982b) and in both translocation breakpoints in cystadenocarcinoma of the ovary (Wake et al., 1980). Both carcinoma of the lung and ovary are relatively common cancers. Common fragile sites may be associated with common cancers (Hecht and Glover, 1984).

1.1.9 Aphidicolin-Induced Common Fragile Sites

The newest class of fragile sites is termed common fragile sites. These fragile sites can be induced by aphidicolin, an inhibitor of DNA polymerase (Glover et al., 1984). Common fragile sites occur with the highest frequency in the human karyotype as compared to the other classes of fragile sites such as the folative sensitive sites, the distamycin-A-inducible sites and the BrdU requiring fragile sites (Hecht and Glover, 1984; de la Chapelle and Berger, 1984).

Certain aphidicolin-induced fragile sites occur in almost all normal individuals studied. These sites include 2q31, 3p14, 6q26, 7q32, 16q23 and Xp22 (Glover et al., 1984). These sites were visible in a high proportion of lymphocytes from each individual. Sites seen less often with aphidicolin included 1p22, 1p32, 1p36, 1q25, 2p13, 2p24, 2q33, 3p24, 3q27, 5q31, 6q21, 7p13, 7q22, 8q22, 9q32, 11p13, 14q24, 22q12 and Xq22 (Glover et al., 1984). Centromeric stretching events were not regarded as a cytologic phenomena associated with aphidicolin.

The locations of these common fragile sites were compared with the locations of 50 accepted cancer chromosome breakpoints (de la Chapelle and Berger, 1984). This comparison indicated that at least eight sites induced with aphidicolin are in chromosome bands containing breakpoints leading to chromosome rearrangements in cancer cells. These eight

aphidicolin-induced fragile sites are: 3p24, 3p14, 3q27, 6q21, 8q22, 11p13, 14p24 and 22q12. The statistical relationship between the locations of fragile sites and cancer chromosome breakpoints (Hecht and Sutherland, 1984) extends beyond the established fragile sites and also includes the common aphidicolin-induced fragile sites. Aphidicolin-induced fragile sites can be viewed now as potentially important to cancer cytogenetics.

1.1.10 Relationship Between Cancer-Specific Rearrangements, Fragile Sites and Oncogenes

A restricted number of chromosomal regions have been implicated in the etiology of human cancer (Mitelman, 1984). In particular, a total of 83 out of 440 bands have been found to be specifically involved in primary structural rearrangements in cancer (Heim and Mitelman, 1987).

In a study based on a computer analysis of cytogenetic data on 17,000 cases of leukemia and lymphoma reported in the literature, the majority of fragile sites have been shown to be associated with a higher rate of structural rearrangements (Braekeleer et al., 1985). Le Beau and Rowley (1984) demonstrated that seven fragile sites are involved in non-random chromosomal abnormalities observed in leukemia and lymphoma. A relationship between fragile sites and leukemias has also been reported by Yunis (1983) and Yunis and Soreng (1984).

It has been recently shown that 72% of breakpoints present in lymphomas were found at sites to which either transformation related genes or fragile sites have been mapped (Chaganti and Koduru, 1987). Furthermore, a comparison of the distribution of cancer-specific breakpoints with the chromosomal sites of the 26 cellular oncogenes

revealed that 19 of the 26 oncogenes were localized in cancer-associated chromosomal breakpoints (Heim and Mitelman, 1987). Genes of importance for the transformation of a normal cell to a cancerous one seem to be located in a restricted number of chromosomal regions (Mitelman, 1984).

1.2 The Relationship Between Fragile Sites, Gaps, and Breaks

Fragile sites are nonstaining areas on chromosomes. A discrete chromatid connection is usually seen throughout the length of the fragile site. A chromatid gap is distinguished from a fragile site because it does not have a discrete chromatid connection. The gap may be present on one chromatid and is termed a single chromatid gap. A double chromatid gap, also termed an isochromatid gap, is present on both chromatids. Both fragile sites and gaps are characterized by chromatid alignment. In contrast, a chromosome break is represented by unequal chromatid alignment. Breaks may occur in one chromatid (ie. single chromatid break) or in both chromatids (ie. isochromatid breaks). Breaks can be clearly distinguished from both gaps and fragile sites under the microscope.

1.3 Characterization of Oncogenes sis and abl

The most significant oncogenes in CML are *sis* and *abl*. The *sis* protein product serves as a growth factor in the cell; the *abl* protein product serves as a tyrosine-specific protein kinase.

The discovery that the oncogene (v-*sis*) of the simian sarcoma virus (SSV) encodes a protein closely related to a major component of human platelet-derived growth factor (PDGF) provided the first evidence that

oncogene products were involved in growth factor-mediated proliferative pathways (Doolittle et al., 1983; Waterfield et al., 1983). Human PDGF preparations contain two related but distinct polypeptide chains, only one of which, PDGF-2, is homologous to the sis gene product. Like human PDGF, the processed dimeric forms of the sis/PDGF-2 gene product have been shown to bind to the PDGF receptor, trigger its phosphorylation at tyrosine residues, and specifically stimulate DNA synthesis of cells possessing such receptors (Leal et al., 1985). Moreover, only those cell types possessing PDGF receptors are susceptible to growth alterations induced by the sis transforming gene (Leal et al., 1985). These findings have indicated that expression of this human growth factor-like oncogene product in a cell responsive to its growth-promoting activity can lead to transformation.

The human sis proto-oncogene is a unique gene (Chiu et al., 1984) and has been localized within the human genome at chromosome 22q11 (Swan et al., 1982). The coding regions of this gene are encompassed within the first six exons (Rao et al., 1986). The great majority of the first exon, as well as the entire seventh exon, is comprised of noncoding sequences. The long lengths of the 5' and 3' untranslated region of the transcript are unusual among most eukaryotic genes. However, there is evidence that some of the genes involved in the pathways by which growth factors stimulate normal cellular proliferation may exhibit long 5' and 3' noncoding sequences (Battey et al., 1983; Dull et al., 1984).

PDGF is found in the α-granules of platelets and presumably is synthesized in their megakaryocyte precursors. Expression of the sis/PDGF-2 gene is present in a variety of tissues including normal

endothelial cells (Barrett et al., 1984), placental trophoblasts (Goustin et al., 1985) and activated monocytes (Martinet et al., 1986). Expression of the normal human sis/PDGF-2 coding sequence in assay cells responsive to this growth factor can cause neoplastic transformation (Gazit et al., 1984). Moreover, the sis-PDGF-2 mRNA has been detected in tumors such as glioblastomas and fibrosarcomas (Eva et al., 1982), whose normal counterparts possess PDGF receptors and are responsive to the growth-promoting action of the sis/PDGF-2 gene product (Heldin, 1981; Bowen-Pope and Ross, 1982). Major mRNA start sites of sis/PDGF-2 transcripts derived from normal placenta and from a human tumor cell line were identical, excluding the possibility that sis/PDGF-2 expression in this tumor reflects the altered transcriptional initiation of this gene (Rao et al., 1986). Further investigation of the role of flanking sequences that may affect sis/PDGF-2 gene expression in tumor cells expressing the sis/PDGF-2 transcript may help to elucidate the mechanism of oncogene activation.

Although relatively more is known about abl, its normal cellular function has not been established. The v-abl oncogene is derived from the Abelson moloney murine leukemia virus and is located at 9q34 (McAlpine et al., 1987). The v-abl oncogene can be divided into two distinct regions, of which the aminoterminal 1.2 kb encodes the tryosine-specific protein kinase (Wang et al., 1984). Human abl contains exons homologous to and colinear with this domain of v-abl (Groffen et al., 1983); the tyrosine phosphorylation acceptor site sequence of v-abl is also found within the human abl oncogene and is identical in amino acid sequences (Groffen et al., 1983). In addition

one exon designated A which is non-v-abl homologous, contains sequence homology to v-src (Groffen et al., 1983). The deduced amino acid sequence of v-abl indicates that it is a member of a family of tyrosine-specific protein kinases including v-src, v-yes, v-fes, v-fgr, v-erb , v-ras and v-fms (for review see Bishop, 1985). It has been reported that the v-abl protein is unlikely to be a transmembrane receptor because it does not appear to span the plasma membrane (Ben-Neriah et al., 1986). All breakpoints identified in different patients are 5' of the most 5' v-abl homologous exon (Heisterkamp et al., 1983).

1.4 Oncogene Activation

The recent work in molecular oncology is based on the proposal that a neoplastic cell develops from its normal progenitor as a consequence of changes in a restricted set of cellular genes. The mutant genes are known as oncogenes, and corresponding wild-type alleles are called proto-oncogenes (or normal cellular genes) (Varmus, 1984). Proto-oncogenes can be activated as oncogenes in human cells by a variety of mechanisms independent of retroviral involvement. These include chromosomal rearrangements (Dalla-Favera et al., 1982; Shen-Ong et al., 1982; Taub et al., 1982; Klein, 1983), gene amplification (Alitalo et al., 1983; Little et al., 1983; Schwab et al., 1983), and alternations as subtle as point mutations in their coding sequences (Reddy et al., 1982; Tabin et al., 1982; Taparowsky et al., 1982). Among these mechanisms, the most significant modes of oncogene activation in leukemia are chromosomal translocation and gene

amplification (Yunis, 1983).

Clues to oncogene deregulation are provided by microscopic damage to the chromosomes of cancer cells. Translocations have typically affected proto-oncogenes. For example, the breakpoints where portions of two chromosomes are joined together by translocations can lie within or adjacent to proto-oncogenes (Leder et al., 1983; Nowell et al., 1985). Several translocations affecting proto-oncogenes are already known from the study of retroviruses (Varmus, 1984; Bishop, 1985). In other cases, the DNA that adjoins breakpoints may include new proto-oncogenes (Tsujimoto et al., 1984; Erikson et al., 1985; Bakhshi et al., 1985). For example, two such candidates are the bcl-1 and bcl-2 oncogenes.

Chromosomal translocations in cancer cells can affect either the expression or biochemical function of proto-oncogenes. Effects on expression are seen in the translocations that join c-myc to various immunoglobulin genes in Burkitt's lymphoma (Leder et al., 1983; Nowell et al., 1985). The Ph'-chromosome typifies the translocation that affects biochemical function (refer to section 1.1.6).

Chromosomal translocation of proto-oncogenes can play a definite role in tumorigenesis. For example, some translocations occur with great consistency in particular tumors (Yunis, 1983) and can affect the same proto-oncogene in different species, such as c-myc in B-cell tumors (Varmus, 1984). Three of the proto-oncogenes first recognized during the study of retroviral oncogenes (c-abl, c-ets and c-myb) have now been involved in translocations seen in various forms of malignancy (Leder et al., 1983; Shtivelman et al., 1985; Grosveld et al., 1986). Chromosomal

translocation of a proto-oncogene can damage both the structure and function of the gene resulting in similarity to the oncogenic form of the same gene (Davis et al., 1985). Furthermore, mice carrying an experimentally introduced facsimile of translocated c-myc in their germinal DNA develop lymphoid tumors (Adams et al., 1985).

Gene amplification is an unusual aberration in mammals often identified by two karyotypic abnormalities, namely, double-minute chromosomes and homogeneously staining regions (HSR). HSRs disrupt the normal banding patterns of chromosomes. Untreated cancer cells often contain amplified DNA that can include proto-oncogenes. Amplification of proto-oncogenes has been found as an occasional feature of diverse tumors (Varmus, 1984; Alitalo and Schwab, 1986) and as a recurrent aberration involving specific proto-oncogenes in particular tumors (Schwab et al., 1985; Schwab, 1985; Escot et al., 1986; Wong et al., 1986). For example, L-myc and N-myc are proto-oncogenes that serve as important components of amplified DNA in several types of human tumors. The cause and mechanisms involved in gene amplification remain unresolved. The phenomenon generally has been found in cells that are proceeding toward neoplastic growth (Stark and Wahl, 1984). Whether or not gene amplification occurs in normal cells is a controversial issue (Srivastava et al., 1985). Usually, it is after the onset of neoplasia that amplification of proto-oncogenes has been shown (Schwab et al., 1985; Schwab, 1985; Escot et al., 1986; Wong et al., 1986).

In general, there are three theories in favor of gene amplication as a factor in neoplasia. Firstly, amplification affects proto-oncogenes that alter cell proliferation (Varmus, 1984; Alitalo and

Schwab, 1986; Schwab et al., 1985). Secondly, amplification of a proto-oncogene sometimes correlates with specific characteristics of cancer cells, as if it were the cause and the effect (Schwab et al., 1985; Schwab, 1985; Escot et al., 1986; Wong et al., 1986). Lastly, amplified DNA persists in mammalian cells only if it provides a selective growth advantage to the cells (Stark and Wahl, 1984; Schimke, 1984).

1.5 Objectives of the Investigation

Human leukemia is a disorder that affects chromosomal constitution and oncogene regulation. In this investigation, chromosomes from untreated leukemia patients were studied with the following objectives: i) to identify the location of cancer-specific chromosomal breakpoints, ii) to investigate the frequency and variation of fragile site expression in normal cells, and iii) to examine the movement of oncogenes in the karyotype by *in situ* hybridization in non-leukemic and leukemic individuals.

2.0 MATERIALS AND METHODS

2.1 Materials

2.1.1 Non-Leukemic Population

Patient 1 - RUS

RUS is an 18 year old male. This patient's karyotype is 46,XY,t(9;11)(q12;p13). Since neither parent possessed the translocation, it occurred in a de novo manner. The patient ranked above the 95th percentile in height, the 75th percentile weight and the 25th percentile with respect to head circumference. Patient RUS has no striking dysmorphic features; however, he has behavorial problems.

Patient 2 - RUM

This patient's karyotype involves the translocation t(12;14). The translocation represents a partial trisomy 12p syndrome (non-mosaic) originating de novo in a first born child to healthy 26 year old non-consanguineous parents (Ray et al., 1985). The karyotype is 46,XX,-14,+t(12;14)(p11;p11). The trisomy 12p syndrome has been confirmed by clinical, cytogenetic and gene dosage studies.

RUM presented with the common features of trisomy 12p: normal birth weight and physical development, hypotonia, hypertelorism, epicanthal folds, downward slanting palpebral fissures, a broad flat nasal bridge, a short upturned nose with anteverted nares, elongated philtrum and a short neck. The patient's translocation results in partial trisomy for the short arm of chromosome 12. The parents have normal karyotypes, confirming the de novo origin of the abnormality in the patient. The gene dosage studies involved the determination of

lactate dehydrogenase (LDH) levels in erythrocyte hemolysates because LDH-B is assigned to 12p12.1. The results demonstrate a gene dosage effect providing further confirmation of the trisomy 12p syndrome.

Patient 3 - GO

The patient is a 36 year old female with no significant medical history. This patient's karyotype is 46,XX,inv(9)(q32q34). She has two previous children. An amniocentesis was performed with the last pregnancy due to advanced maternal age.

Patient 4 - WO

Patient WO is a 7 year old male. He has an abnormal karyotype that is 47,XY,+i(12p) in fibroblast cells exclusively. WO has multiple congenital anomalies. Bilateral opacification of the lens was also apparent. The patient had frequent seizures. Patient WO has coarse facial features that include hypertelorism, a flat nasal bridge, short upturned nose and long philtrum. The patient also had generalized hypotonia, roving eye movements, large ears and a high, narrow palate. The patient's trunk had unusual pigmentation. Patient WO was diagnosed to have Pallister-Killian Syndrome. This syndrome is associated with severe psychomotor retardation and recurring seizures.

Patient 5 - RH

This patient is a 4 year old female. Her karyotype is 46,XX,rec(9),dup(9)(q33q34), dir ins(9)(q22;q33q34)pat leading to 9q34 syndrome. Height is less than the 25th percentile for age; weight is less than the 5th percentile and head circumference is less than the 5th percentile. By definition, there is evidence for microcephaly. Patient RH has dysmorphic features. RH has learning difficulties due to some

degree of mental retardation. The patient has tremors and a squint. Neurologic examination has revealed features of "spastic ataxia." All these features are the result of partial 9q trisomy. The unbalanced chromosome complement in RH is due to an unequal cross over event in a germ cell of her father who carries a balanced chromosome rearrangement, 46,XY,dir ins(9)(q22;q33q34). Two large Newfoundland families have the identical chromosome rearrangement as RH (Allderdice, 1983).

2.1.2 Leukemic Population

2.1.2.1 Subgroup 1

This group of leukemia patients was used to study chromosomal rearrangements. It included 72 untreated patients that were karyotyped during the past three years (1984-1987) in the clinical cytogenetic laboratory, Health Sciences Centre, Winnipeg, Manitoba. The patients were diagnosed with ALL (5 cases), AML (5 cases), CGL (2 cases), and CML (59 cases).

2.1.2.2 Subgroup 2

This group of untreated leukemia patients was used to study oncogene mobility. In each case, the diagnosis has been confirmed by an oncologist at the Manitoba Cancer Treatment and Research Foundation, Winnipeg, Manitoba. The diagnosis was based on morphologic, hematologic and clinical criteria.

There were four patients diagnosed with ALL. Patient DI and MUR have a normal karyotype, 46,XY, whereas patients MA and MC have abnormal karyotypes, namely, 46,XY,t(11;19) and 45,X,-16,-Y,+2, respectively.

One patient, namely MU, was diagnosed with AML. Patient MU has a

translocation, 46,XY,t(16;16)(q22;q24).

LAD, the only patient diagnosed with CGL, has the karyotype, 46,XY,$9q^-$,$11q^-$,$14q^+$,$22q^+$.

The majority of the patients were diagnosed with CML. BJ, NI, WR, NIS, FRA and JA have the karyotype, 46,XY. One CML patient, LA, had the karyotype, 46,XY,t(9;22)(q34;q11).

Patient HA was diagnosed with aplastic anemia. Particular cases of aplastic anemia can also be classified as a form of pre-leukemia. This patient has the karyotype, 46,XY,-7,+G.

2.1.2.3 Subgroup 3

This group of individuals was used to study aphidicolin-induced fragile site expression. It included two non-leukemic or control individuals and eight untreated leukemic individuals. The control individuals were in good health although they were not age-matched to the leukemic individuals. The eight leukemic individuals were MU, MA, BJ, NI, MC, WR, NIS and DI as previously described. A comparable number of individuals was used for the expression of aphidicolin-induced fragile sites by Glover et al. (1984).

2.2 Methods

2.2.1 Chromosomal Rearrangements

2.2.1.1 Blood Cultures/Harvest/Slide Preparation

Chromosome analyses for the study of chromosomal rearrangements in leukemic patients were performed on peripheral blood and bone marrow cells. The blood and bone marrow were set up in McCoy's 5A medium with

10% fetal calf serum without PHA for either 24 or 48 hours. Only the cancer cells are able to grow and proliferate without PHA. The cultures were harvested following colchicine treatment (0.05 ug/ml) and the cells were fixed with three parts methanol and one part acetic acid (3:1) following hypotonic treatment with 0.075M KCL. The cells were spread on wet microscope slides and air dried.

2.2.1.2 Chromosome Banding/Nomenclature

Metaphase chromosomes were stained with quinacrine mustard (100 ug/ml) according to the method of Caspersson et al. (1971). Chromosomes showed intercalary banding upon observation with a fluorescent light source. Printed metaphase spreads were analyzed for the establishment of a karyotype. Karyotypes of patients were described with the cytogenetic nomenclature documented in ISCN, 1978.

2.2.2 Fragile Sites

2.2.2.1 Blood Cultures/Fragile Site Induction

Chromosome analyses for the study of fragile sites in leukemic patients and controls were performed on peripheral blood cells. The blood was set up in McCoy's 5A medium with 10% fetal calf serum for 72 hours with PHA. The normal cells are able to grow and proliferate in the presence of PHA but not the cancer cells. Common fragile sites were induced by the addition of 0.2 uM aphidicolin during the last 24 hours of culture (Glover et al., 1984). Cultures were harvested and fixed and slides were prepared as previously described.

2.2.2.2 Chromosome Banding/Scoring/Statistical Analysis

Metaphase chromosomes were examined with light microscopy by solid staining with 4% Giemsa (2 ml Harleco Giemsa: 48 ml Gurr's buffer with pH 6.8) in order to photograph fragile sites. The chromosomes were destained in three parts methanol and one part acetic acid for five minutes. G-banding according to the method of Seabright (1971) was then performed on the chromosomes demonstrating fragile sites. These chromosomes were re-photographed following G-banding. Fifty metaphases from each individual were analyzed microscopically (Glover and Stein, 1987).

The fragile sites, gaps and breaks were scored in accordance with the definitions presented in section 1.2. Since the maximum yield of metaphase cells per leukemia patient closely approximated 50 cells, the majority of cells were scored from each patient. For practical reasons, prior knowledge of the identity of each patient studied could not be avoided.

The average frequency of fragile sites per cell in the non-leukemic individuals (i.e. controls) was used as an estimate of the "expected" frequency of fragile sites in the statistical analysis. The frequency of fragile sites in each leukemic individual was statistically compared to the average frequency of fragile sites in the controls by the Poisson distribution (Armitage, 1971).

2.2.3 In Situ Hybridization

2.2.3.1 Theory and Applications of In Situ Hybridization

In situ hybridization allows the visualization of nucleic acid

sequences directly on cytologic preparations. As a result, it is a powerful technique for studying nucleic acid sequence organization and function in a wide variety of cell types and tissues. In 1969, this technique was first used for the chromosomal localization of DNA sequences of relatively high copy number (Gall and Pardue, 1969; Birnstiel and Jones, 1969). In situ hybridization now can be used to detect chromosomal sequences present in only one or two copies per cell (Harper and Saunders, 1981; Harper et al., 1981) or RNA present at less than 20 transcripts per cell (Harper et al., 1986) after short autoradiographic exposure. High sensitivity is attained by hybridization of cloned nucleic acid probes radiolabelled with ^{3}H or ^{35}S using high specific activity nucleotide triphosphates. Slide preparations are coated with nuclear track emulsions exposed for several days to several weeks, developed, and visualized under the microscope.

In situ hybridization offers several advantages as a technique for detecting and localizing nucleic acid sequences in eukaryotic cells. It is a direct method which is rapid and requires small tissue samples. Low abundance sequences can be detected readily and the number of sequences can be reasonably quantitated (for review see Henderson, 1982; Harper and Marselle, 1986).

The technique has been widely applied in cancer cytogenetics. It has been used to map the location of oncogenes and proto-oncogenes such as c-abl and c-sis (Jhanwar et al., 1984), c-ets (de Taisne et al., 1984), c-fos (Barker et al., 1984), c-ras (Jhanwar et al., 1983), and c-mos (Neel et al., 1982) along with many others. In situ hybridization has the capability to directly detect repositioning of sequences within

the karyotype as a result of chromosomal rearrangements (Dalla-Favera et al., 1982; Taub et al., 1982; Bartram et al., 1983; Le Beau et al., 1985; Diaz et al., 1985). Also, small rearrangements not detectable by standard karyotypic analysis may be uncovered by this technique (Le Beau et al., 1985; Ohyashiki et al., 1987; Bartram et al., 1985b; Hagemeijer et al., 1984; Morris et al., 1986). In situ hybridization has also been used to detect both oncogene deletion (Eccles et al., 1984) and oncogene amplification (Wolman et al., 1985). Another application is detection and characterization of breakpoints using probes for defined sequences. For example, the metallothionein gene cluster is split by chromosome 16 rearrangement in myelomonocytic leukemia (Le Beau et al., 1985). Therefore, in situ hybridization should be useful in the investigation of oncogenes involved in cancer-specific chromosomal anomalies.

2.2.3.2 DNA Probes

DNA probes mapping to particular oncogenes in the human genome were used in this investigation. The v-abl and v-sis oncogenes map to 9q34 and 22q12 -> q13 respectively (McAlpine et al., 1987). The human oncogenes, K-ras-2 and bcr, map to 12p12 and 22q11 respectively (Human Gene Mapping Workshop 9).

The v-abl probe is specific for the p102 coding region of the v-abl gene (Srinivasan et al., 1981). The plasmid, pK2, contains a 1.6 kb insert derived from the Abelson murine leukemia virus genomic clone AM-1. Viral derived abl probes have been commonly used for in situ hybridization (Morris et al., 1986) in CML.

The proto-oncogene c-sis appears to encode a protein with partial homology to a subunit of platelet-derived growth factor (Doolittle et

al., 1983). The plasmid, pR81, contains a 1.2 kb insert derived from the Simian sarcoma virus genomic clone SSV-12 (Robbins et al., 1981). This probe has been previously used *in situ* hybridization (Thiele et al., 1987).

The human K-*ras*-2 probe encodes an intron from the human K-*ras* locus (McCoy et al., 1983). The plasmid, p640, contains a 0.64 kb insert from the SW-2-3 cell line. This line is a tertiary transfectant cell line of NIH 3T3 cells transformed by human colon carcinoma DNA. The K-*ras*-2 probe has been recommended for *in situ* hybridization by the distributing company (Catalogue of recombinant DNA collections, 1986).

The human *bcr* probe encodes the b3 exon which is located 5' to the chromosomal breakpoint at 22q11 (Grosveld et al.,1986). The 0.5 kb insert was derived from the cDNA clone V1-3. This probe has been used for *in situ* hybridization by Dr. C. C. Lin (personal communication).

The following probes were obtained from American Type Tissue Culture Collection: v-*abl*, v-*sis* and K-*ras*-2. The human *bcr* probe was obtained from Dr. C. C. Lin, University of Calgary.

2.2.3.3 Treatment of Slides Prior to Hybridization

Metaphase chromosome spreads were treated with pancreatic ribonuclease A (Sigma), 100 ug/ml in 2X SSC, pH 7.0 at 37°C for 1 h to remove endogenous RNA. The slides were then rinsed three times with 2X SSC (pH 7.0), dehydrated for 15 minutes each in successive solutions of 50%, 75% and 95% ethanol and air dried for 3 h. The chromosomal DNA was denatured by immersion of slides in 70% (vol/vol) deionized formamide/2X SSC (pH 7.0) at 70°C for 2 min. The slides were then dehydrated in cold (4°C) ethanol as described above and air dried overnight.

2.2.3.4 Radioactive Labelling of Probes

The probes were labelled by nick translation or oligo-labelling.

The plasmids were nick translated according to the technique of Zabel et al. (1983) with a few modifications. Plasmid DNA (100 ng) was nick translated in a reaction volume of 10 ul with 6 uM of three labelled nucleotides (Amersham), namely, [^{3}H] dTTP (46 Ci/mmol), [^{3}H] dCTP (50 Ci/mmol) and [^{3}H] dATP (29 Ci/mmol) and with 60 uM of unlabelled dGTP in buffer (50 mM Tris-HCl pH 7.8, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 5 ug of bovine serum albumin per ml). Five pg of DNase I (bovine pancreas grade 1, Boehringer Mannheim) and 10 units of Escherichia coli polymerase I (Boehringer Mannheim) were added for about 2 h at 15° C.

Plasmids were also oligo-labelled according to the technique of Feinberg and Vogelstein (1983, 1984). This procedure involves using random oligonucleotides as primers and polymerizing with the large subunit of DNA polymerase I (Klenow fragment). Some modifications were made with tritium labelling according to Lin et al. (1985). The labelled nucleotides (Amersham) have been described previously. Plasmid DNA was denatured at 100°C for 5 min. and cooled on ice. 500 ng of plasmid DNA was oligo-labelled in a reaction volume of 50 ul with 0.75 nmol each of ^{3}H-dNTP (^{3}H-dATP, ^{3}H-dCTP and ^{3}H-dTTP), 96.8 uM of unlabelled dGTP in oligo-labeling buffer (Feinberg and Vogelstein, 1984), bovine serum albumin (10 mg/ml) and 4 units of Klenow fragment (Boehringer Mannheim). The reaction was incubated at room temperature for 4 h.

Both types of labelling reactions were stopped by adding 100 mM EDTA (4°C). The labelled DNA was separated from free nucleotides on a Sephadex G-50 column in 3X SSC. Sonicated salmon sperm DNA (2.5 ul of a 10 mg/ml solution for each 10 ul of eluate) was used as a carrier. The DNA was precipitated by 2 vol of 95% ethanol and incubated overnight at -20°C. The specific activity from nick-translation was 2.5 to 5.3×10^7 cpm/ug while the specific activity from oligo-labelling was 1.3 to 4.8×10^8 cpm/ug.

2.2.3.5 Probe Preparation for Hybridization

The radiolabelled pellet was washed in 70% ethanol, air dried and redissolved in hybridization buffer (50% deionized formamide, 10% dextran sulfate, 2X SSC, 40 mM NaH_2PO_4, 0.1% SDS, 1X Denhardt's solution, final pH 7.0). The probe was hybridized at final concentration of 100 ng/ml. Before *in situ* hybridization, the radiolabelled probe was denatured by heating at 70°C for 10 min. and cooled in ice.

2.2.3.6 In Situ Hybridization

This technique is a modification of the procedures of Gerhard et al. (1981) and of Harper and Saunders (1981). The radiolabeled probe was placed on chromosome spreads (2.5 ul) under a coverslip, and incubated at 42°C in a 50% formamide/2X SSC saturated environment. After 18 h, the slides were dipped in 50% formamide/2X SSC at 40°C to remove the coverslip and then washed three times for 10 min. in 50% formamide/2X SSC, pH 7.0, at 40°C to remove nonspecifically bound DNA. The slides were then washed three times for 10 min. in 2X SSC, pH 7.0,

at 40°C and three times for 10 min. in 2X SSC pH 7.0, at room temperature. After this, they were washed for 1 h in 0.1X SSC, pH 7.0, at room temperature, and for 1 h in 0.1X SSC, pH 7.0, at 4°C. The slides were finally dehydrated four times in cold (4°C) ethanol at successive concentrations of 25%, 50%, 75% and 95% and air dried overnight.

2.2.3.7 Autoradiography

Hybridizing slides were dipped in Kodak emulsion diluted 1:1 with water at 42°C. After being air dried in the dark for 2 h, the slides were sealed in bakelite boxes containing Drierite dessicant and exposed for 9 days at 4°C.

The slides were developed for 75s in Kodak Dektol at 20°C, fixed for 30s in Kodak fixer (3 water:1 fixative), rinsed in water, and air dried overnight.

2.2.3.8 Staining and Banding

Staining and banding were performed after hybridization and autoradiography. Banding techniques included the G-banding and Q-banding. Chromosome banding in leukemic chromosomes was particularly difficult to obtain because of their indistinct morphology and poor ability to pick up stain compared with chromosomes of normal cells (Hossfeld and Weh, 1984). These characteristics of leukemic chromosomes appear to be intimately related to the leukemic process (Hossfeld and Weh, 1984).

G-banding was obtained using the method of Perry and Wolff (1974). The slides were stained for 15 min. with 33258 Hoechst dissolved in

water (150 ug/ml), briefly rinsed, and air dried. The slides were then mounted under a coverslip in 2X SSC, pH 7.0, and illuminated for 30 min. by a UV lamp at a distance that maintained a temperature of 47°C to 50°C at slide level. Slides were then rinsed and stained in 4% Giemsa solution (pH 6.8) for 5 min. An example of this banding method used for in situ hybridization is provided by Zabel et al. (1983).

Q-banding was obtained using a modification of the method of Caspersson et al. (1971). The slides were stained with quinacrine mustard and photographed with a fluorescent microscope using a UV light source (Laurie and Gosden, 1980; Kirsch and Morton, 1982; Sakai et al., 1985; Yang-Feng et al., 1986).

2.2.3.9 Analysis of Grain Distribution

The distribution of grains in the karyotype was determined by analyzing at least 25 photographic prints of metaphase spreads (Muller et al., 1987). A metaphase spread was photographed if it had a complete chromosome constitution along with a distinct morphology and/or banding pattern. Due to the limited number of cells available, all such cells per patient were scored for grains.

Silver grains commonly "slip" during in situ hybridization (Mattei et al., 1985). Such slipping of the grains may be due to agitation during the developing process or due to unequal shrinking of the emulsion during developing and fixing. Grains were scored if they were observed on or "near" a chromosome (Lin et al., 1985) to account for such "slippage" of the grains. A grain located "near" a chromosome may touch the chromosome in question. Alternatively, it may be located at some distance from any chromosome in which case it is attributed to

the chromosome in closest proximity to it.

Statistical analysis by the Poisson distribution with the number of grains per chromosome band adjusted for the relative size of the band in a 400-band idiogram was used to reveal significant grains accumulations (Armitage, 1971; Bartram et al., 1985).

3.0 RESULTS

3.1 Chromosomal Rearrangements in 72 Leukemia Patients

The cytogenetic and clinical diagnosis of the 72 patients with chromosomal rearrangements are presented in Table 1. These patients were karyotped in the clinical cytogenetic laboratory mainly by N. Christie and D. Riordan during 1984 to 1987. From a review of the cytogenetic files, four main types of aberrations were revealed - translocations, deletions, duplications and insertions. The majority of the patients were diagnosed as CML. All rearrangements in these patients have been observed from cultures without PHA. The cultures with PHA were found mostly to have a normal karyotype. The rearrangements in unstimulated cultures were distributed in 100% of the metaphase cells. The breakpoints in the chromosomal rearrangements were found to correspond with the map location of both fragile sites and oncogenes reported in the literature.

Figure 1 illustrates partial karyotypes of various chromosomal rearrangements observed in the leukemias. Eleven cases of CML and four cases of ALL have revealed the Philadelphia translocation, namely, t(9;22)(q34;q11). Other aberrations illustrated include an insertion, ins(10;11)(p13;q23q25), a translocation, t(4;11)(q21;q23), and a translocation, t(16;16)(q22;q24), in three AML patients, and a deletion, del(5)(q31) in an ALL patient.

Although the CML patient is typically characterized by a Ph' translocation, five of the 60 CML cases revealed cells with a variant Ph' translocation. The variant Ph' translocation resulted from a

complex translocation that involved chromosomes 9 and/or 22 along with extra material from chromosome 2, 5, 11, 15, 17 or 21. In 41 of the 60 CML cases characterized by a deletion, the breakpoint at band q11 in chromosome 22 was the only visible cytogenetic aberration. It is possible that the deleted part of chromosome 22 is translocated onto a terminal chromosomal band, such as 9q34. Translocations involving the terminal band of a chromosome are difficult to detect without the use of a high resolution reverse-banding technique or by in situ hybridization (Hagemeijer et al., 1984). Therefore, the CML and CGL patients characterized by a deletion at band q11 in chromosome 22 represent cases that require specialized cytogenetic analysis.

The relationship between cancer specific breakpoints, fragile sites and oncogenes in 72 leukemia patients is depicted in Figure 2. The criterion used to define a relationship between these three genetic events is their chromosomal band location in a 400-band karyotype. Either "exact" band concordance or "close" band concordance among the locations of the breakpoints, fragile sites and oncogenes was determined. "Close" band concordance was defined to be within two chromosomal bands. A similar definition was used to review heritable fragile sites in cancer by Le Beau and Rowley (1984). Sixty-six of the 72 leukemia patients in this study were characterized by aberrations involving chromosome 9 or chromosome 22. The remaining six cases did not reveal aberrations involving chromosomes 9 and 22.

Ninety-eight of the 108 breakpoints in Figure 2 either "exactly" map or "closely" map to oncogenes. Similarly, 99 of the 108 breakpoints either "exactly" or "closely" map to fragile sites. The "close"

association of breakpoints to corresponding fragile sites and oncogenes is seen in the following chromosomes: 1, 2, 5, 7, 8, 9, 17 and 22. There was no chromosomal band association observed in chromosomes 4, 10, 15 and 21. "Exact" band association between a chromosomal breakpoint and an oncogene is seen in 20 patients for chromosome 9 and in 3 patients for chromosome 11.

Table 1: *List of the structural rearrangements seen in 72 cases of hematologic malignancies and the corresponding fragile sites and oncogenes.

Structural Rearrangement	No. of Cases/Type of Malignancy	Distribution in Cell (%)	Associated Fragile Sites	Associated Oncogenes
46,XX,del(7)(p12p15),t(9;22)(q34;q11)	1/CML	100	7p11,7p13, 9q32,22q12	erb-B1,abl, sis
46,XX,t(9;15;22)(q34;q15;q11)	1/CML	100	9q32,22q12	abl,sis
46,XX,t(8;9)(q21;q34)	1/CML	100	9q32	abl
46,XY,t(11;17)(q23;p13),t(17;22)(q25;q11)	1/CML	100	11q23,17p12, 22q12	ets-1,p53, sis
46,XY,t(1;21)(q32;q11)(t(11;17)(q13;p13),t(17;22)(q25;q11)	1/CML	100	1q32,11q13,, 17p12,22q12	ski,ets-1, p53,sis
47,XY,+8,t(9;22)(q34;q11)	1/CML	100	9q32,22q12	abl,sis
46,XY,t(5;9;22)(q31;q34;q11)	1/CML	100	5q31,9q32, 22q12	fms,abl,sis
46,XX,del(2)(p13p23),t[dup9(q13q34);22(q34;q11)]	1/CML	100	2p13,9q32, 22q12	N-myc,abl, sis
46,XX or XY,t(9;22)(q34;q11)	11/CML	100	9q32,22q12	abl,sis
46,XY,ins(10;11)(p13;q23q25)	1/AML	100	11q23	ets-1
**46,XX or XY,del(22)(q11)	41/CML 2/CGL	100	22q12	sis
46,XX or XY,t(9;22)(q34;q11)	4/ALL	100	9q32,22q12	abl,sis
46,XY,del(5)(q31)	1/ALL	100	5q31	fms
46,XY,del(8)(q21),del(9)(q21)	1/AML	100	8q22	mos
46,XY,t(4;11)(q21;q23)	1/AML	100	11q23	ets-1
46,XY,t(15;17)(q22;q21)	1/AML	100		fes,erb
46,XY,t(16;16)(q22;q24)	1/AML	100	16q22	

* Karyotypic analysis was performed mainly by N. Christie and D. Riordan in the clinical cytogenetic laboratory during 1984 to 1987.

** These deletions represent the Ph' chromosome (see 4.1).

Figure 1: Partial karyotypes identified in five leukemia patients: t(9;22)(q34;q11), ins(10;11)(p13;q23q25), t(16;16)(q22;q24), t(4;11)(q21;q23) and del(5)(q31). The arrows on the right indicate the abnormal chromosome homologue.

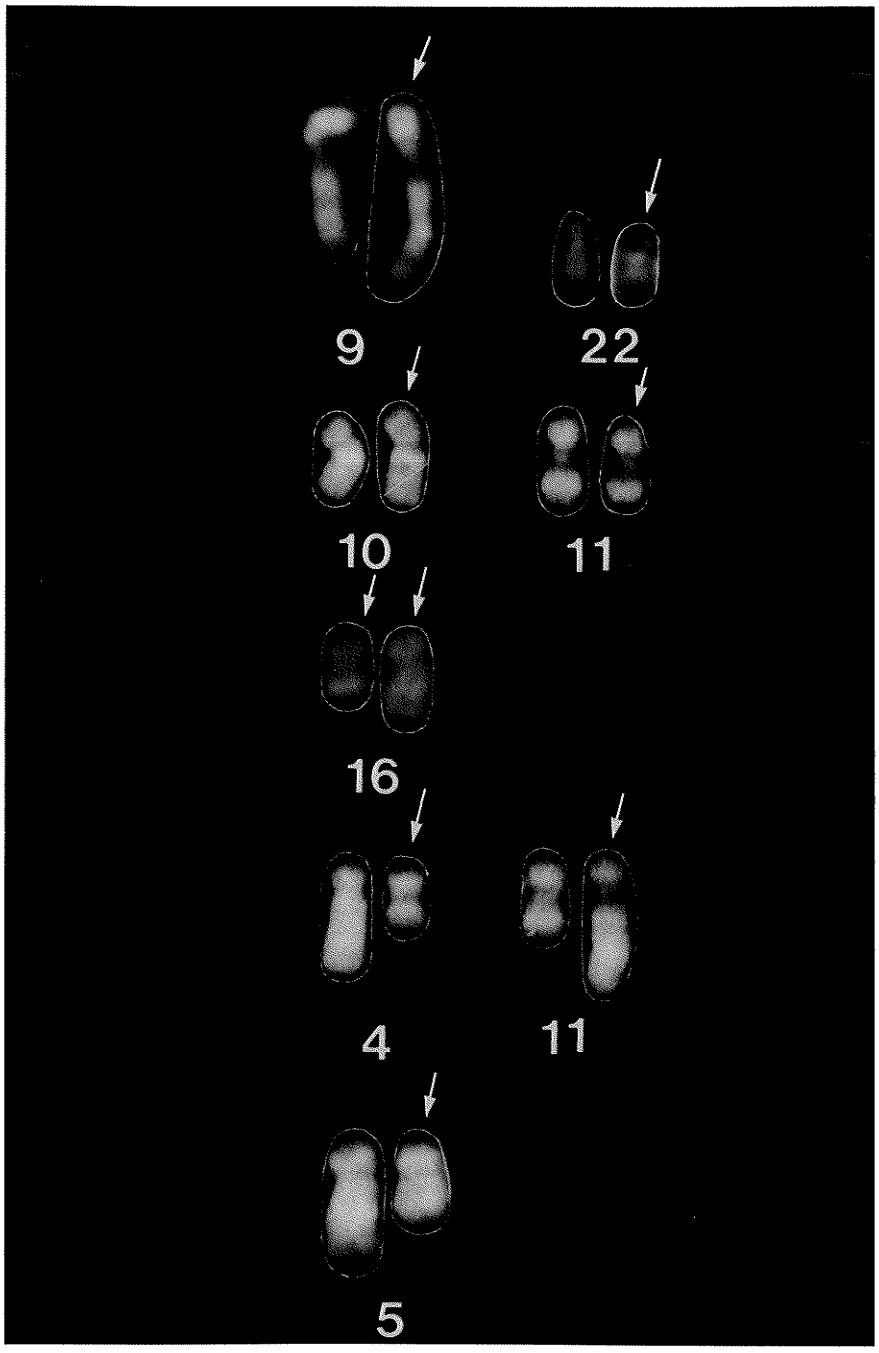
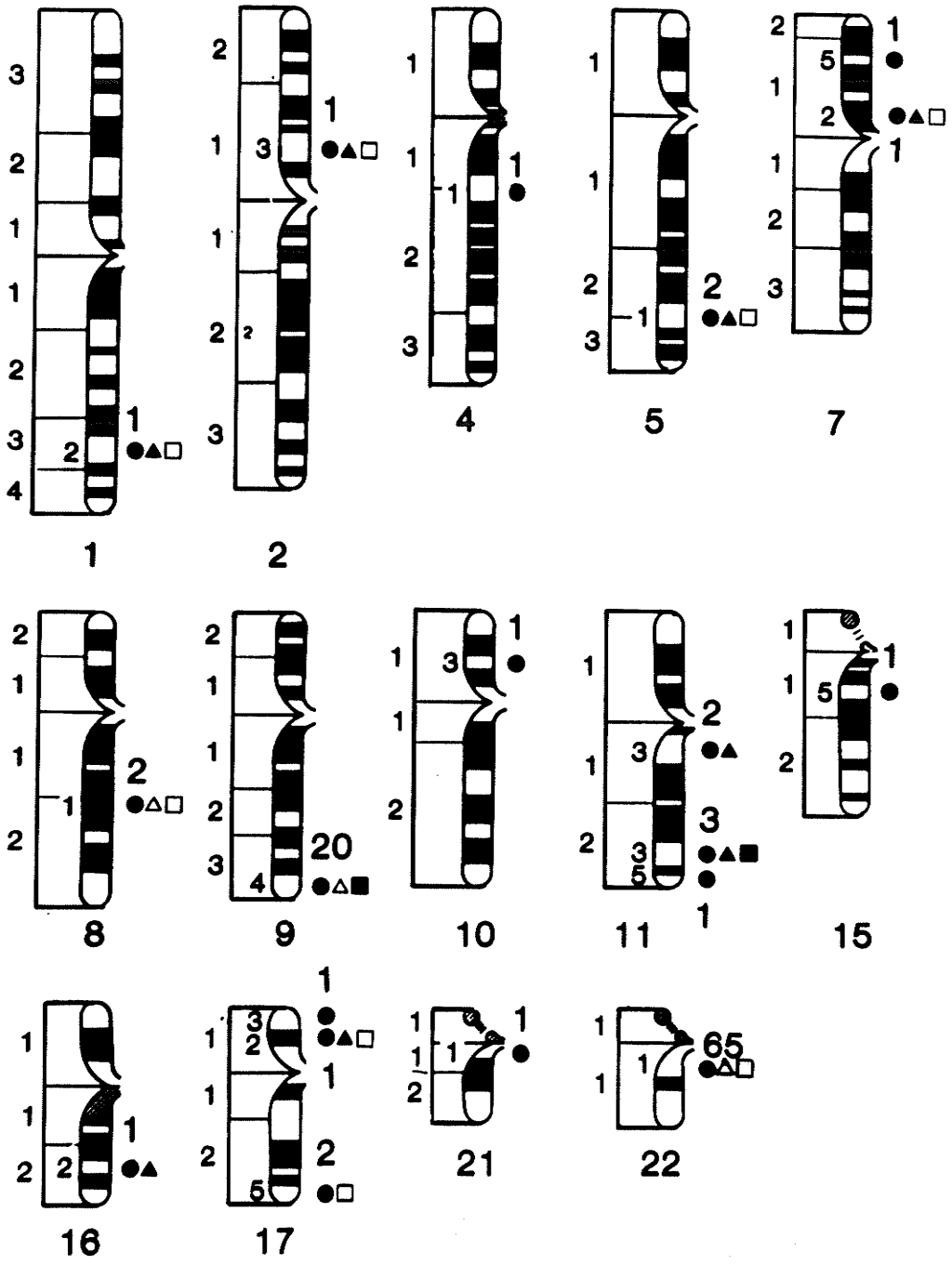

9
22
10
11
16
4
11
5

Figure 2: Diagram of chromosomes indicating the relationship between cancer-specific chromosomal breakpoints, fragile sites and oncogenes. The symbols to the right represent either "exact" map location among breakpoints (●), fragile sites (▲), and oncogenes (■), or "close" map location among breakpoints (●), fragile sites (△), and oncogenes (□). The numbers above the symbols indicate the number of patients in which a breakpoint occurred.


1
2
4
5
7
8
9
10
11
15
16
17
21
22

3.2 Fragile Sites

The expression of aphidicolin-induced fragile sites and centromeric stretches in two non-leukemic and eight untreated leukemic individuals is presented in Table 2. In this table, a number of metaphase spreads had at least 20 fragile sites and are referred to as "fragmented" spreads. A fragmented metaphase spread is depicted in Figure 3. The frequencies of fragile sites and centromeric stretches from Table 2 are summarized in Tables 3 and 4, respectively. The average frequency of fragile sites per cell in the controls is 0.11. Although no centromeric stretches were observed in the controls, a frequency of 0.02, corresponding to one fragile site per cell, was assumed to simplify statistical analysis. The frequencies of fragile sites per cell and centromeric stretches per cell were used as estimates of the "expected" frequencies in the Poisson distribution. As indicated in Table 3, seven out of eight leukemic individuals have a statistically significant increase in the frequency of fragile sites ($p < 10^{-6}$) as compared to the controls. If this phenomenon were to occur by chance alone in seven out of eight leukemia patients, assuming a 50% probability of success, the expected percentage for this occurrence would be less than 2%. Similarly, all eight leukemic individuals demonstrated a statistically significant increase in the frequency of centromeric stretches as compared to controls. The level of significance (p) was less than 0.19 for patient MA, less than 0.004 for patient MC and less than 1.0×10^{-6} for the remaining leukemic individuals. If this increase in centromeric stretches were to occur in eight out of eight leukemic

individuals by chance alone, assuming a 50% probability of success, the expected percentage for this occurrence would be less than 0.4%. The cytogenetic data shows a statistically significant increase in the expression of both fragile sites and centromeric stretches in leukemia patients as compared to controls.

The distribution of fragile sites in leukemic patients is particularly important when these patients carry a translocation. For example, patient MU carries a translocation in his cancer cells (PHA unstimulated cultures) but does not have fragile sites in his normal cells that correspond with the breakpoints of the translocation. The fact that the fragile site is found in PHA-stimulated lymphocytes means that it is constitutional and not acquired by the cancer cells so that these fragile sites might still predispose cancer patients to chromosomal rearrangements (Yunis, 1983).

Examples of common fragile sites expressed in chromosome one are depicted in Figure 4. Fragile sites usually occurred at the junction of Giemsa-negative and Giemsa-positive bands or in Giemsa-negative bands close to the junction. The location of fragile sites is important because the bulk of the structural genes are in Giemsa-negative bands. A few chromosomes, such as 1, 2, 3, 6, 10 and 11, have a variety of fragile sites. Others have only one or two fragile sites, such as chromosomes 5, 7, 8, 13, 14, 16, 17 and 18. Examples of centromere stretching are depicted in Figure 5.

Fragile sites and centromeric stretching events both cluster to a few chromosomes in the cancer karyotype. For example, highly expressed fragile sites in leukemic patients cluster on chromosomes 1, 2, 3 and 5

(Figure 6). Furthermore, the map location of these highly expressed fragile sites corresponds to oncogenes. In Figure 6, fragile site 1p22 corresponds to oncogenes ngf-α and N-ras on chromosome 1, fragile site 2p24 corresponds with N-myc, fragile site 2p13 and 2p11 correspond with tgf-α, fragile site 3p24 corresponds with raf-1 and fragile site 5q31 corresponds with fms. Centromeric stretching also appears to cluster on chromosomes 1 and 2 as depicted in Figure 7. Chromosome 2 does not contain as much constitutive heterochromatin flanking the centromere as compared with chromosome 1. Therefore, centromeric stretching present in chromosome 2 involves the euchromatin which is abundant in structural genes.

The cytogenetic data with respect to fragile sites can be summarized as follows:

1. Common fragile sites are present in the normal cells of leukemic patients at an increased frequency as compared to non-leukemic patients.

2. Highly expressed fragile sites cluster on chromosome 1, 2, 3 and 5.

3. The map location of highly expressed fragile sites corresponds with oncogenes.

Table 2: Expression of fragile sites in control and leukemia patients. The total number of fragile sites and centromeric stretches found in 50 cells is indicated below each patient column.

Control 1	Non-leukemic	Control 2	Non-leukemic
Fragile Site	No./50 Cells	Fragile Site	No./50 Cells
1p32	1	2q11	1
2p	1	2q31	1
2q33	1	3p14	1
3p21	2	6p21	1
3q	1	6p27	1
Fragile sites = 6		Fragile sites = 5	
Centromeric stretches = 0		Centromeric stretches = 0	

Table 2 (Cont'd)

MA-ALL 46,XY,t(11;19)		DI-ALL 46,XY			
Fragile Site	No./50 Cells	Fragile Site	No./50 Cells	Fragile Sites	No./50 Cells
1q11	2	1p22	1	8q22	1
1q32	1	1p32	2	9p21	1
cs-1	1	1p36	1	9q32	1
2p11	1	1q25	3	10q23	2
2q11	5	1q32	2	10q25	2
cs-2	2	cs-1	7	11p13	3
3p21	2	2p11	3	11q23	3
3q21	1	2p13	1	cs-C	2
4q12	1	2p24	3	Cq	15
4q21	1	2q11	1	16q12	1
5q31	2	2q13	1	16q22	1
cs-5	1	2q31	5	16q23	2
6q27	1	2q33	2	cs-16	1
9p21	1	cs-2	2	17p12	1
11q12	1	3p14	3	Fragmented Cells	5 x 20 = 100
18q21	1	3p24	8		
		3q27	1		
		cs-3	3		
		cs-4	3		
		5q31	7		
		cs-5	1		
		Bq	9		
		6p23	2		
		6q21	1		
		7p11	4		
		7q22	1		

Fragile sites	= 20	Fragile sites	= 194
Centromeric stretches	= 4	Centromeric stretches	= 18

Table 2 (Cont'd)

MU-AML t(16;16;)(q22;q24)		BJ-CML 46,XY		NI-CML 46,XY	
Fragile Site	No./50 Cells	Fragile Site	No./50 Cells	Fragile Site	No./50 Cells
cs-1	8	1p22	2	1q32	1
2q11	1	1q11	4	1p36	1
cs-2	5	cs-1	18	1q11	1
3p21	2	2p11	1	1q32	3
cs-3	1	2q11	9	1q44	1
cs-4	3	2q31	1	cs-1	7
cs-5	2	2q33	1	2p11	9
cs-7	3	cs-2	9	2p13	1
cs-11	1	3p14	4	2p21	1
		3q11	1	2q11	2
		cs-3	1	3p21	1
		5q31	1	3p23	1
		5q35	1	3q11	1
		6p23	1	cs-3	1
		7p13	1	cs-4	2
		cs-7	1	5q15	1
		16p11	2	5q31	1
		17p12	1	cs-5	2
		17p21	1	6q21	1
		18q23	1	6q27	1
				8q22	2
				10q22	1
				12q13	1
				13q34	2
				14q24	1
				15q15	1
				18q23	1
Fragile sites = 3		Fragile sites = 23		Fragile sites = 37	
Centromeric stretches = 23		Centromeric stretches = 29		Centromeric stretches = 10	

Table 2 (Cont'd)

MC-ALL 45,X,-16,-Y,+2				WR-CML 46,XY		NIS-CML 46,XY	
Fragile Sites	No./50 Cells	Fragile Sites	No./50 Cells	Fragile Site	No./50 Cells	Fragile Site	No./50 Cells
1p22	4	11p13	2	1p32	8	1p22	8
1p32	2	11q23	2	1p32	5	1p32	2
1q32	4	12q13	1	1p36	4	1q36	4
cs-1	2	14q24	2	1q25	4	1q11	1
2p11	11	20p11	1	2p11	3	1q32	5
2p13	8	22q12	1	2q11	1	cs-1	1
2p24	4	18q	1	2q31	10	2p11	5
2q11	1	Cq	32	2p	1	2p13	1
2q13	1			cs-2	2	2p24	7
2q31	5			3p14	7	2q31	7
2q33	3			3p24	3	3p24	2
3p14	3			3q27	3	3p14	5
3p24	3			cs-3	2	cs-2	1
3q27	4			5q31	7	cs-3	1
cs-4	1			7q32	1	5q31	8
				8q22	1	Bp	1
5q31	9			9p21	2	Bq	6
5q35	2			9q13	1	B-cs	7
cs-5	1			9q33	1	6p23	2
5q	1			11p13	2	10q23	1
Bq	3			12p13	2	10q25	1
6p23	5			14q24	1	11q23	1
6q25	2			Bq	20	Cp	8
cs-6	1			B-cs	2	Cq	34
7p11	3			Cp	1	cs-C	11
7p13	2			Cq	49	Dq	1
7p21	2			cs-C	2	E	1
7q32	1			Dq	2	F	2
10q23	2			cs-10	1	14q24	2
10q25	1					Fragmented cells 1 x 20 = 20	
Fragile sites = 129 Centromeric stretches = 5				Fragile sites = 139 Centromeric stretches = 9		Fragile sites = 142 Centromeric stretches = 21	

Table 3: Frequency of fragile sites per cell in control and leukemia patients. The patients are listed in increasing order of fragile site expression. The level of significance (p) is indicated in brackets.

Patient	Frequency Fragile Sites /Cell	
control #1 46,XX	0.12	
control #2 46,XY	0.10	
MU AML t(16;16)(q22;q24)	0.06	
MA ALL 46,XY,t(11;19)	0.40	($p < 10^{-6}$)
BJ CML 46,XY	0.46	($p < 10^{-6}$)
NI CML 46,XY	0.74	($p < 10^{-6}$)
MC ALL 45,X,-16,-Y,+2	2.58	($p < 10^{-6}$)
WR CML 46,XY	2.78	($p < 10^{-6}$)
NIS CML 46,XY	2.84	($p < 10^{-6}$)
DI ALL 46,XY	3.88	($p < 10^{-6}$)

Table 4: Frequency of centromeric stretches per cell in control and leukemia patients. The patients are listed in increasing order of centromere stretching expression. The level of significance (p) is indicated in brackets.

Patient	Frequency Centromere Stretches/Cell	
control #1 46,XX	0.00	
control #2 46,XY	0.00	
MA ALL 46,XY,t(11;19)	0.08	($p < .019$)
MC ALL 45,X,-16,-Y,+2	0.10	($p < .004$)
WR CML 46,XY	0.18	($p < 10^{-6}$)
NI CML 46,XY	0.20	($p < 10^{-6}$)
DI ALL 46,XY	0.36	($p < 10^{-6}$)
NIS CML 46,XY	0.42	($p < 10^{-6}$)
MU AML t(16;16)(q22;q24)	0.46	($p < 10^{-6}$)
BJ CML 46,XY	0.58	($p < 10^{-6}$)

Figure 3: Photograph of solid stained chromosomes from ALL patient DI. The arrows indicate fragile sites in the chromosomes.

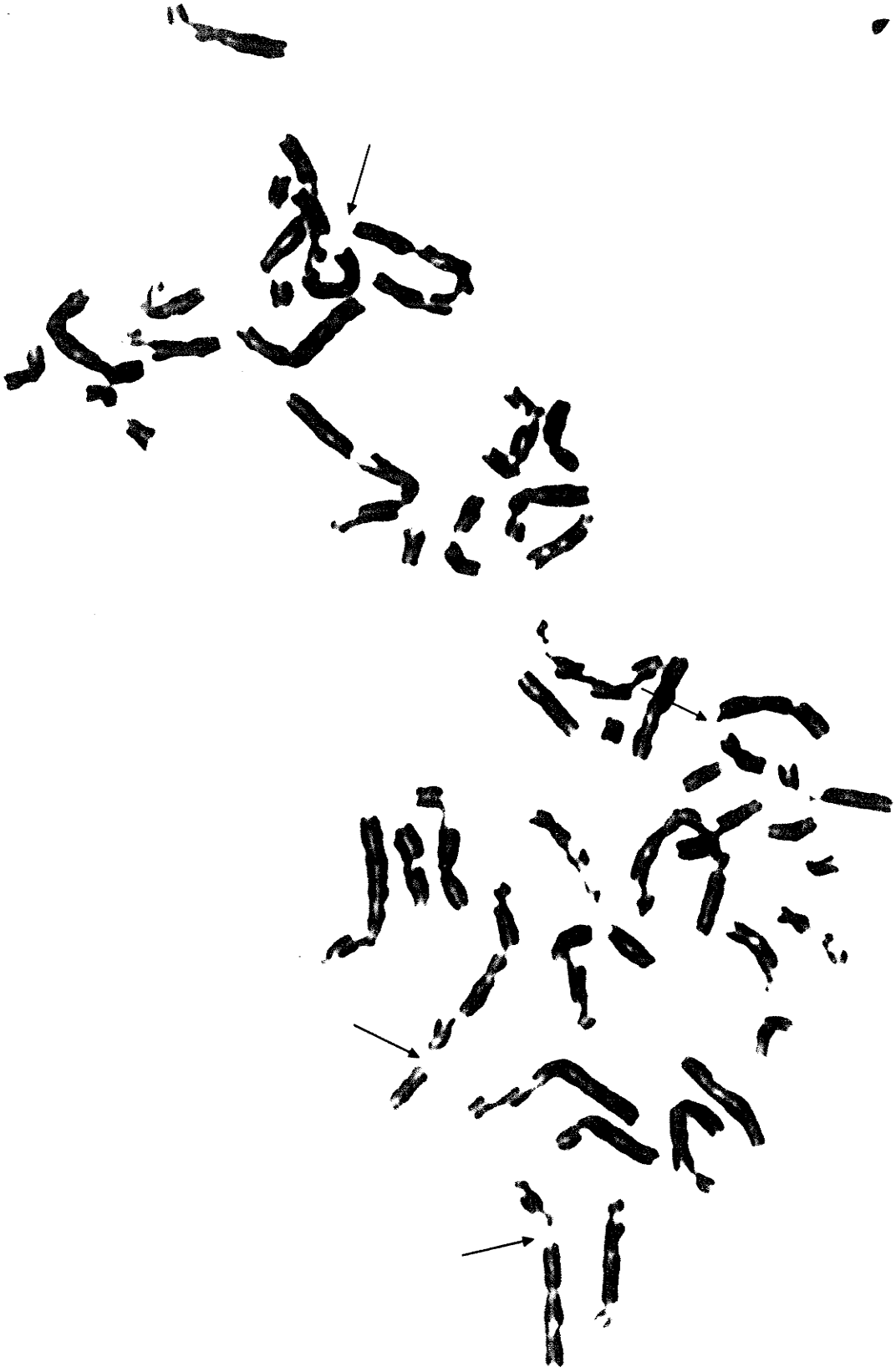


Figure 4: Examples of fragile sites identified in chromosome one from eight leukemia patients. The arrows indicate the fragile site in the solid stained chromosome on the left. The chromosome on the right has been G-banded in order to confirm the exact location of the fragile site.

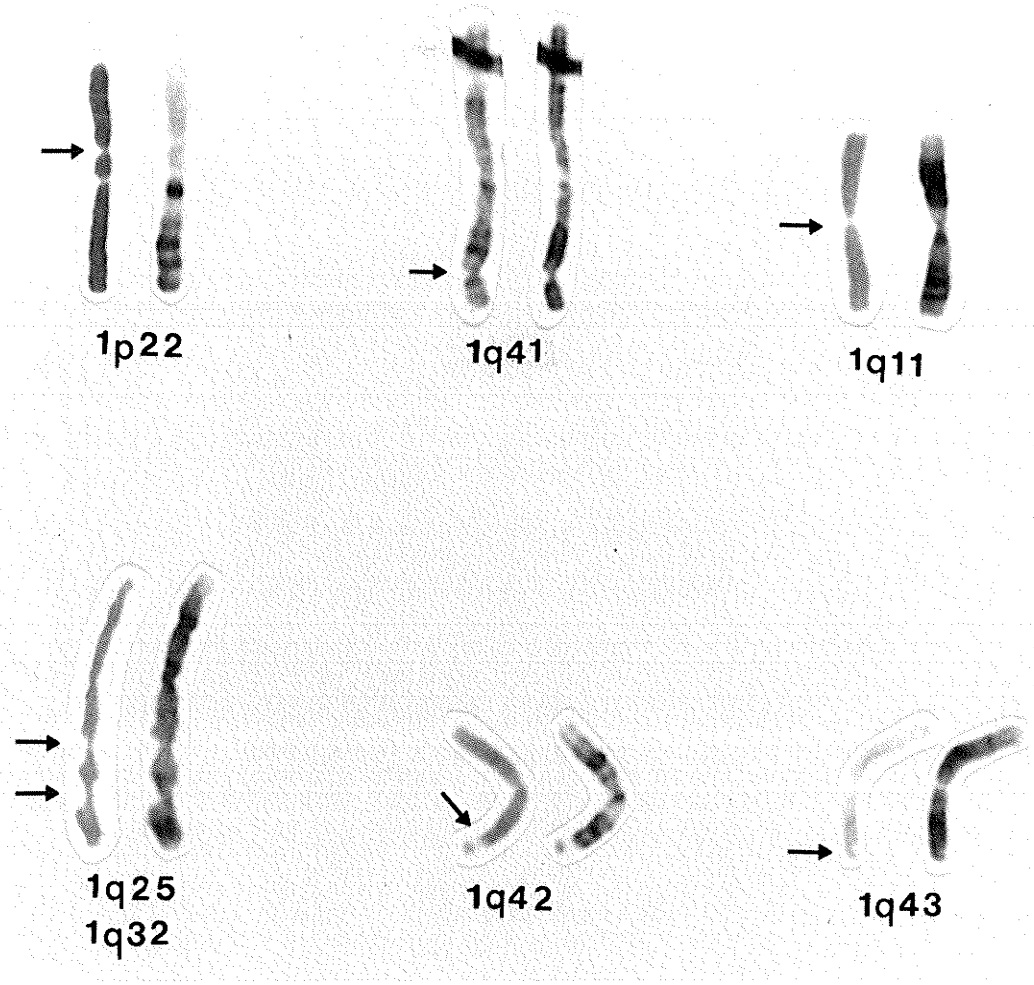

1p22
1q41
1q11
1q25
1q32
1q42
1q43

Figure 5: Centromeric stretches identified in chromosomes from eight leukemia patients. The arrows indicate the centromeric stretch in the solid stained chromosome on the left. The chromosome on the right has been G-banded in order to confirm the chromosome number.

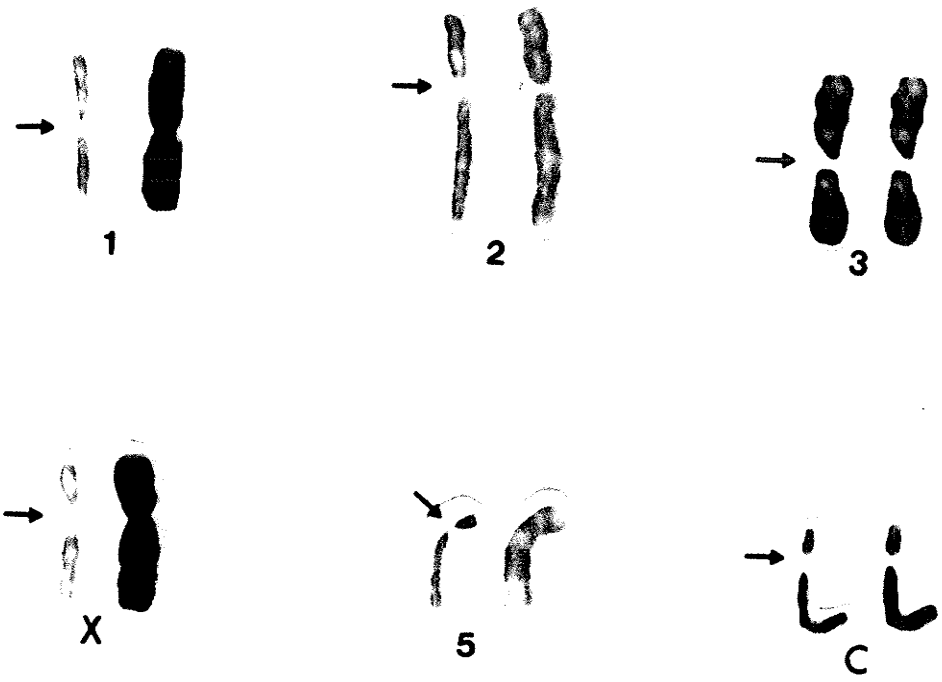
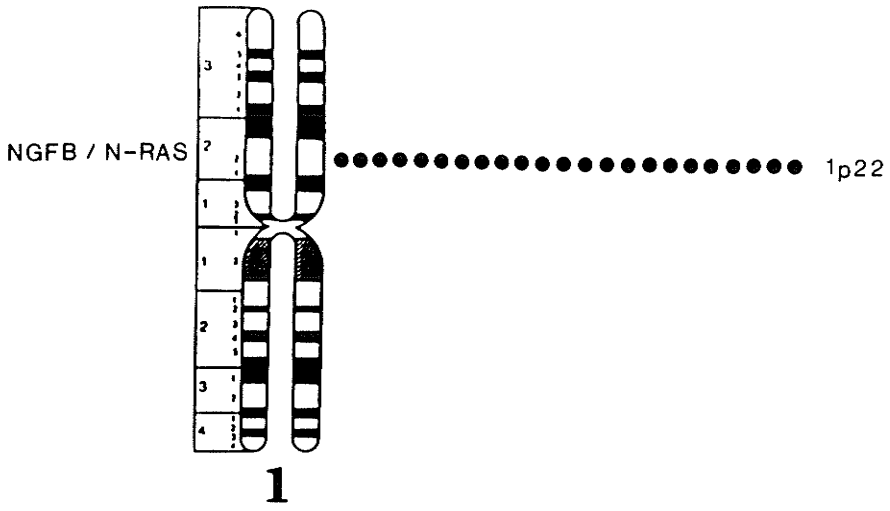
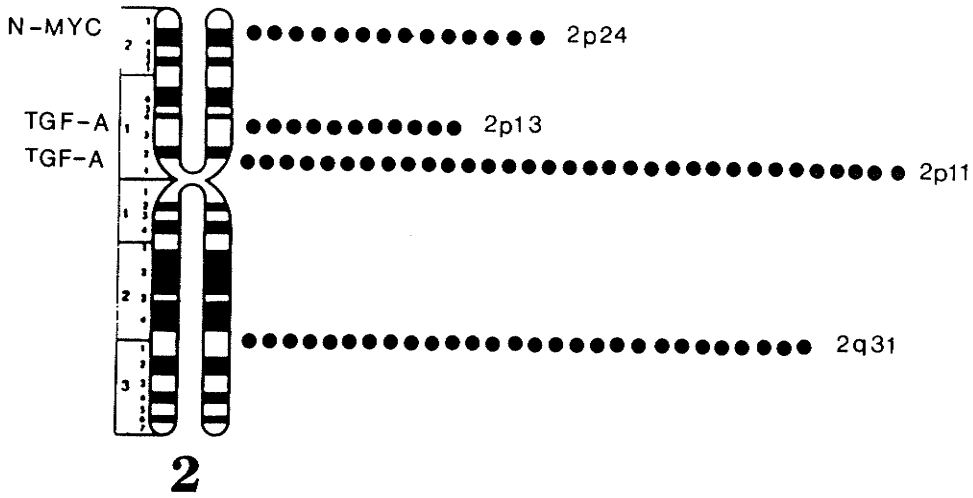
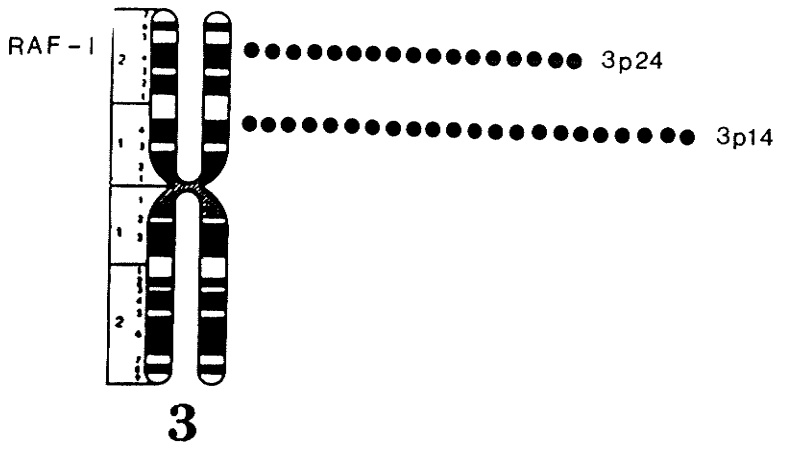

1
2
3
X
5
C
11

Figure 6: Diagram of chromosomes indicating the relationship between highly expressed fragile sites (●), indicated on the right of each chromosome, and oncogenes, indicated on the left. The total number of dots corresponds to the total number of a particular fragile site seen in the eight leukemia patients.


NGFB / N-RAS
1p22
1


N-MYC
2p24
TGF-A
2p13
TGF-A
2p11
2q31
2


RAF-1
3p24
3p14
3

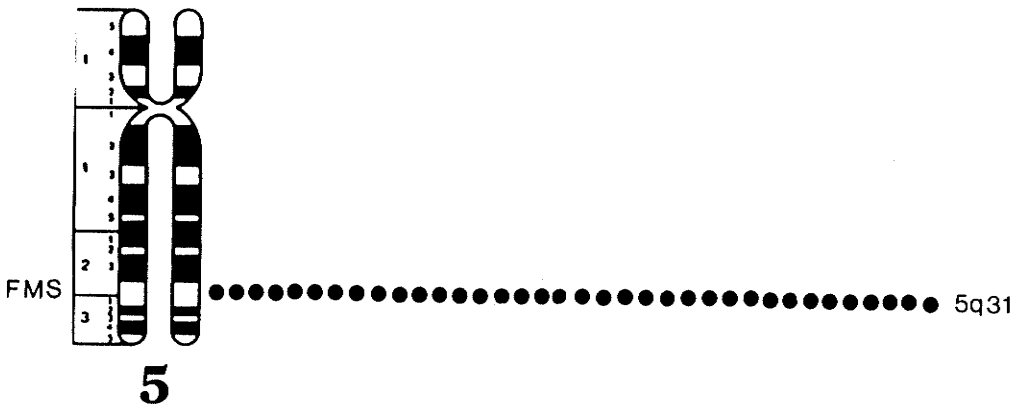
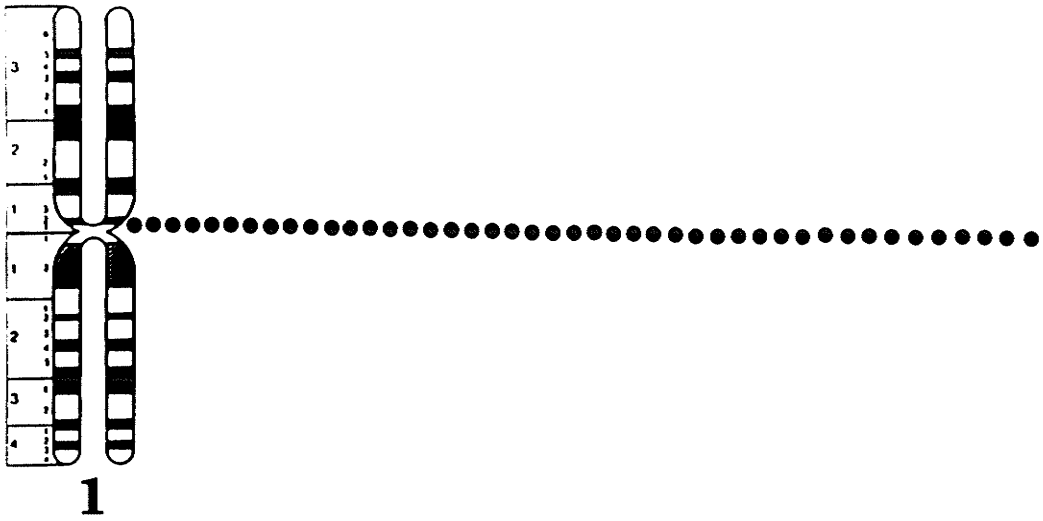
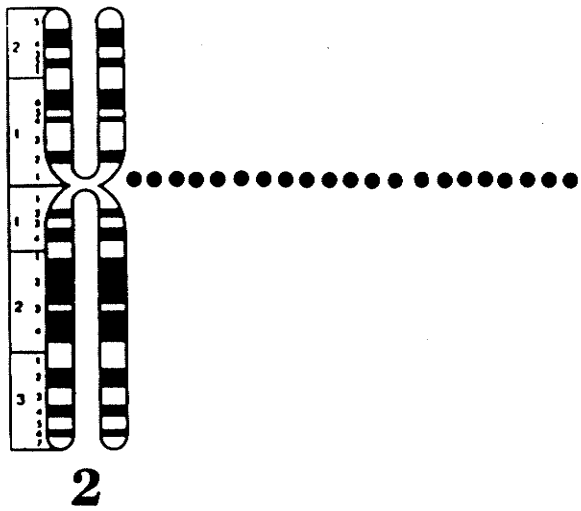

FMS
5q31
5

Figure 7: Diagram of chromosomes indicating the highly expressed centromeric stretches present in the eight leukemia patients. The total number of dots corresponds with the total number of centromeric stretching events on a chromosome.



1



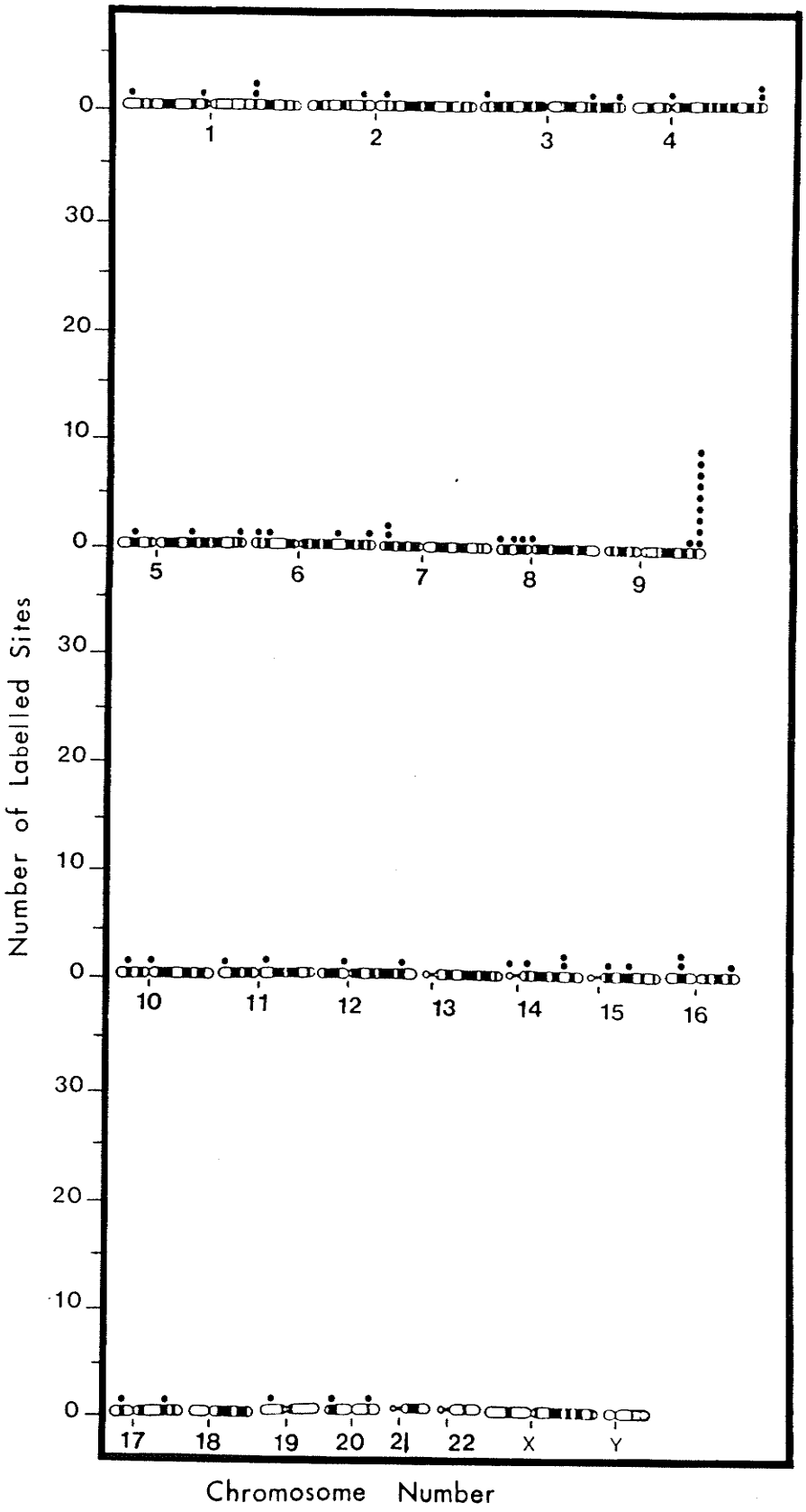
2

3.3 Oncogene Mobility

3.3.1 Controls

The efficiency of hybridization obtained with three probes, abl, bcr and sis was determined. The abl probe was radiolabelled by nick translation. The grain distribution for this oncogene was constructed after analyzing 45 metaphase spreads (Figure 8a). From these cells, 19% of all grains hybridized to 9q34 (Figure 8b). This efficiency of hybridization is highly significant as indicated by the very low p value (ie. $p < 10^{-6}$). Using random oligonucleotides as primers, the probes for bcr and sis were radiolabelled with the large fragment of DNA polymerase I. The number of cells counted was 45 for both probes. The grain distributions, represented in Figure 9a and 10a, reveal highly significant hybridization efficiencies, that is, 22% ($p < 10^{-6}$) and 18% ($p < 10^{-6}$), respectively. Photographs of representative metaphase spreads are shown in Figures 9b and 10b. All three probes are characterized by a low amount of non-specific hybridization to random chromosomes.

Figure 8a: Distribution of abl hybridization grains in 45 metaphase spreads from a control individual. Nine of 76 total grains or 19% of grains are located at 9q34.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13
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16
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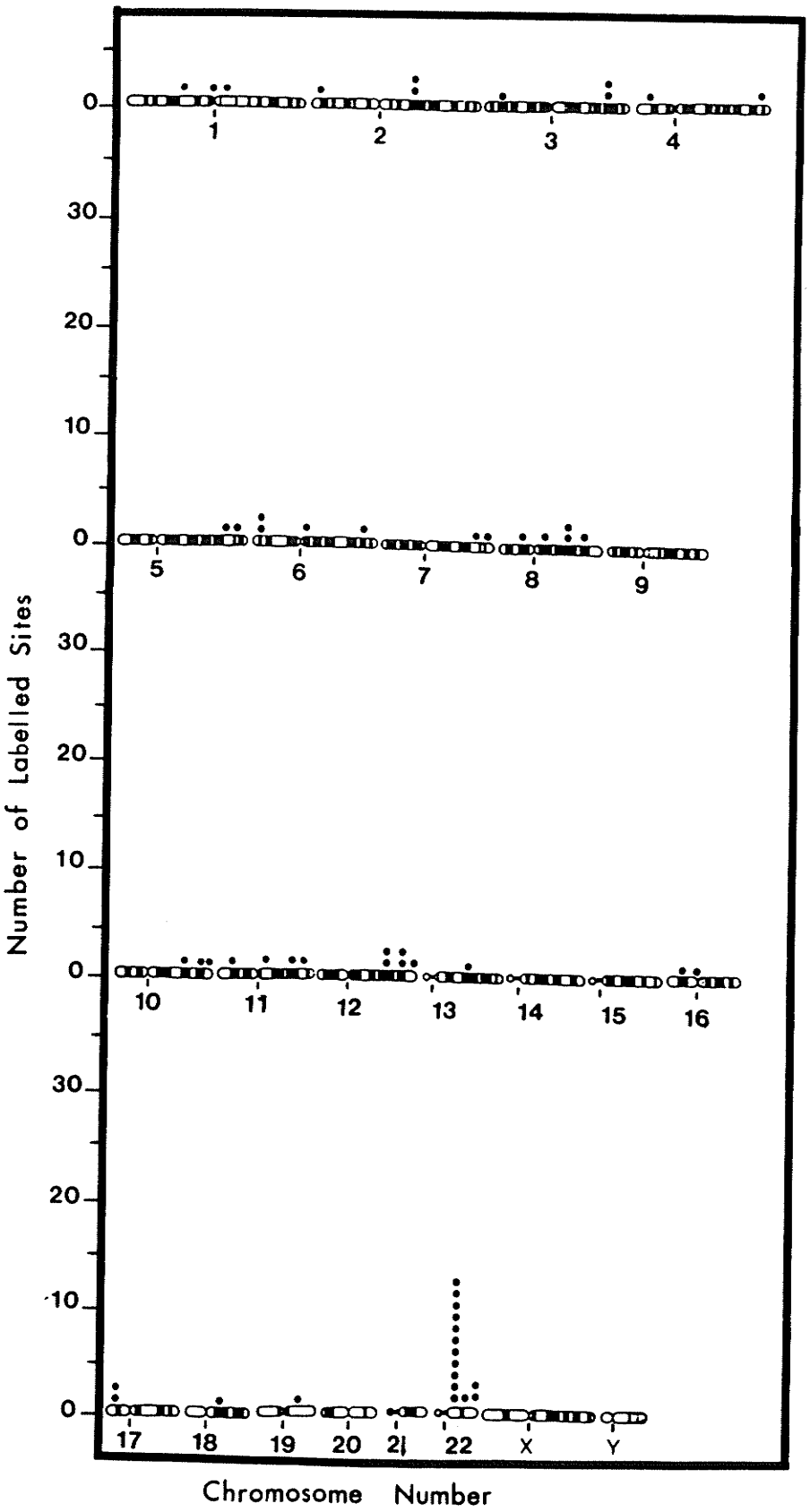
18
19
20
21
22
X
Y
Chromosome Number

Figure 8b: Photograph of a representative metaphase spread hybridized with a ^{3}H-labelled abl probe. The arrow indicates the localization of the grain on chromosome 9. The chromosomes are G-banded.

Figure 9a: Distribution of bcr hybridization grains in 45 metaphase spreads from a control individual. Thirteen of 59 total grains or 22% of grains are located at 22q11.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11
12
13
14
15
16
17
18
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21
22
X
Y
Chromosome Number

Figure 9b: Photograph of a representative metaphase spread hybridized with a ^{3}H-labelled bcr probe. The G-group chromosomes are identified. The arrow shows that the localization of the grain is on chromosome 22. The chromosomes are Q-banded.

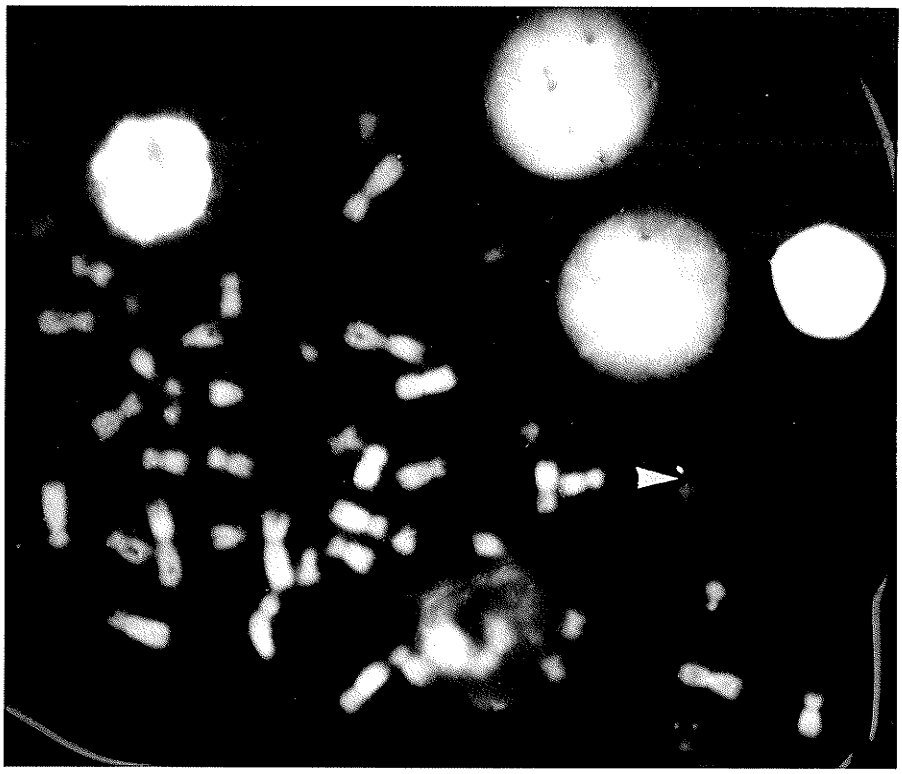
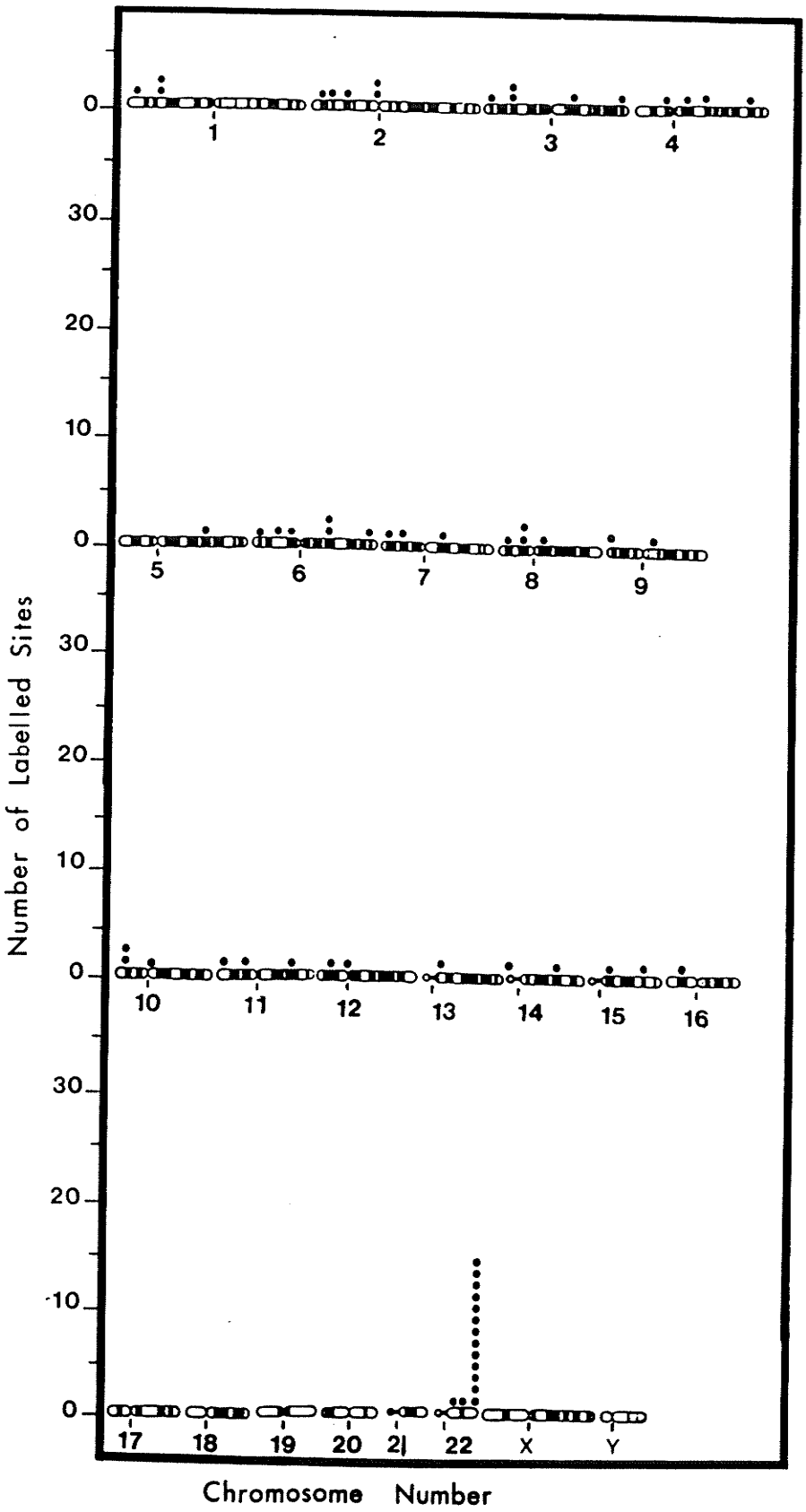


Figure 10a: Distribution of sis hybridization grains in 45 metaphase spreads from a control individual. Eleven of 62 total grains or 18% of grains are located at 22q13.


Number of Labelled Sites
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13
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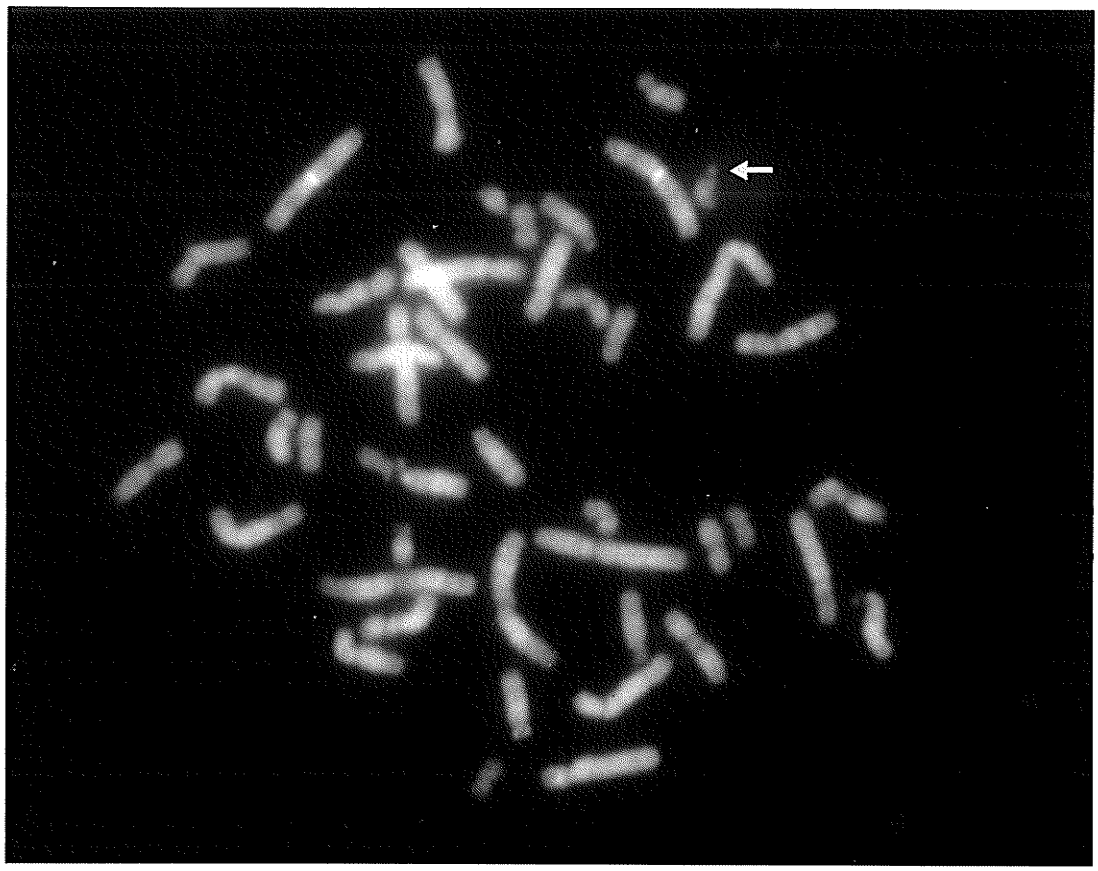
21
22
X
Y
Chromosome Number

Figure 10b: Photograph of a representative metaphase spread hybridized with a ^{3}H-labelled sis probe. The arrow shows that the localization of the grain is on chromosome 22. The chromosomes are Q-banded.



3.3.2 Non-Leukemic Individuals

Patient RUS is characterized by a reciprocal translocation between chromosome 9 and 11, namely, 46,XX,t(9;11)(q22;p15). RUS has two translocation chromosomes, a normal homologue of chromosome 9, and a normal homologue of chromosome 11 (Figure 11a). This aberration translocates the region of chromosome 9 from 9q22 to the terminus onto chromosome 11 at 11p15. Thirty cells were used to determine the *abl* grain distribution as illustrated in Figure 11b. The 33% hybridization efficiency is highly significant ($p < 10^{-6}$). The majority of *abl* hybridization grains are located on the translocation chromosome, t(9;11). Four *abl* grains remain on the normal chromosome 9 at 9q34 but this accumulation is not significant. A photograph of a representative ^{3}H-labelled metaphase cell is shown in Figure 11c.

Patient RUM is characterized by a unbalanced translocation between chromosome 12 and 14, 46,XX,-14,+t(12;14)(p11;p11). In the genesis of the translocation chromosome, the region of chromosome 12 from 12p11 to the terminus is rearranged to the short arm of chromosome 14. This patient has two normal homologues for chromosome 12 and one normal homologue for chromosome 14. The translocated region of chromosome 12 includes the K-*ras*-2 oncogene. The grain distribution resulting from 40 metaphase spreads is seen in Figure 12a. Fourteen of 128 total grains (11%) are on 12p12 and 24 of 128 grains (19%) are on the translocation chromosome. These hybridization efficiencies are significant ($p < 10^{-6}$). A representative ^{3}H-labelled metaphase cell is shown in Figure 12b.

Patient GO is characterized by an inversion in chromosome 9, namely, 46,XX,inv(9)(q32q34) as seen in Figure 13a. This patient has a inverted homologue of chromosome 9 along with a normal homologue. The 9q34 breakpoint of this inversion corresponds exactly to the breakpoint in the Ph' translocation. The abl grain distribution was constructed after analyzing 30 metaphase spreads. The majority of hybridization grains (13/70) in the inverted homologue are not located at 9q34 but instead are located more proximal to the centromere as shown in Figure 13b. The 19% hybridization efficiency to the chromosomal region above 9q34 in the inverted homologue of chromosome 9 is highly significant ($p < .00003$). A small peak at 9q34 is characterized by 2/70 hybridization grains in the inverted homologue. This peak is not a significant hybridization event as compared to non-specific hybridization. Hybridization to the normal homologue of chromosome 9 at q34 demonstrated by 7/70 grains (10%). This hybridization efficiency is significant at $p < .00003$. A representative ^{3}H-labelled metaphase cell for patient GO is given in Figure 13c.

Patient WO has an isochromosome for the short arm of chromosome 12 which includes the K-ras-2 oncogene. The karyotype is 47,XY,i(12p). The patient has two normal homologues of chromosome 12 in addition to the isochromosome. The grains were distributed after analysis of 31 metaphase spreads. There are 47/154 K-ras-2 hybridization grains on the short arm of chromosome 12, especially at 12p12, and 61/154 grains on the isochromosome (Figure 14a). The total hybridization efficiency to the short arms of chromosome 12 is 70% and is highly significant ($p < 10^{-6}$). The hybridization events on the isochromosome were mainly

seen as double grains of equal size. The majority (43/61) of grains were present on one terminus of the isochromosome (Figure 14b) although 9/61 grains were present on both arms of the isochromosome. The double grains were recorded as two hybridization events since they occurred consistently.

Patient RH has a duplication of the terminus of chromosome 9. The karyotype is 46,XX,dup(9)(q33q34) as seen in Figure 15a. There is one abnormal homologue of chromosome 9 and one normal homologue. The breakpoint of this duplication at 9q34 corresponds exactly to the breakpoint of the Ph' translocation. Fifty cells were used to construct the abl grain distribution as shown in Figure 15b. It was found that double hybridization grains were present in many of these cells. In these cells, either one grain was very large as compared to the second or both grains were of equal size. The double grains were counted as separate hybridization events since they occurred in a consistent manner rather than as a spontaneous event. The number of grains was used in addition to chromosomal banding to distinguish the duplicated homologue of chromosome 9 from the normal homologue. There are 14/106 abl grains on chromosome 9 at band q34, and 48/106 grains on the duplicated homologue of chromosome 9. As a result, the 58% hybridization efficiency to chromosome 9 from both the duplicated and normal homologues was highly significant ($p < 10^{-6}$). The major hybridization peaks occurred at 9q33 and 9q34. A photograph of a ^{3}H-labelled metaphase cell is in Figure 15c.

The results from in situ hybridization in non-leukemic individuals with karyotypic abnormalities are twofold:

1. Oncogenes may be rearranged as a result of chromosomal translocation or inversion.

2. Oncogenes may be present in more than one copy per chromosome as a result of chromosomal duplication or isochromosome formation. This is indicated by high hybridization efficiences (58-70%).

Figure 11a: Partial karyotype from patient RUS. This patient's complete karyotype is, 46,XY,t(9;11)(q22;p15). The chromosomes are G-banded. The arrows indicate the abnormal chromosomes.

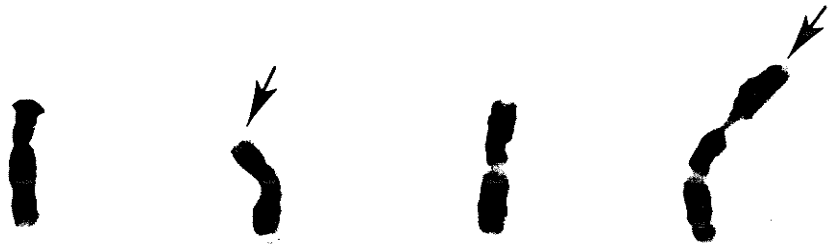
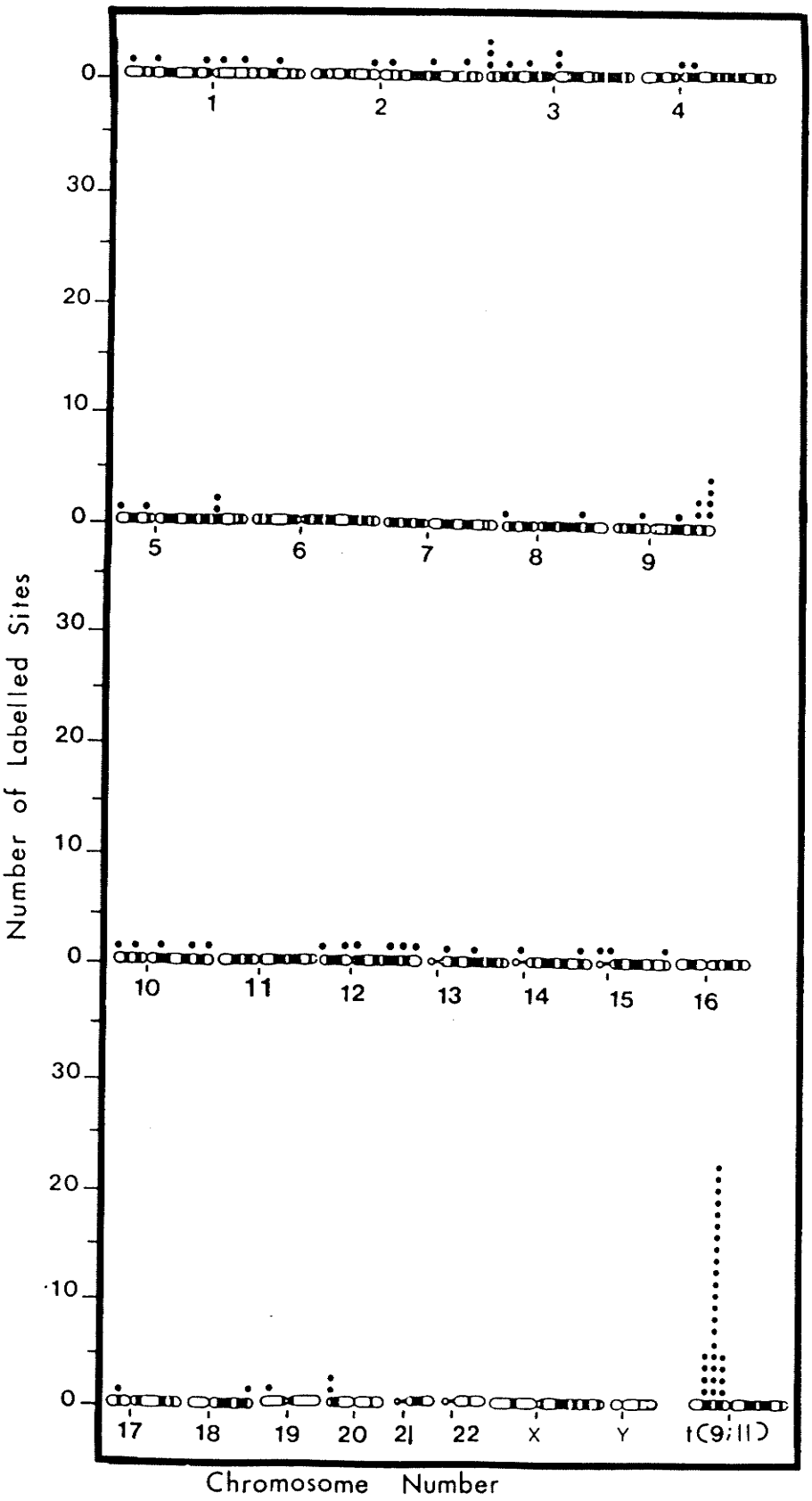

9
11

Figure 11b: Distribution of abl hybridization grains on 30 cells from non-leukemic patient RUS with a translocation, t(9;11)(q12;p13). Twenty-eight out of 84 total grains (33%) were located on the translocation chromosome.


Number of Labelled Sites
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20
21
22
X
Y
t(9;11)
Chromosome Number

Figure 11c: Photograph of a representative metaphase spread from RUS hybridized with a ^{3}H-labelled abl probe. The translocation chromosome is identified. Its shape is similar to chromosome 3 although it is larger. The arrow indicates the grain on the translocation chromosome. These are G-banded chromosomes.

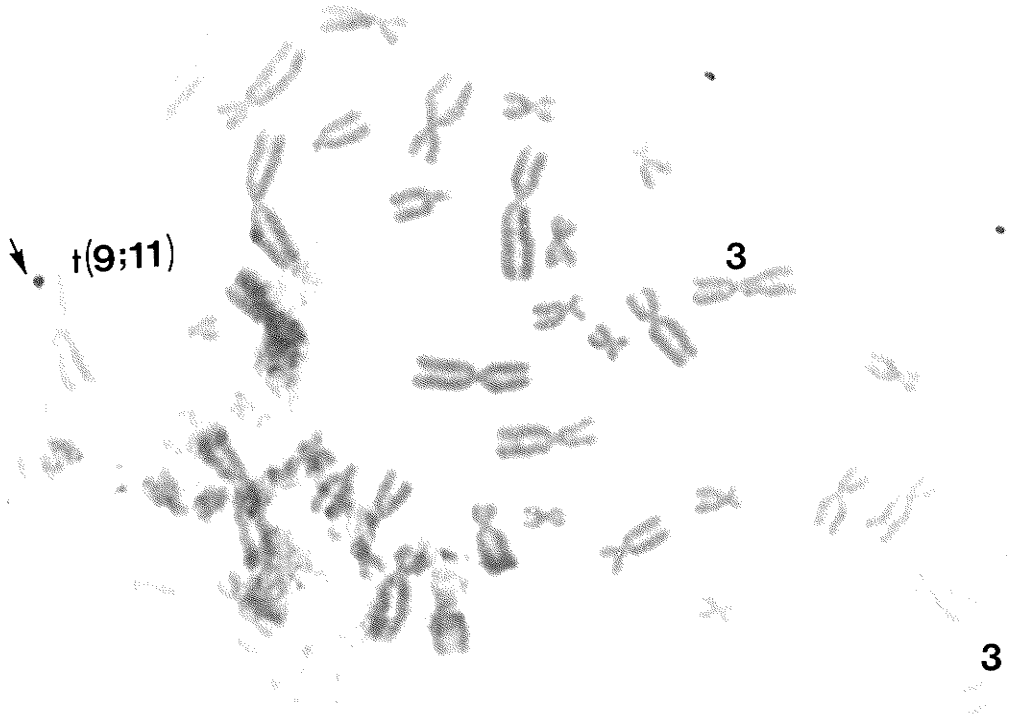
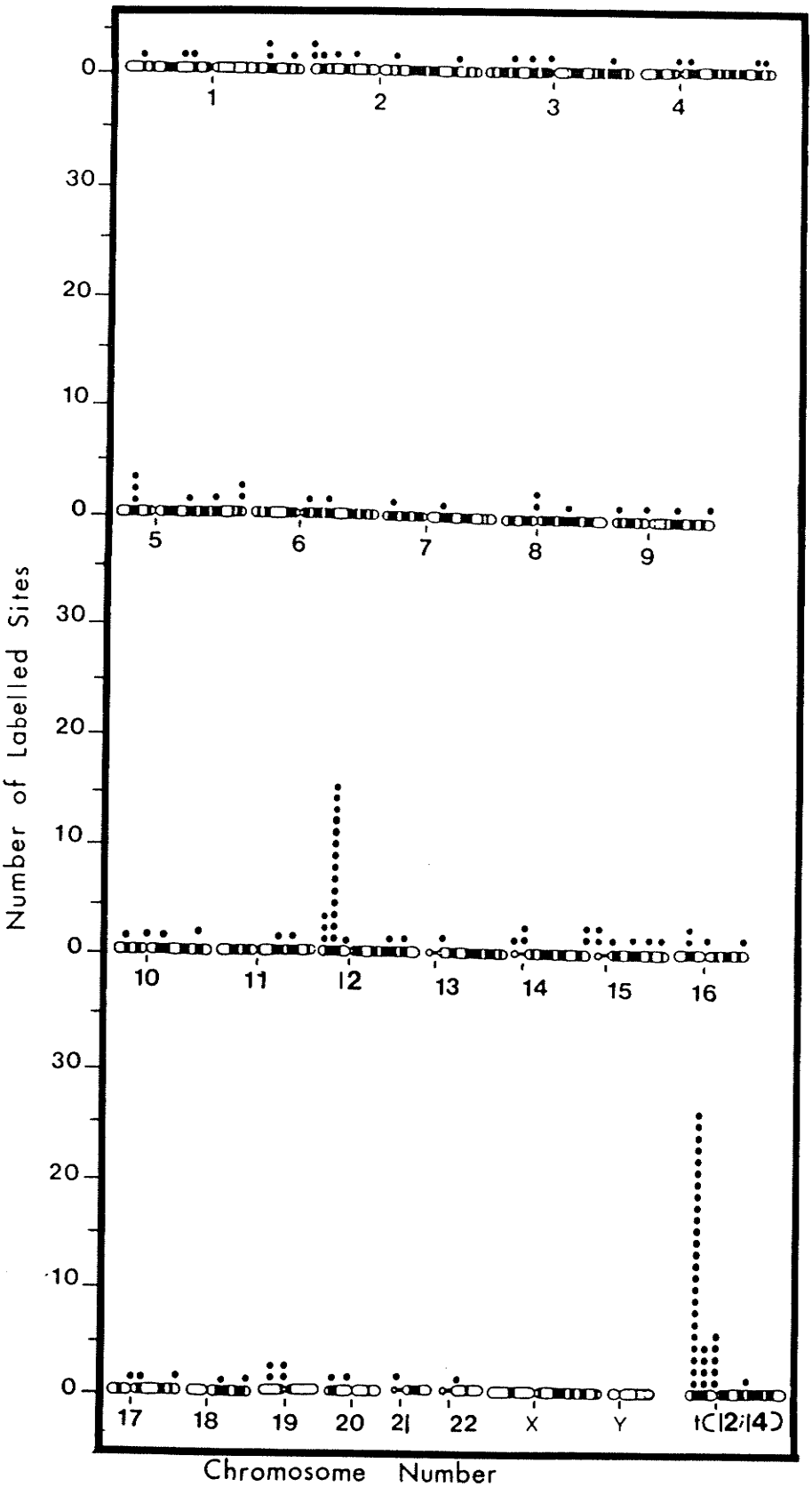

t(9;11)
3
3

Figure 12a: Distribution of K-ras-2 hybridization grains on 40 cells from non-leukemic patient RUM. This patient has a translocation, t(12;14)(p11;p11). Fourteen out of 128 grains (11%) were present on 12p12 and 24 out of 128 grains (19%) were present on the translocation chromosome.


Number of Labelled Sites
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X
Y
t(12;14)
Chromosome Number

Figure 12b: Photograph of a representative metaphase cell from RUM hybridized with a ^{3}H-labelled K-ras-2 probe. The arrows indicate the translocation chromosome with the grain and the normal homologue of chromosome 14. These chromosomes are Q-banded.

Figure 13a: Three partial karyotypes from three different metaphase cells of non-leukemic patient GO showing an inversion, inv(9)(q32q34). The chromosomes are G-banded. The normal homologue of chromosome 9 is on the left and the abnormal homologue of chromosome 9 is on the right.

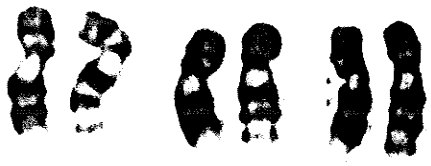
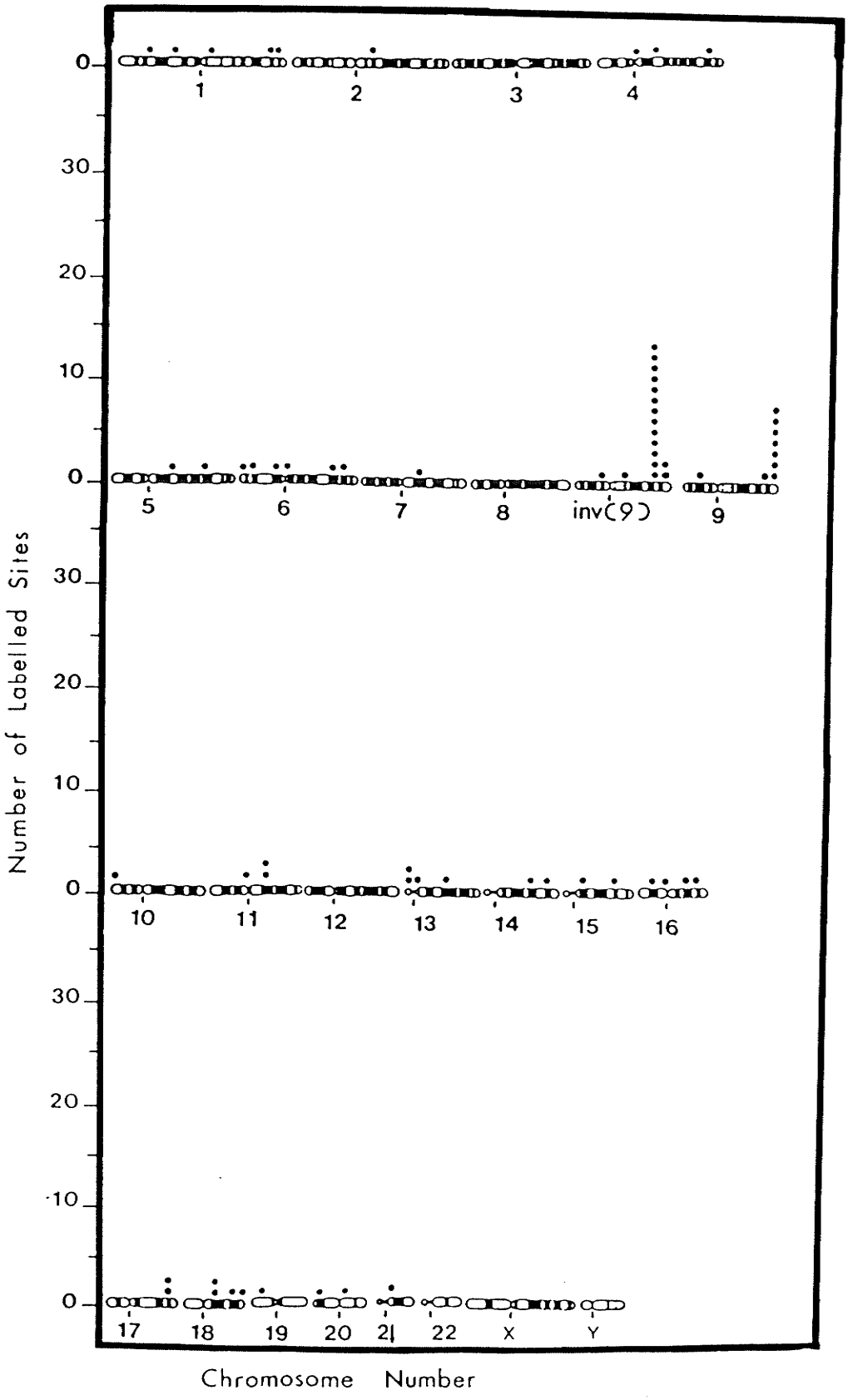


Figure 13b: Distribution of abl hybridization grains in 30 cells from non-leukemic patient GO. Thirteen of 70 total grains (19%) were located proximal to 9q34 on the abnormal homologue of chromosome 9. Seven of 70 grains (10%) were located on the terminus of the normal homologue of chromosome 9.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inv(9)
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11
12
13
14
15
16
17
18
19
20
21
22
X
Y
Chromosome Number

Figure 13c: Photograph of a representative metaphase spread from GO hybridized with a ^{3}H-labelled abl probe. The arrow indicates the location of the grain on the inverted homologue of chromosome 9 above the terminus. The chromosomes are C-banded.

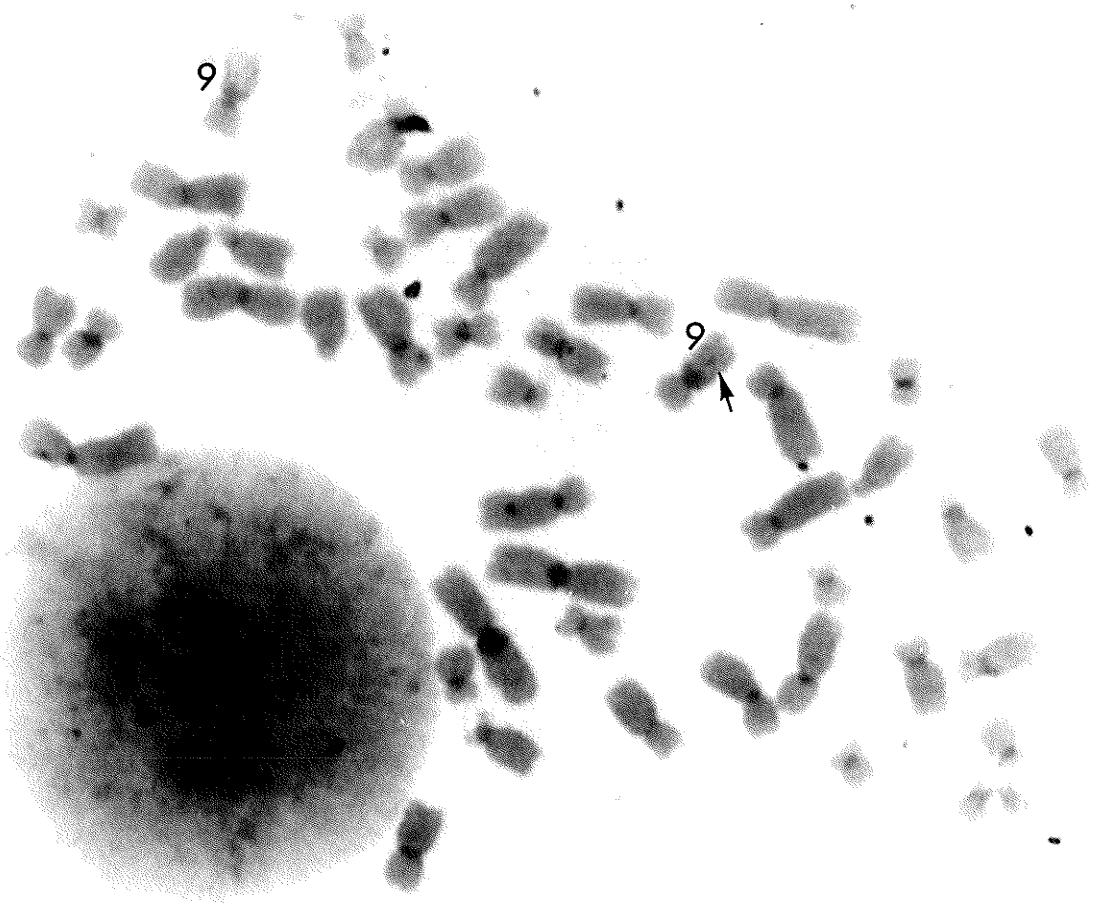
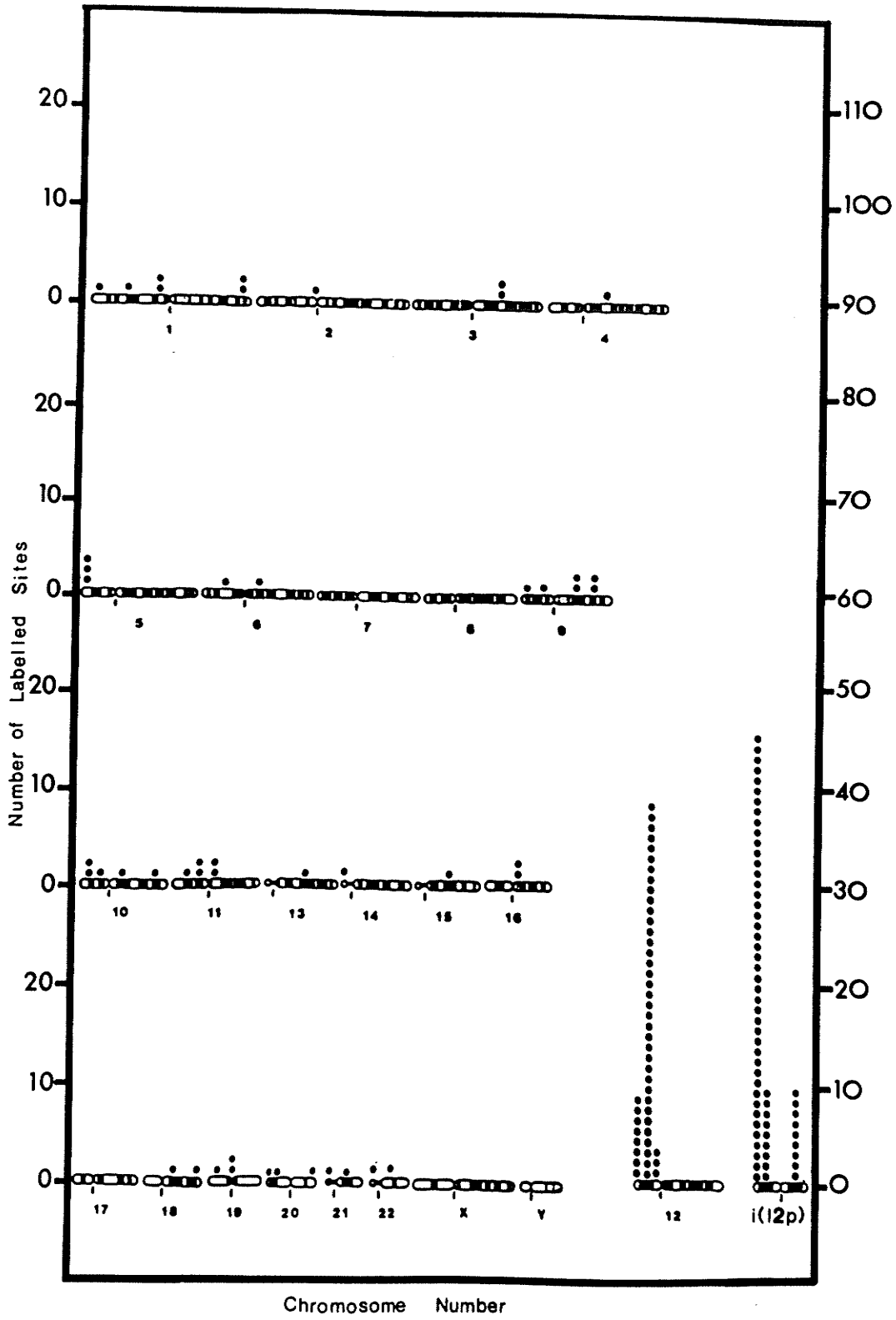

9
9

Figure 14a: Distribution of K-ras-2 hybridization grains on 31 cells from non-leukemic patient WO with an isochromosome, i(12p). One-hundred and eight out of 154 total grains (70%) were localized to the short arms of chromosome 12.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16
17
18
19
20
21
22
X
Y
12
i(12p)
Chromosome Number

Figure 14b: Photograph of a representative metaphase cell from WO hybridized with a ^{3}H-labelled K-ras-2 probe. The arrow indicates a double hybridization event on one terminus of the isochromosome. The chromosomes are Q-banded.

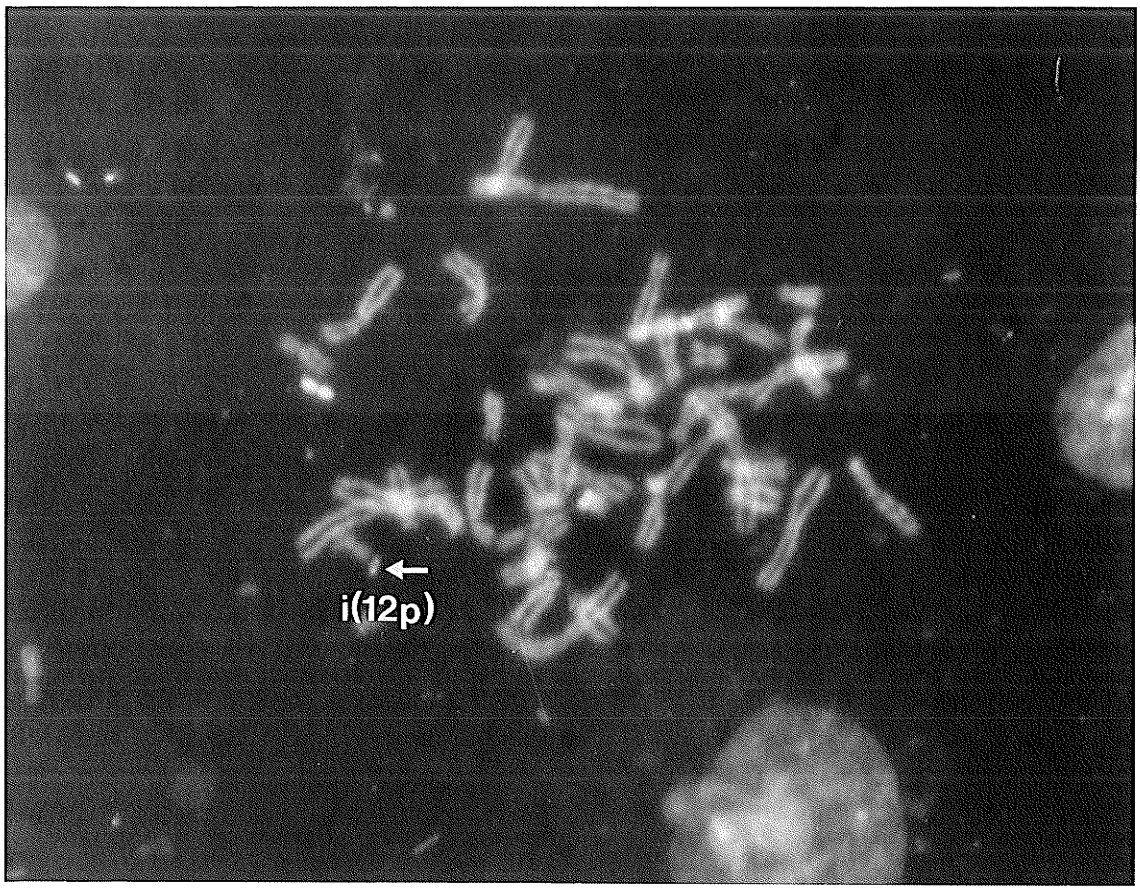

i(12p)

Figure 15a: Five partial karyotypes from five different metaphase cells of non-leukemic patient RH. This patient has the karyotype, 46,XX,dup(9)(q33q34). The upper chromosomes are G-banded and the lower chromosomes are C-banded. The normal homologue is on the left and the abnormal homologue is on the right.

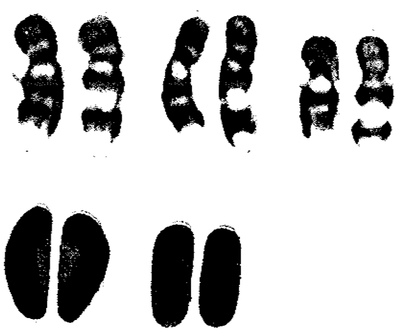
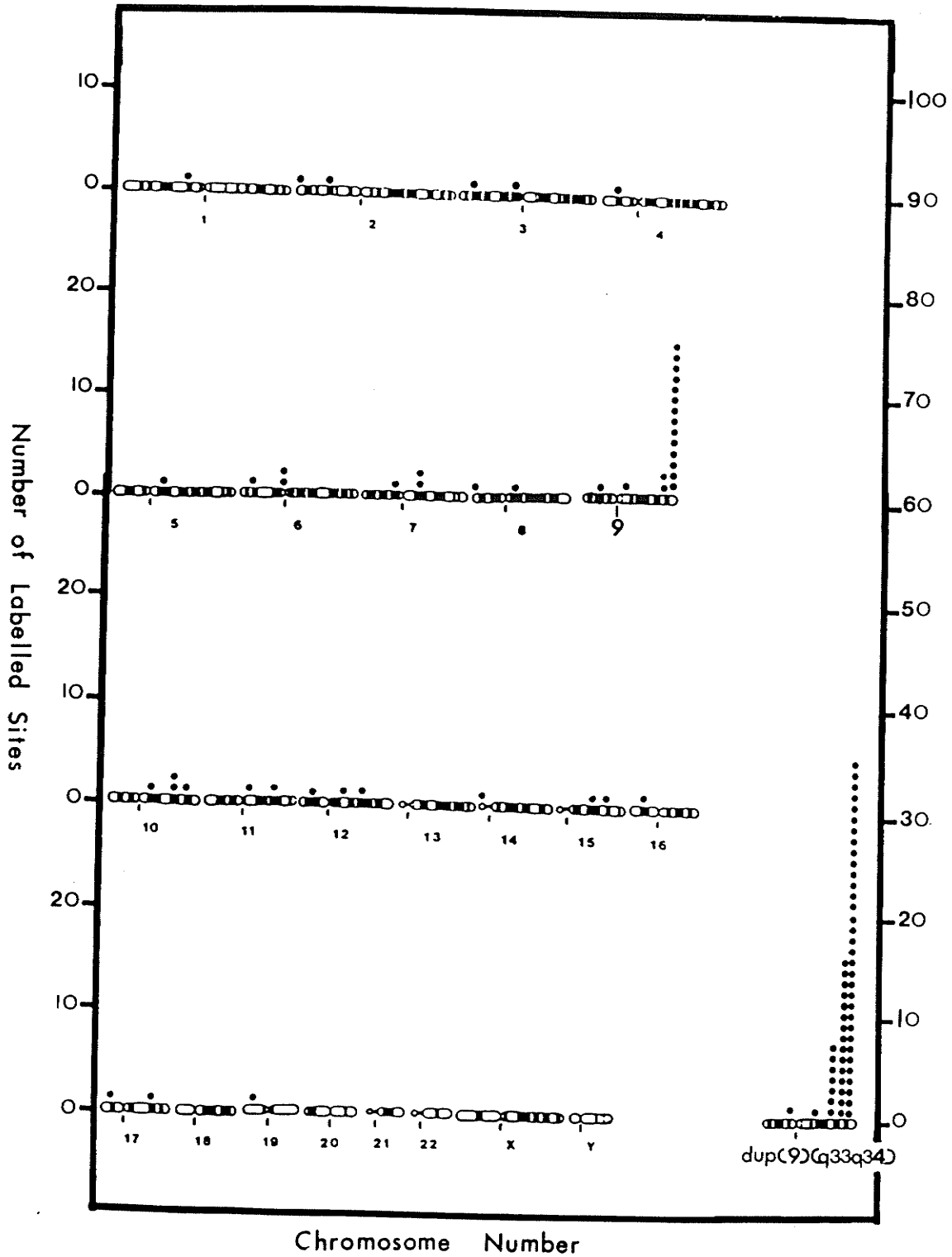


Figure 15b: Distribution of abl hybridization grains in 50 cells from non-leukemic patient RH. Sixty-two out of 106 total grains (58%) were located at 9q33;9q34 when both the duplicated and normal homologues of chromosome 9 were examined.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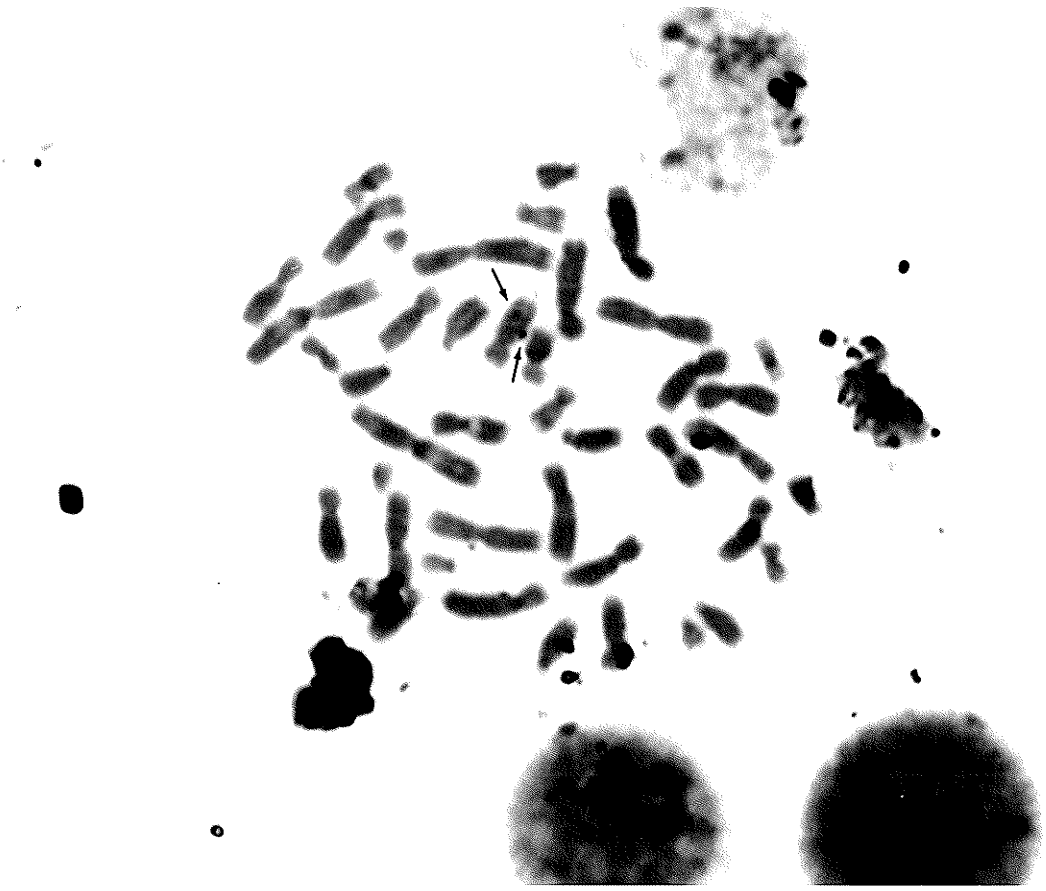
X
Y
dup(9)(q33q34)

Figure 15c: Photograph of a representative metaphase cell from patient RH hybridized with a ^{3}H-labelled abl probe. The arrows indicate the double grains on the duplicated homologue of chromosome 9. These are G-banded chromosomes.



3.3.3 Leukemic Individuals

Eleven untreated leukemia patients were used to investigate abl and sis/bcr mobility by in situ hybridization. The probes were hybridized separately to chromosomes from each patient unless otherwise stated. Ten of the eleven patients lacked a Ph' chromosome. In seven of these patients, abl mobility from chromosome 9 to chromosome 22 was noted. In these patients, either sis or bcr remained on chromosome 22.

CML patient NI has a normal karyotype. From 30 metaphase spreads, an abl grain distribution (Figure 16a) was constructed. The abl oncogene hybridized to chromosome 22 at band q12 (as depicted in Figure 16b). The chromosome 22 hybridization efficiency was 19% and highly significant ($p < .00003$). The preliminary results from in situ hybridization with sis suggest that the majority of grains localize to chromosome 22 in this patient.

CML patients BJ and WR have a normal karyotype. Twenty-seven and twenty-six cells were analyzed respectively. The grain distribution depicted in Figure 17a and 18a indicate that the abl hybridization peak is located at 22q13. Photographs of representative cells are shown in Figure 17b and 18b. The hybridization efficiency for BJ was 16% and WR was 22%. These efficiencies are both highly significant ($p < 10^{-5}$). The abl grains on the WR distribution are very specific for 22q13. In contrast, chromosome 22 of BJ is characterized by minor peaks at 22q11 and 22q12. Hybridization results of sis and bcr were not possible for these two patients, due to an insufficient number of metaphase spreads.

Patient FRA is the CML patient with the highest number of metaphase spreads analyzed (50) and the best abl hybridization efficiency (36%) to chromosome 22. This efficiency is highly significant ($p < 10^{-6}$). From the grain distribution in Figure 19a, the major peak (20/56 grains) occurs at 22q13. A representation metaphase spread is shown in Figure 19b. The preliminary data indicate that the bcr probe localized to chromosome 22 in cells from this patient. Patient FRA has a normal karyotype.

The pattern of abl mobility in CML patients with a normal karyotype was confirmed in patient NIS. NIS is also a Ph'-negative CML patient. Metaphase chromosomes from this patient were hybridized simultaneously with abl and sis. From an analysis of 28 cells, sixteen were characterized by the presence of two distinct grains on chromosome 22. The major hybridization peaks occurred at 22q12 and 22q13 (Figure 20a). The (40%) efficiency of hybridization to chromosome 22 was highly significant ($p < 10^{-6}$). The presence of both an abl hybridization event and a sis hybridization event on chromosome 22 (Figure 20b) confirmed the pattern of abl mobility in the absence of sis mobility in CML patients with a normal karyotype.

In contrast to the CML patients with a normal karyotype, LA has a karyotypic abnormality. This abnormality is the reciprocal translocation, t(9;22)(q34;q11). The abl grain distribution, depicted in Figure 21a, was constructed from 40 metaphase spreads. The abl hybridization peak appeared on the normal homologue of chromosome 22 rather than the abnormal homologue of chromosome 22 or the Ph' chromosome (Figure 21b). Grains were located at bands 22q11 and 22q12

on the normal chromosome 22. The 27% efficiency of hybridization to this chromosome was highly significant ($p < 10^{-6}$). The preliminary results suggest that the sis grains are located on the normal homologue of chromosome 22 in this patient's chromosomes.

Movement of the abl oncogene was also evident in non-CML leukemia patients such as LAD and MUR. Patient LAD has CGL with a complex karyotype, that is, 46,XY,9q-,11q-,14q+,22q+. This patient is missing the long arms of chromosome 9 and 11 and has an extra chromosomal piece on the long arms of chromosome 14 and 22. The normal homologues of chromosome 9,11,14 and 22 are present. Thirty-five cells were used to construct the abl grain distribution in Figure 22a. From this distribution, it can be seen that the major abl hybridization peak occurs at 14q+. The efficiency of hybridization to chromosome 14q+ is 35% and is highly significant ($p < 10^{-6}$). Although abl hybridization to the normal homologue of chromosome 9 was expected, it was not demonstrated. A photograph of a metaphase cell is given in Figure 22b. The sis probe hybridized to the normal chromosome 22 in metaphase cells from this patient in preliminary experiments.

A normal karyotype was present in patient MUR diagnosed with ALL. Forty metaphase cells revealed abl hybridization was concentrated on chromosome 22 (Figure 23a). The major peak occurred at 22q13 and minor peak occurred at 22p11. Hybridization efficiency to chromosome 22 was 19% and was highly significant ($p < 10^{-6}$). A photograph of a metaphase cell is shown in Figure 23b. The bcr probe localized to chromosome 22 in preliminary hybridizations in this patient.

Patient HA has aplastic anemia and an abnormal karyotype

characterized by an absent chromosome 7 and an extra G-group chromosome. This patient was unique because both abl and sis oncogene movement was demonstrated. The abl grain distribution is shown in Figure 24a. The major abl peak is located at 22q11. The 20% efficiency of hybridization to chromosome 22 is significant ($p < .002$). Twenty metaphase spreads were used for both the abl and sis grain distributions. In the sis distribution (Figure 25a), the major peak is located at 9q34. The efficiency of hybridization is 19% and is significant ($p < .005$). Both abl and sis oncogenes are repositioned in this patient in the absence of a Ph' translocation (Figures 24b and 25b).

Although nine leukemia patients revealed abl movement from chromosome 9 to 22, two patients did not. These patients, JA and MC, have been diagnosed with CML and ALL respectively as is indicated in Table 5. Patient JA has a normal karyotype while patient MC has an abnormal karyotype (ie. 46,XY,-16,-Y,+2). In these patients, the abl oncogene consistently hybridized to chromosome 9 and the sis oncogene consistently hybridized to chromosome 22 (Figure 26).

The eleven leukemia patients in this investigation can be divided into two groups based on oncogene mobility (Table 5). The majority of patients are characterized by the absence of a microscopic Ph' translocation. The first group includes nine leukemia patients in which abl has moved from chromosome 9 to 22. This group has the bcr/sis oncogene on chromosome 22. This pattern of mobility was confirmed through the formation of two grains on chromosome 22 after hybridization with abl and sis. The second group includes two leukemia patients in which both abl and sis have not moved.

Figure 16a: Distribution of abl grains in 30 cells from CML patient NI. This patient has a normal karyotype. Twelve out of 63 total grains (19%) are located at 22q12.

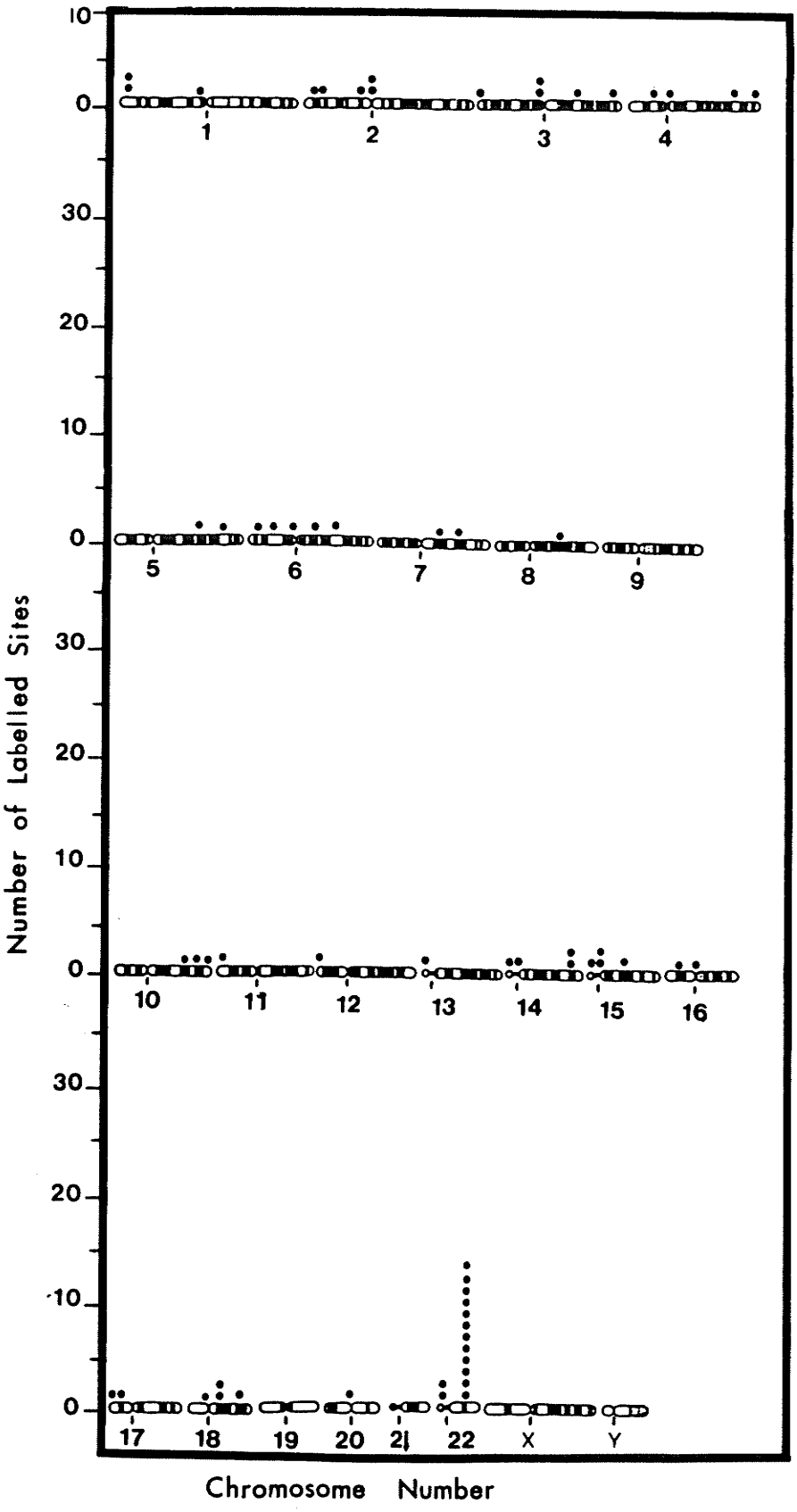

Number of Labelled Sites
10
0
30
20
10
0
30
20
10
0
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y
Chromosome Number

Figure 16b: Photograph of a metaphase spread from CML patient NI. The chromosomes have been hybridized to a ^{3}H-labelled abl probe. The chromosomes are G-banded. The G-group chromosomes are identified. The arrow indicates the hybridization grain on chromosome 22.

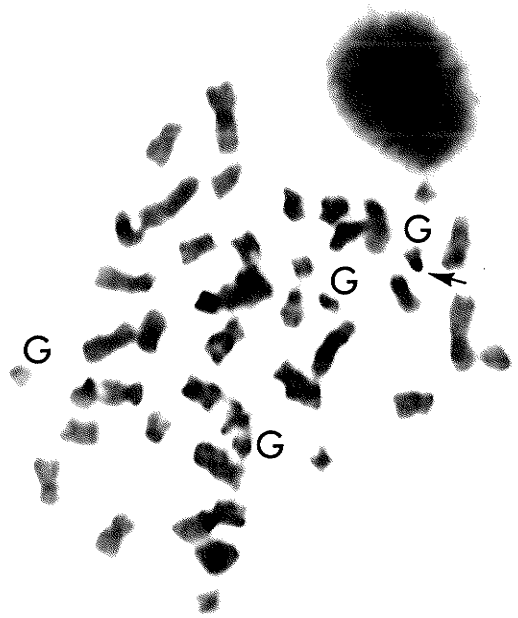

G
G
G
G

Figure 17a: Distribution of abl grains in 27 cells from CML patient BJ. This patient has a normal karyotype. Twenty out of 129 total grains (16%) hybridized to 22q13.

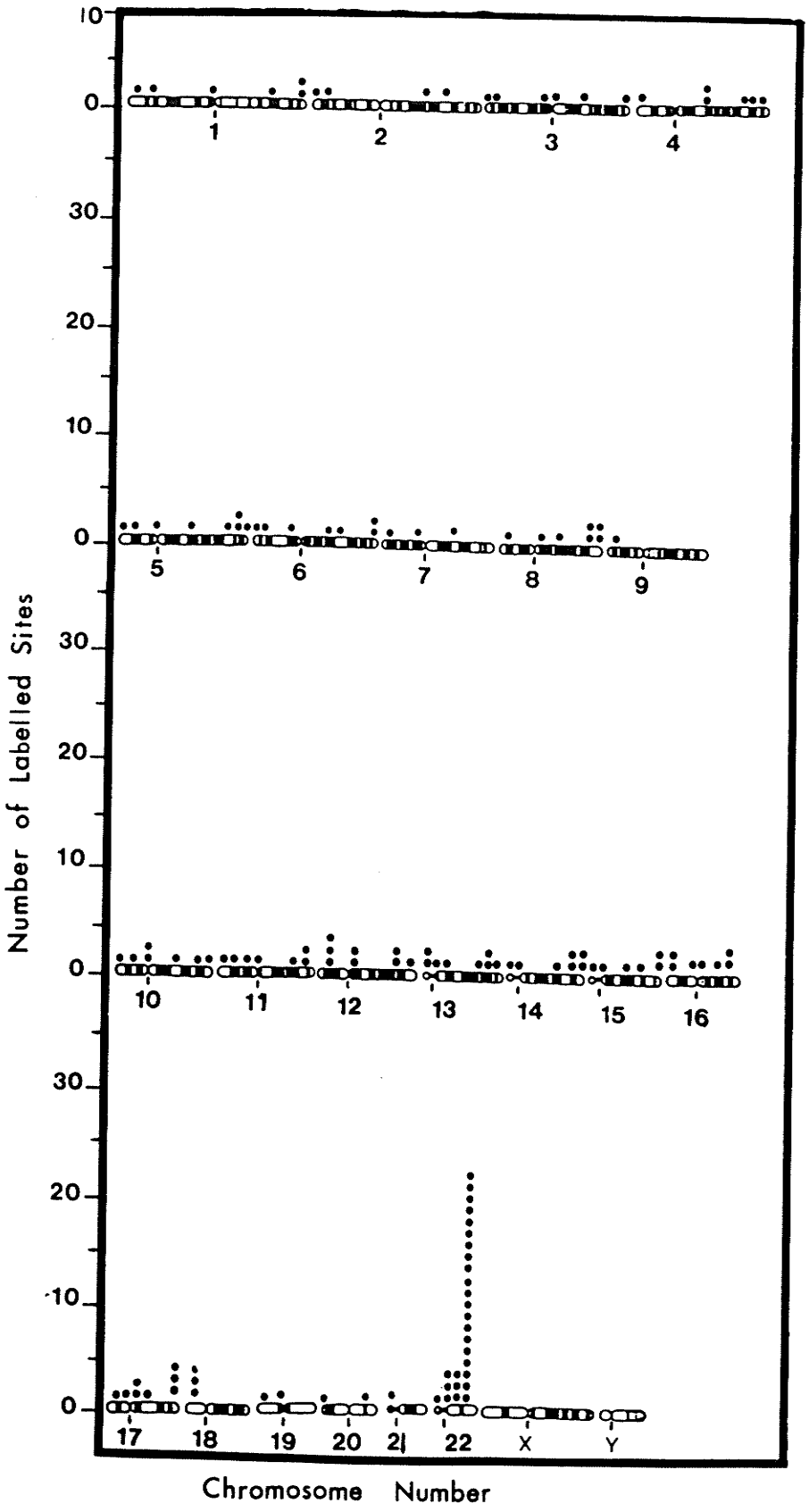

Number of Labelled Sites
10
0
30
20
10
0
30
20
10
0
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y
Chromosome Number

Figure 17b: Photograph of a representative metaphase spread from CML patient BJ. The chromosomes have been hybridized to a ^{3}H-labelled abl probe. The chromosomes are Q-banded. The arrow indicates the hybridization grain on chromosome 22.

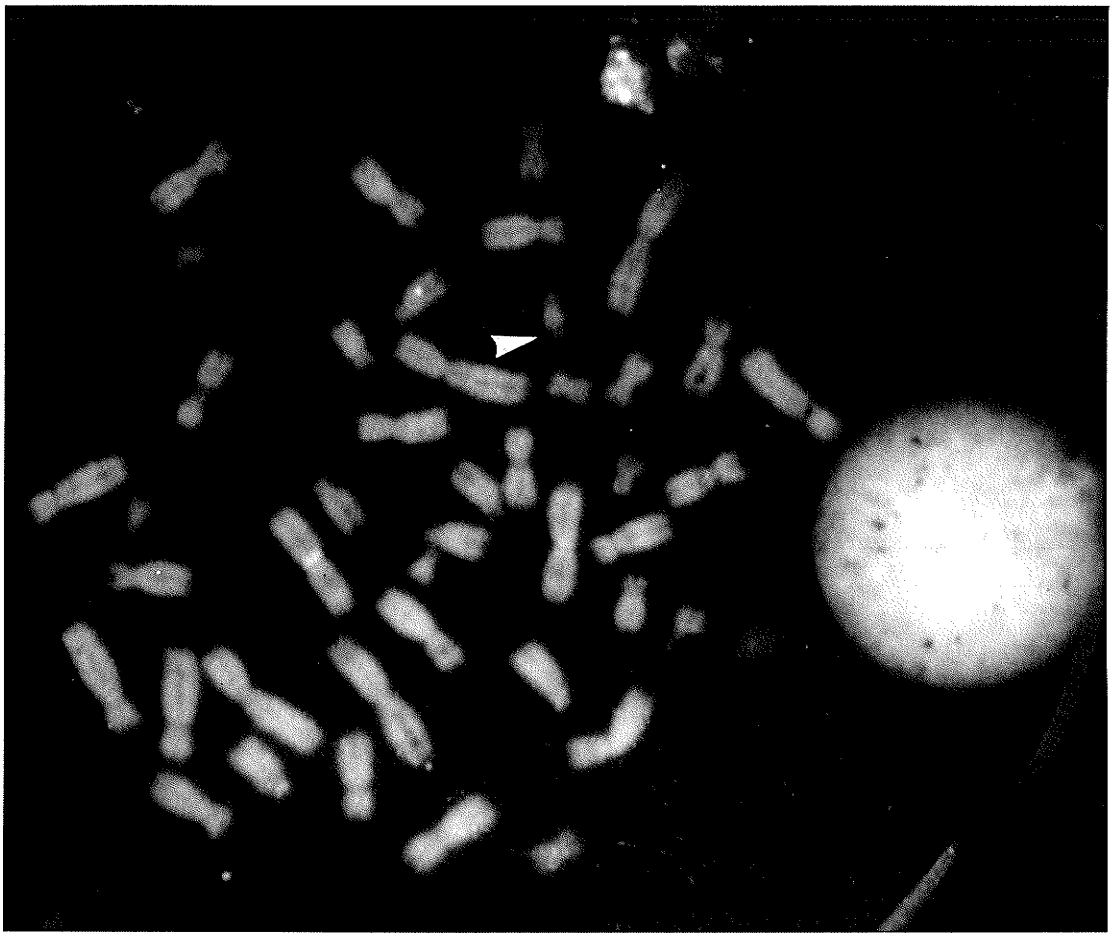


Figure 18a: Distribution of abl grains on 26 cells from CML patient WR. This patient has a normal karyotype. Thirty-one out of 138 total grains (22%) were located at 22q13.

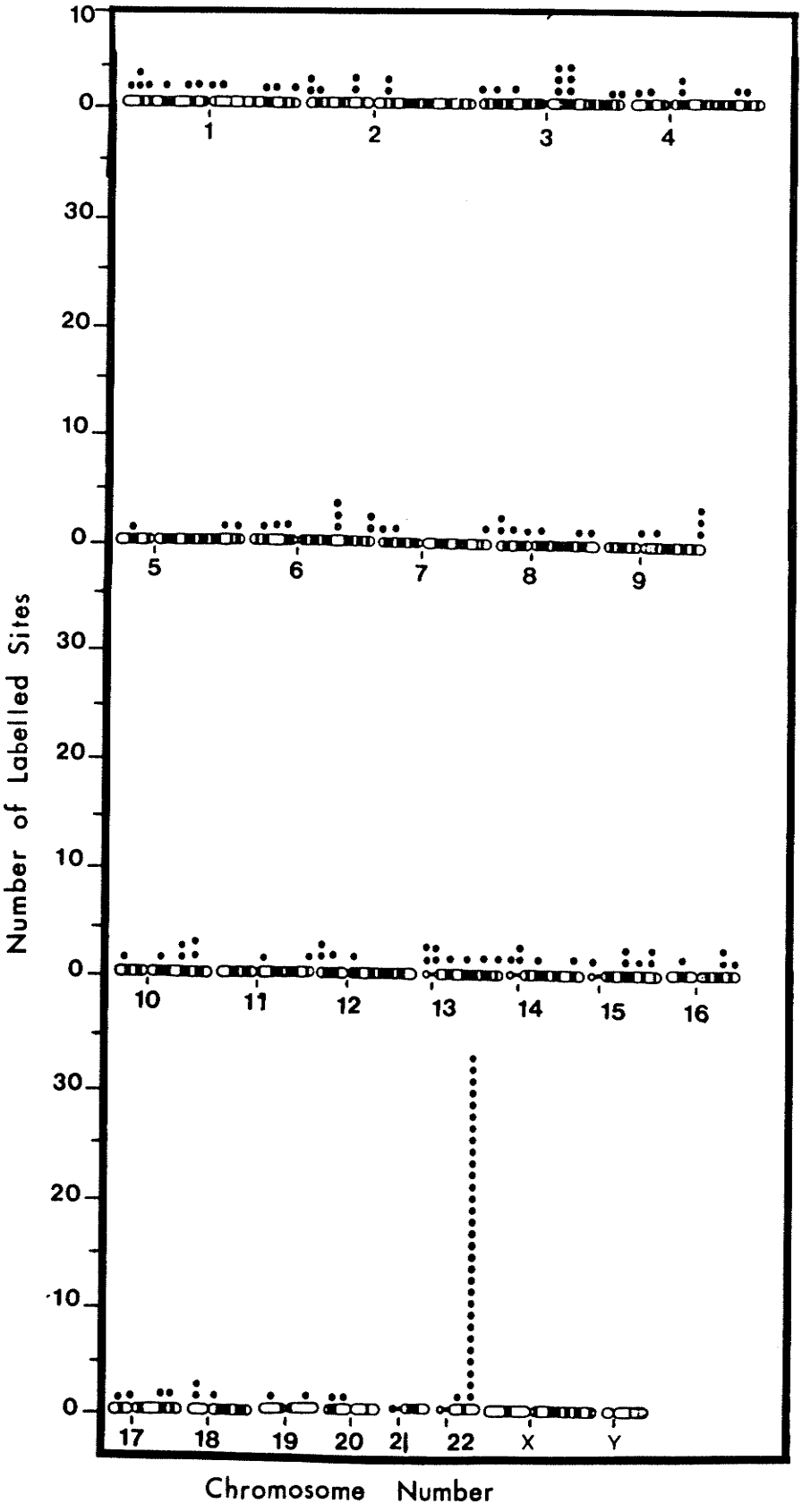

Number of Labelled Sites
10
0
30
20
10
0
30
20
10
0
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y
Chromosome Number

Figure 18b: Photograph of a representative metaphase spread from CML patient WR. The chromosomes have been hybridized to a ^{3}H-labelled abl probe. The chromosomes are Q-banded. The G-group chromosomes have been identified. The arrow indicates the hybridization grain on chromosome 22.

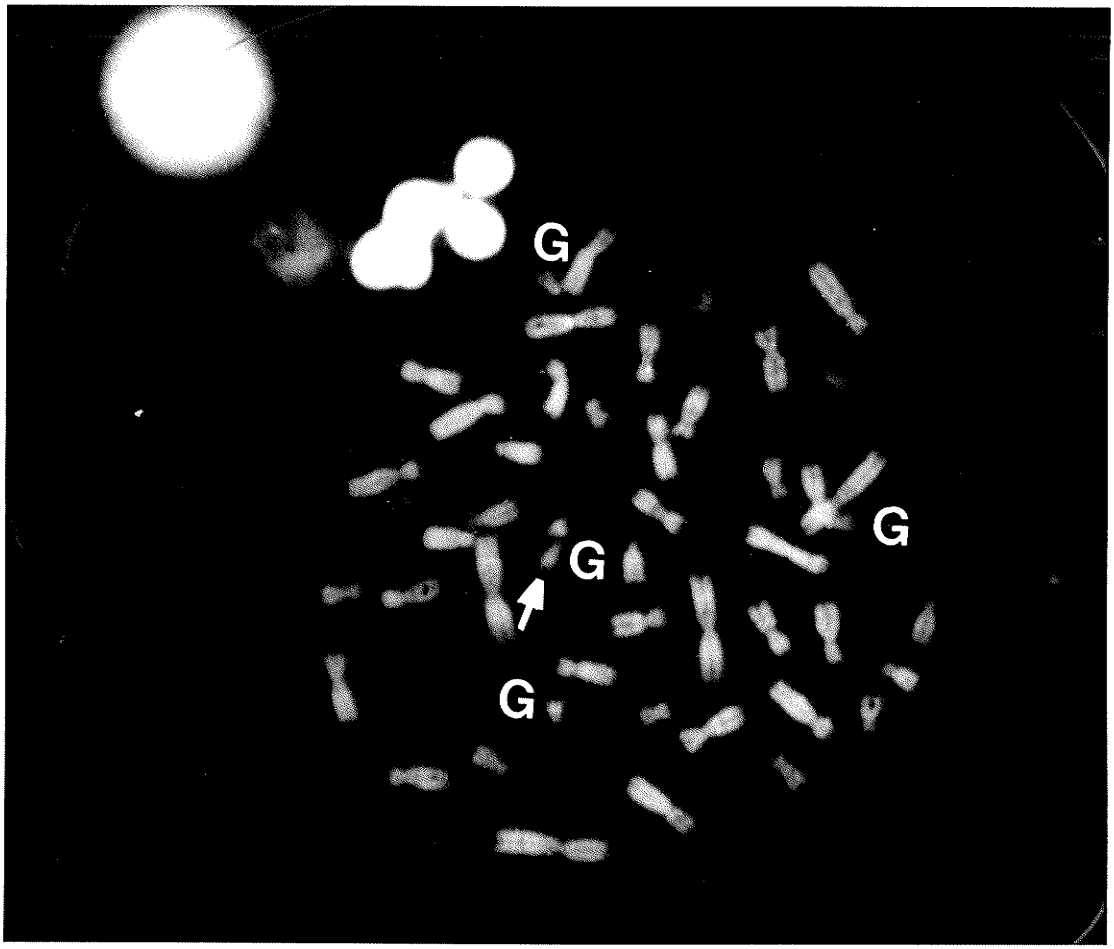

G
G
G
G

Figure 19a: Distribution of abl grains in 45 cells from CML patient FRA. The patient has a normal karyotype. Twenty out of 56 total grains (36%) are located at 22q13.

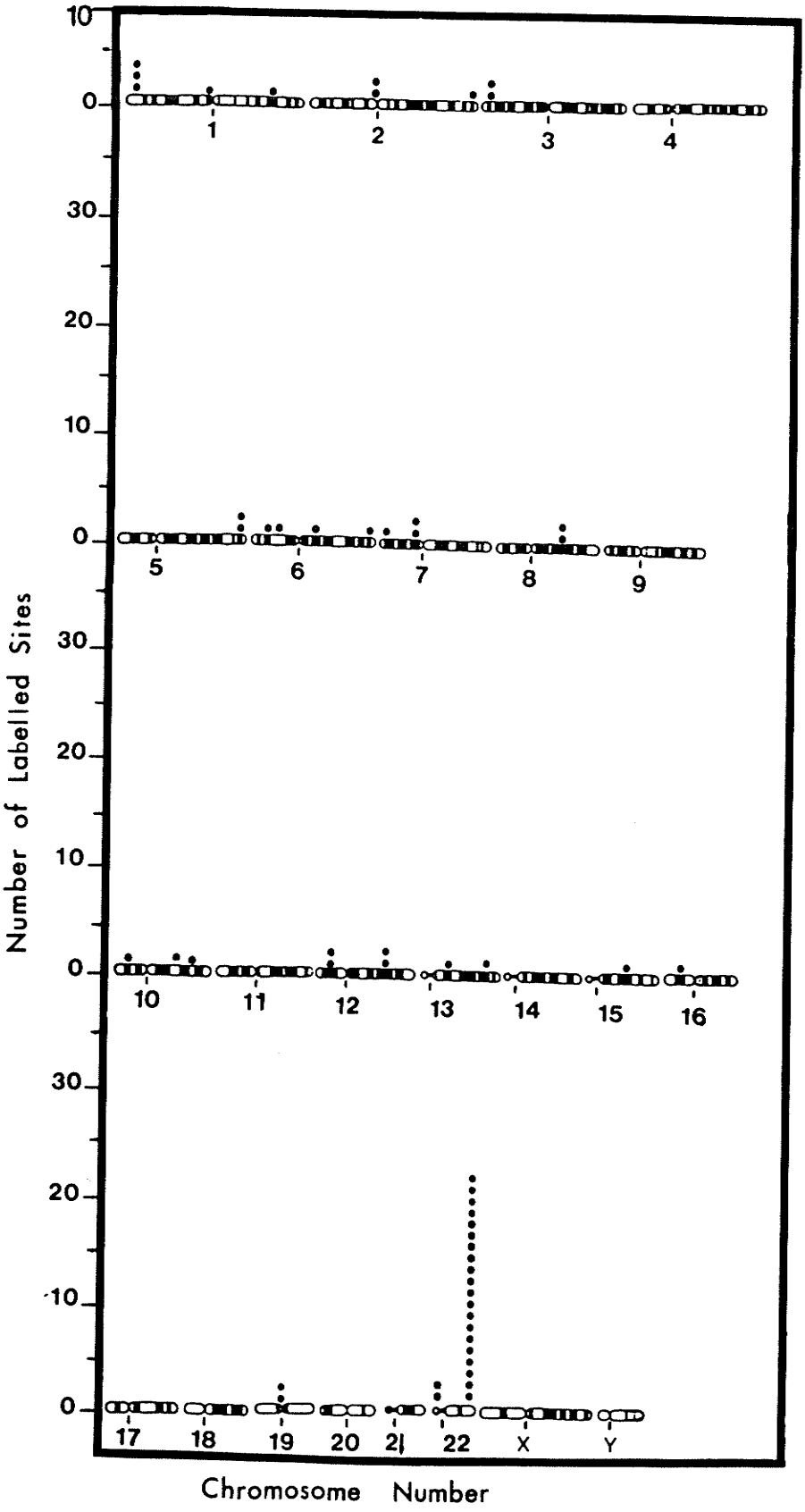

Number of Labelled Sites
10
0
30
20
10
0
30
20
10
0
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y
Chromosome Number

Figure 19b: Photograph of a representative metaphase spread from CML patient FRA. The chromosomes have been hybridized to a ^{3}H-labelled abl probe. The chromosomes are Q-banded. The arrow indicates the hybridization grain on chromosome 22.

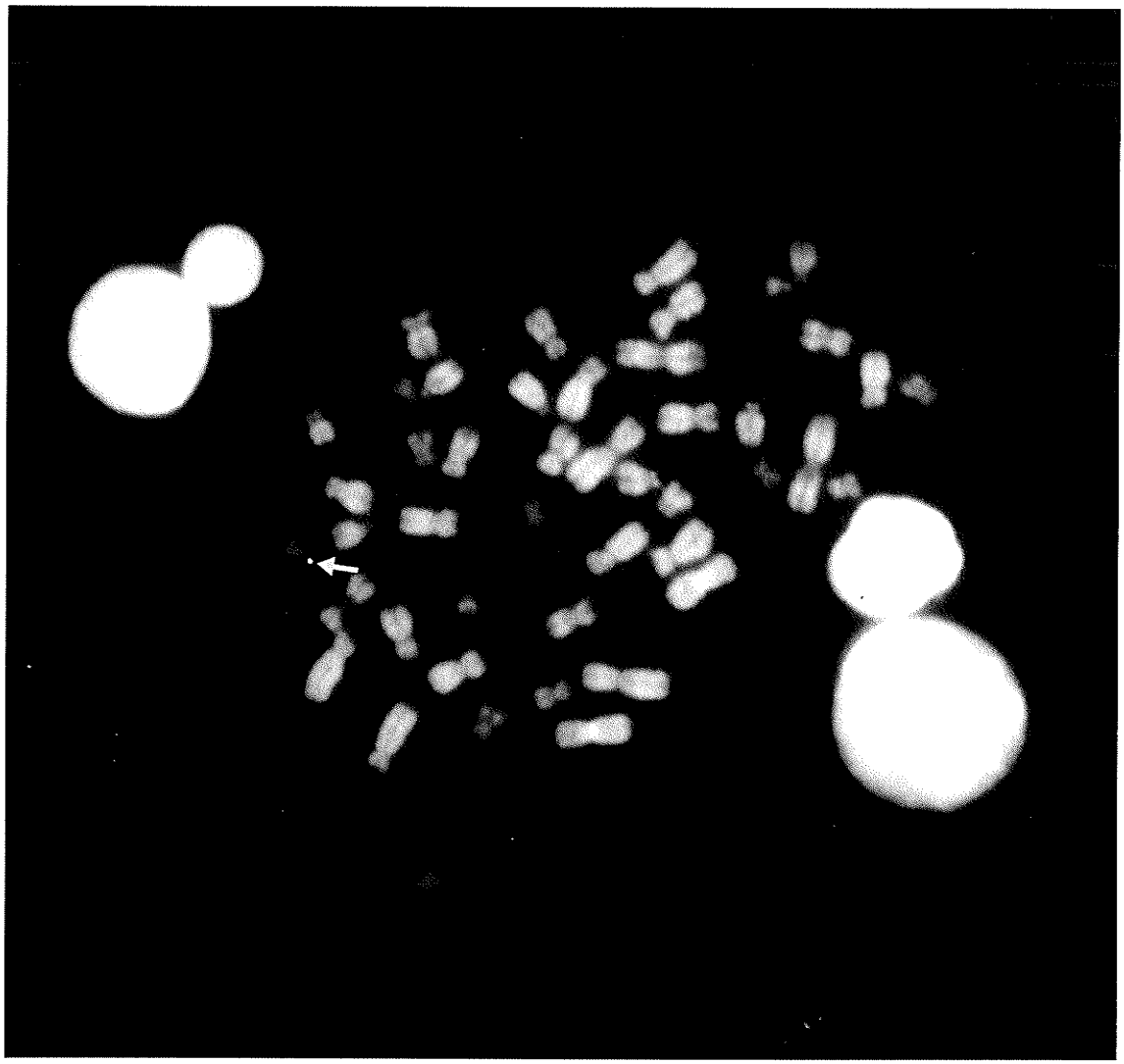


Figure 20a: Distribution of abl and sis grains in 28 cells from CML patient NIS. This patient has a normal karyotype. Seventeen out of 42 total grains (40%) are located at 22q12;22q13.

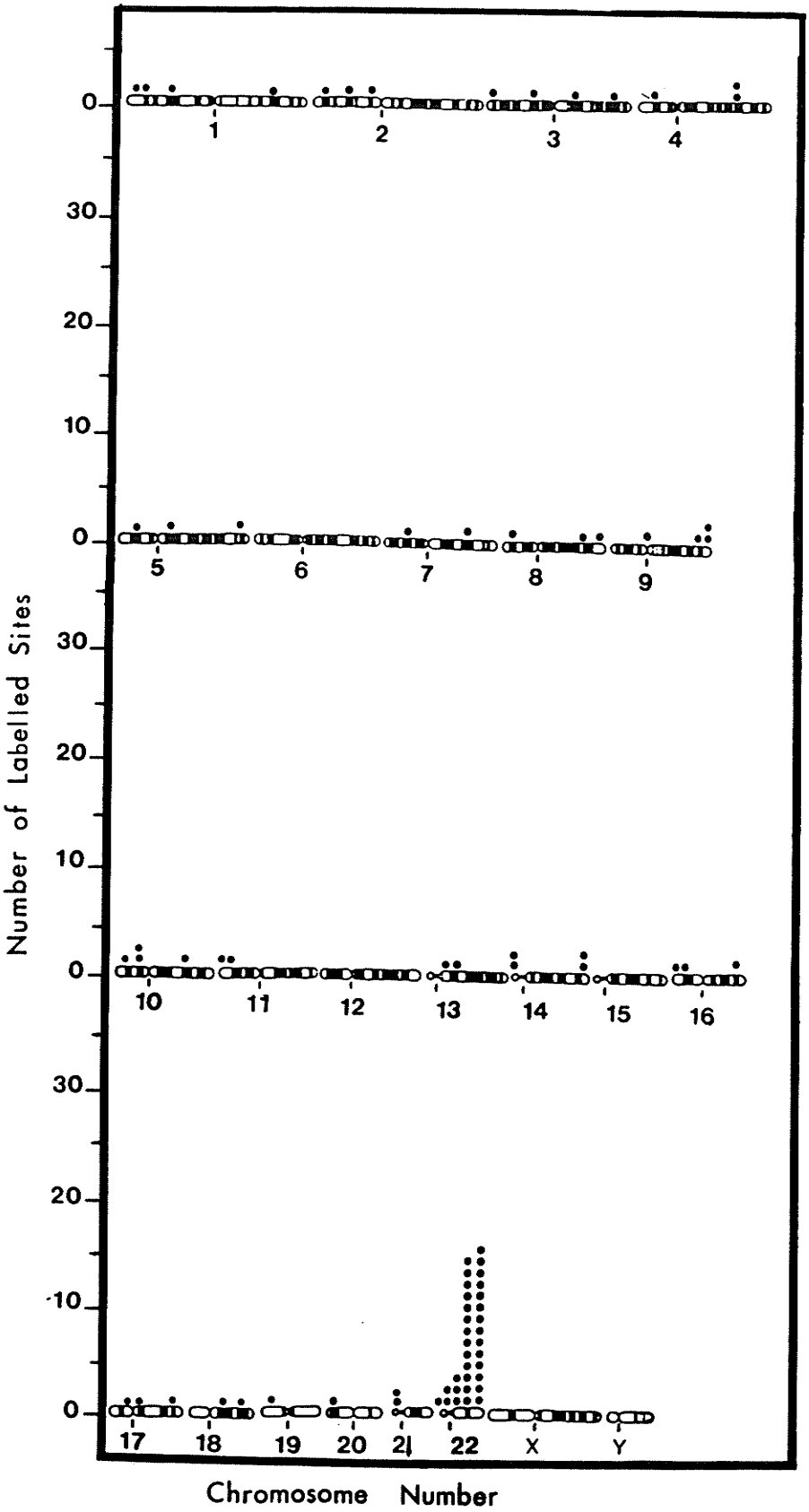

Number of Labelled Sites
0
30
20
10
0
30
20
10
0
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y
Chromosome Number

Figure 20b: Photograph of a representative metaphase spread from CML patient NIS. The chromosomes have been hybridized to a ^{3}H-labelled abl and sis probe. The two grains on chromosome 22 are indicated with an arrow.

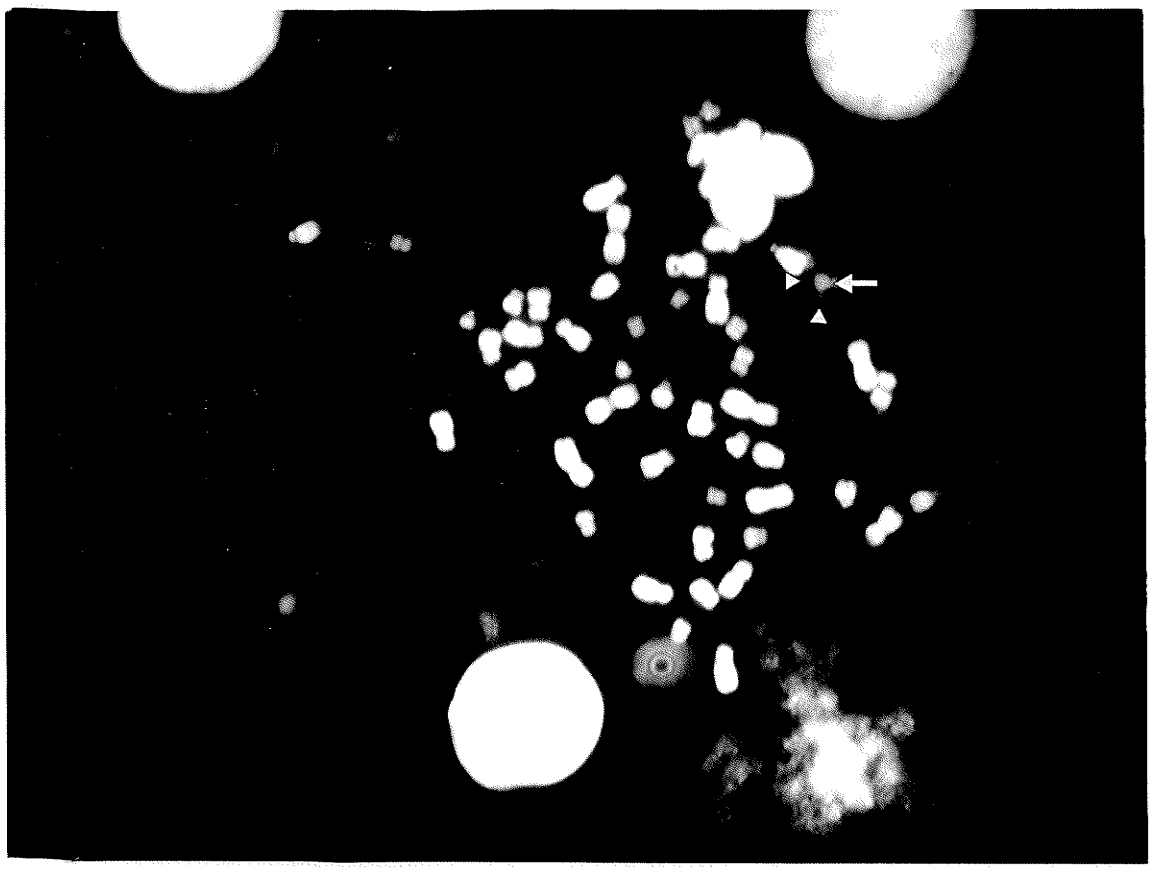


Figure 21a: Distribution of abl grains in 40 cells from CML patient LA. This patient has the translocation, t(9;22)(q34;q11). Nineteen out of 70 total grains (27%) are located at 22q11 22q12.

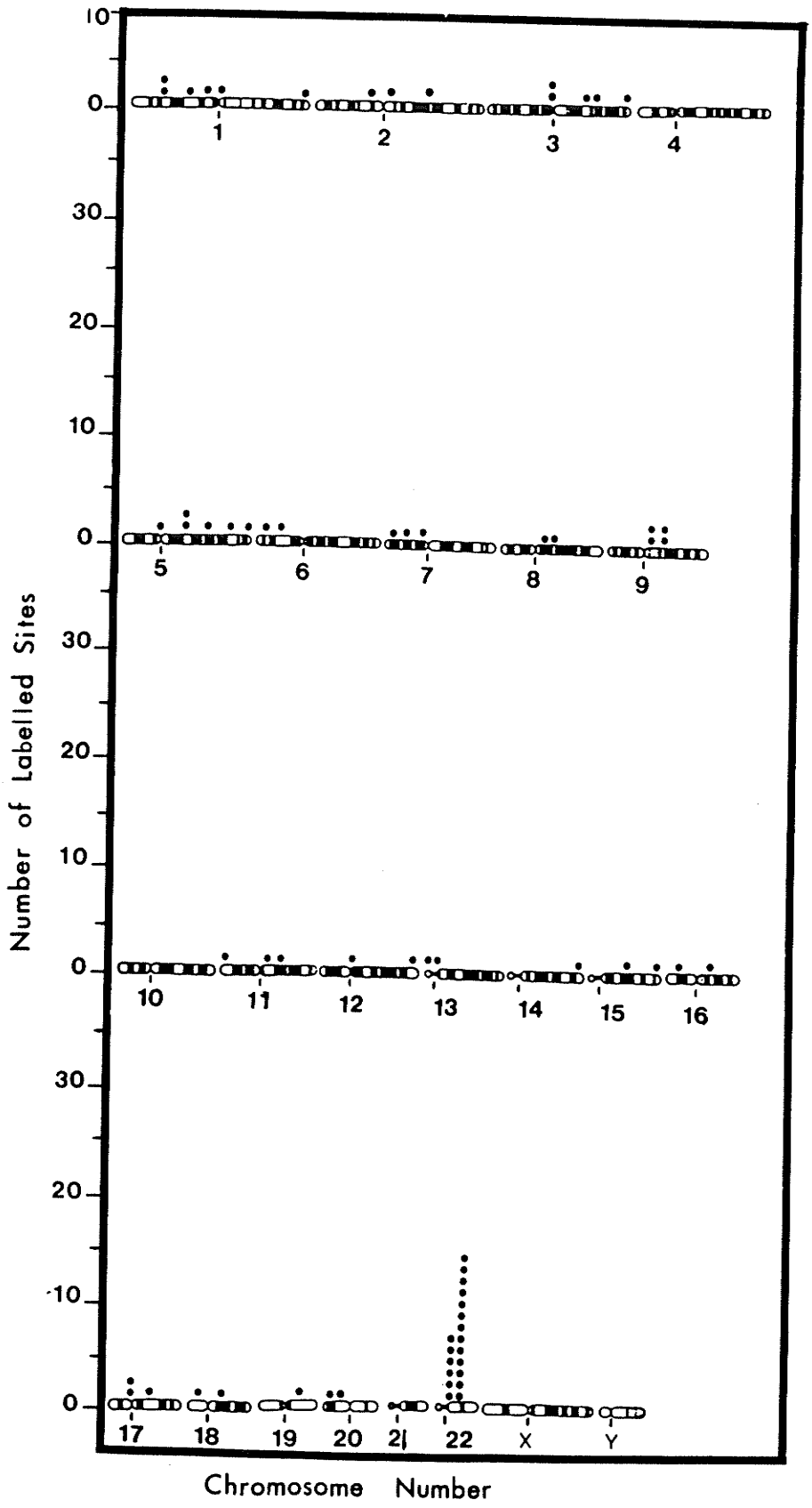

Number of Labelled Sites
10
0
30
20
10
0
30
20
10
0
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y
Chromosome Number

Figure 21b: Photograph of a representative metaphase spread from CML patient LA. The chromosomes have been hybridized to a ^{3}H-labelled abl probe. The Ph' and G-group chromosomes have been identified by G-banding. The arrow indicates the grain on chromosome 22.

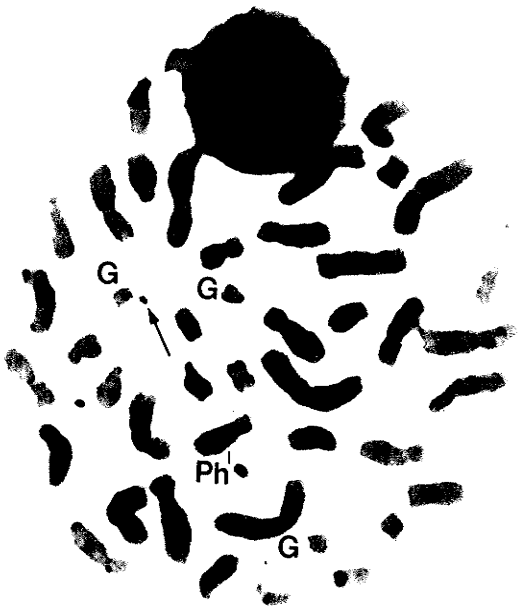
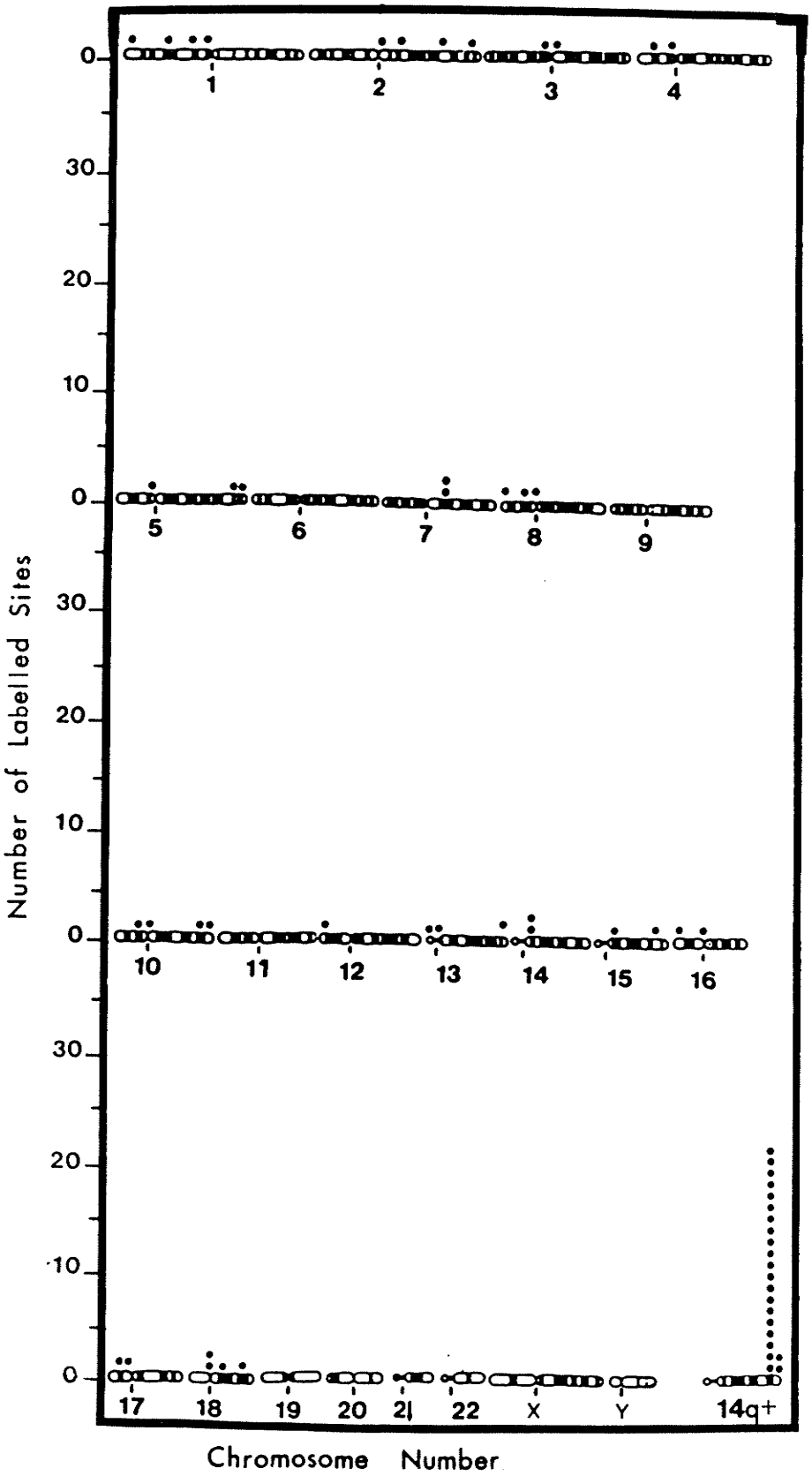

G
G
Ph
G

Figure 22a: Distribution of abl grains in 35 cells from CGL patient LAD. This patient's karyotype is 46,XY,9q-,11q-,14q+,22q+. Twenty-two out of 62 total grains (35%) are located at the terminus of chromosome 14.


Number of Labelled Sites
0
30
20
10
0
30
20
10
0
30
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10
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y
14q+
Chromosome Number

Figure 22b: Photograph of a representative metaphase spread from CGL patient LAD. The chromosomes have been hybridized to a ^{3}H-labelled abl probe. The chromosomes have been G-banded. The arrow indicates the grain on chromosome 14.

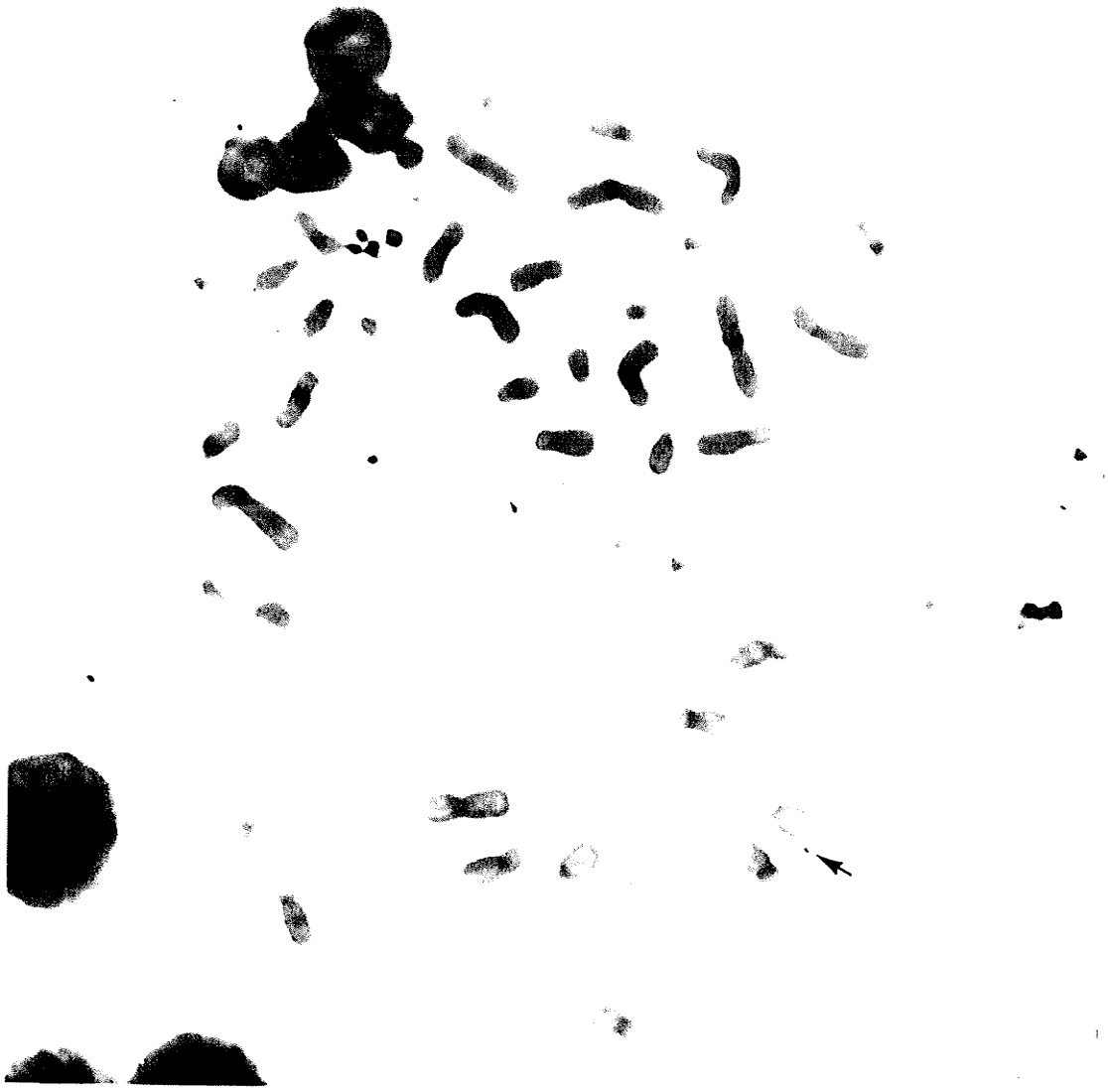
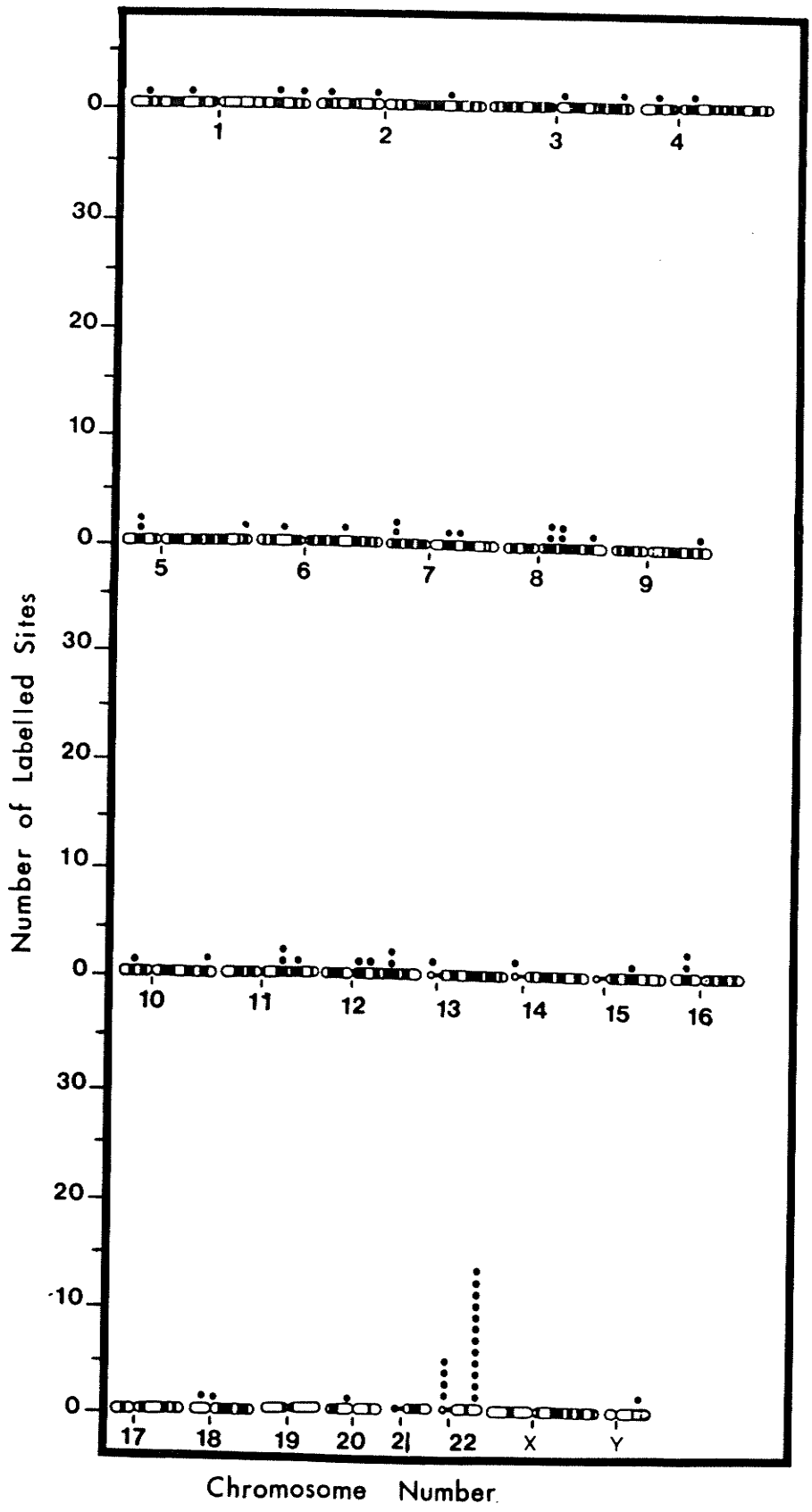


Figure 23a: Distribution of abl grains in 40 cells from ALL patient MUR. The patient has a normal karyotype. Twelve out of 62 total grains (19%) were located at 22q13.


Number of Labelled Sites
0
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20
10
0
30
20
10
0
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y
Chromosome Number

Figure 23b: Photograph of a representative metaphase spread from ALL patient MUR. The chromosomes have been hybridized to a ^{3}H-labelled abl probe. The chromosomes were Q-banded. The arrow indicates the hybridization grain on chromosome 22.

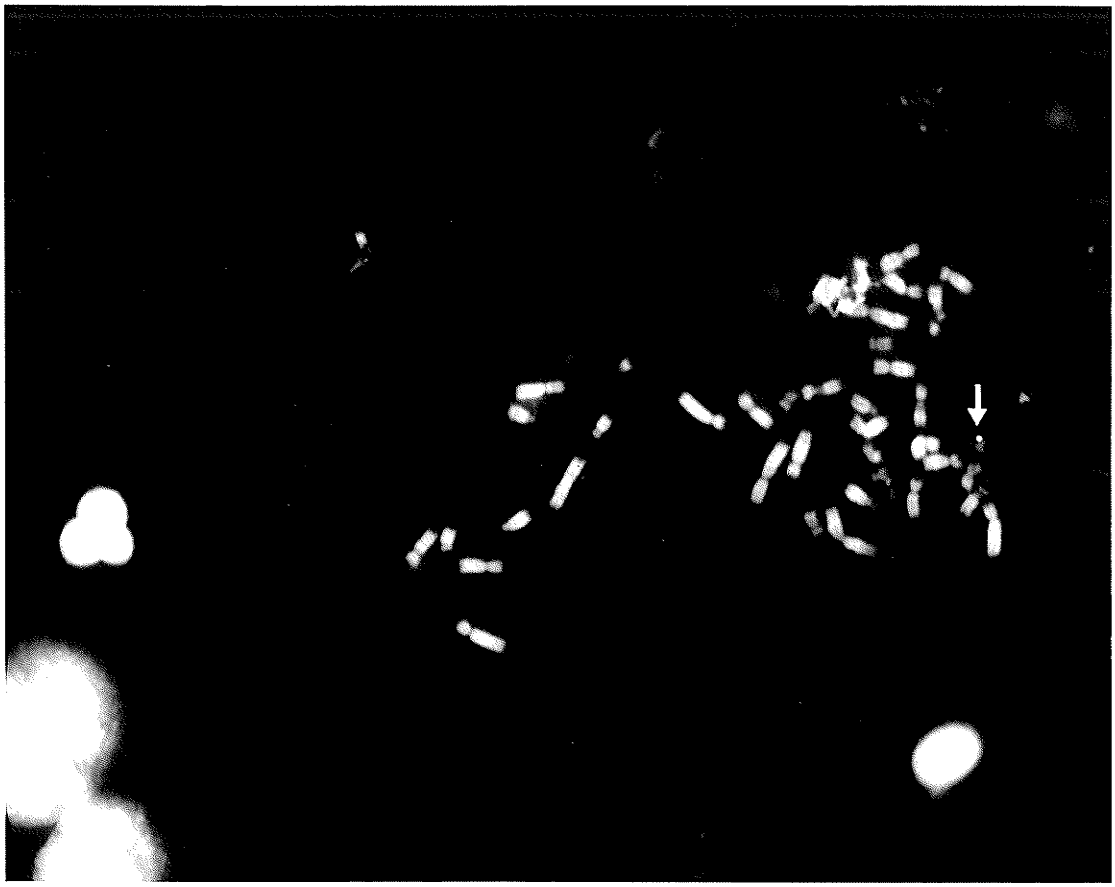
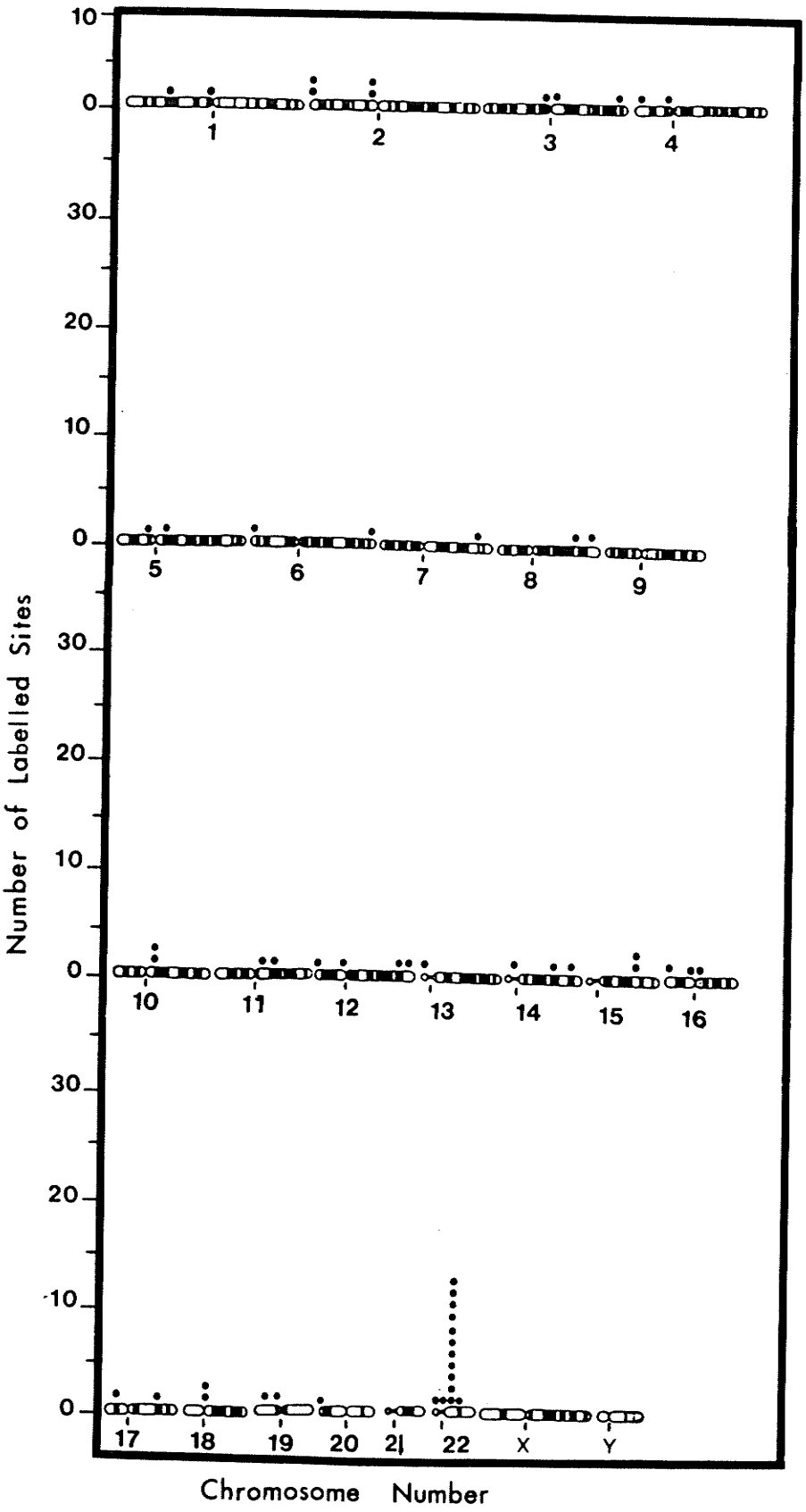


Figure 24a: Distribution of abl grains in 20 cells from aplastic anemia patient HA. The patient's karyotype is 46,XY,-7,+G. Eleven out of 56 total grains (20%) were located at 22q11.


Number of Labelled Sites
10
0
30
20
10
0
30
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10
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30
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10
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y
Chromosome Number

Figure 24b: Photograph of a metaphase cell from aplastic anemia patient HA. The chromosomes have been hybridized to a ^{3}H-labelled *abl* probe. Chromosome 9 and the G-group chromosomes were identified by G-banding. The arrow indicates the hybridization grain on chromosome 22.


9
9
G
G
G
G
G

Figure 25a: Distribution of sis grains in 20 cells from aplastic anemia patient HA. The patient's karyotype is 46,XX,-7,+G. Eight out of 43 total grains (19%) were located at 9q34.

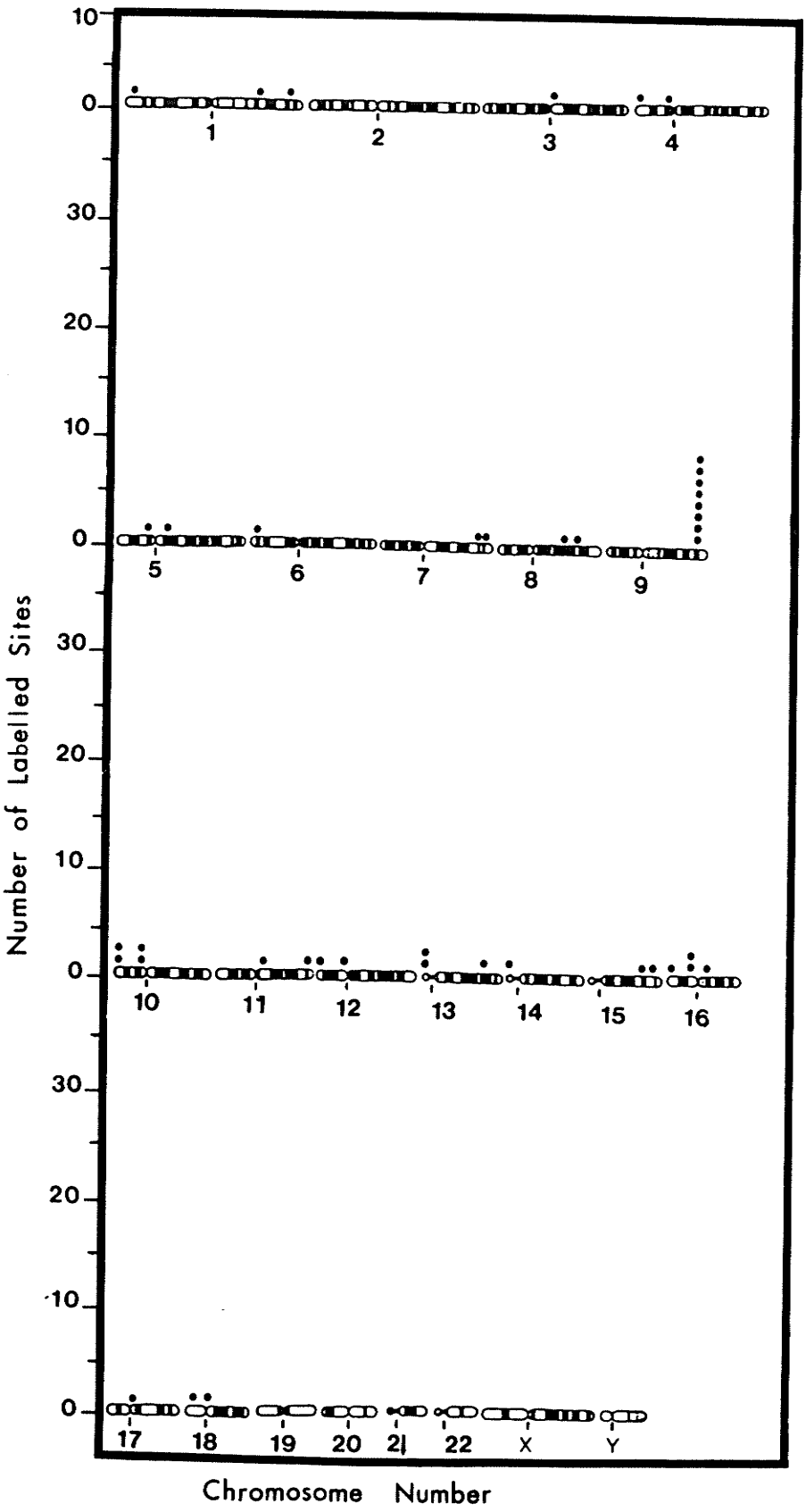

Number of Labelled Sites
10
0
30
20
10
0
30
20
10
0
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y
Chromosome Number

Figure 25b: Photograph of a metaphase cell from aplastic anemia patient HA. The chromosomes have been hybridized to a ^{3}H-labelled *sis* probe. The chromosomes were G-banded. Chromosome 9 and the G-group chromosomes are identified. The arrow indicates the hybridization grain on chromosome 9.

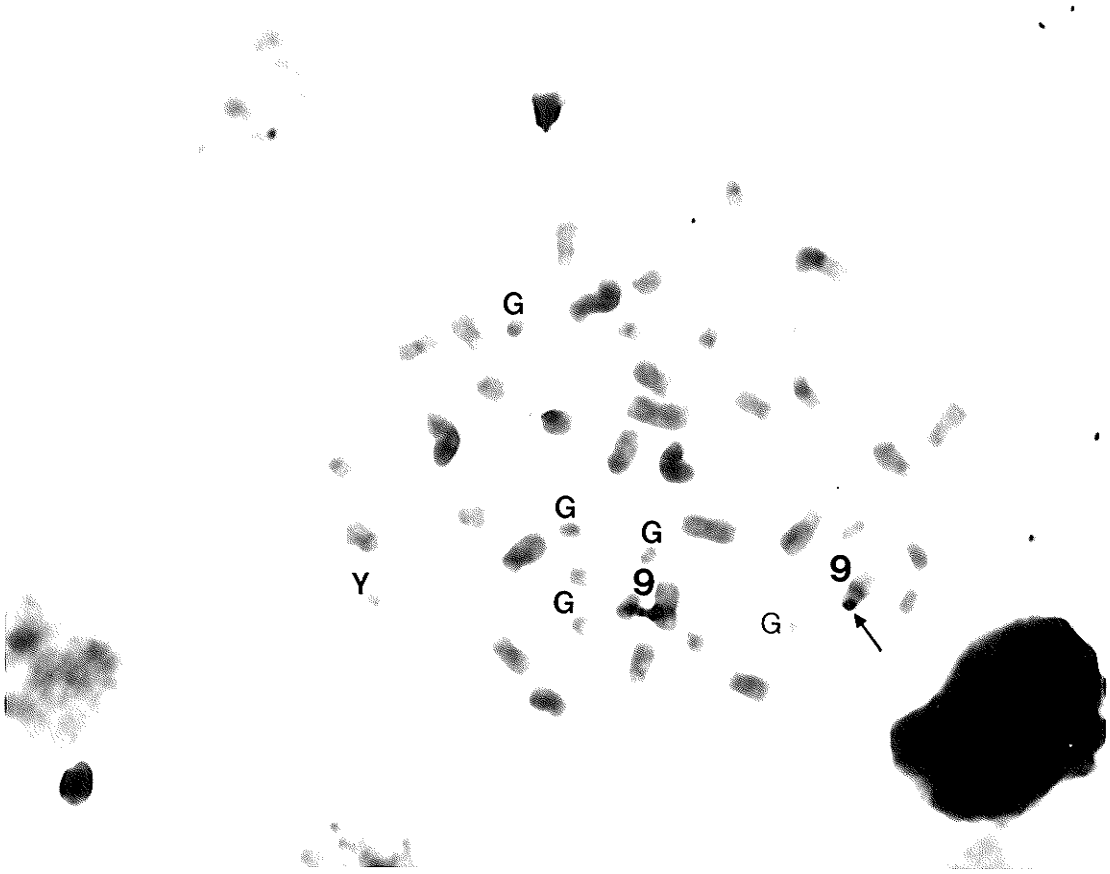
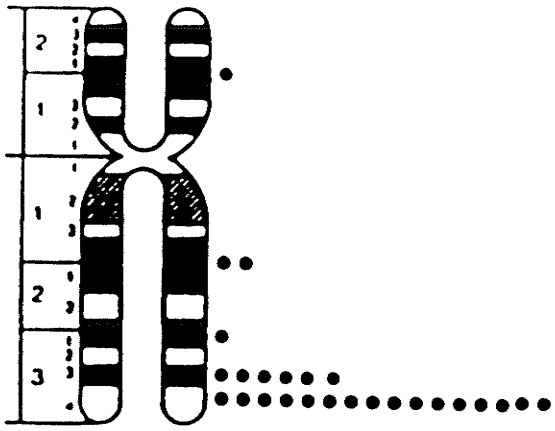
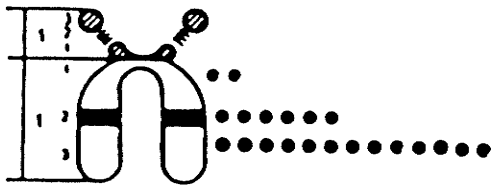

G
G
G
Y
9
G
G
9

Figure 26: Combined distribution of abl grains to chromosome 9 and sis grains to chromosome 22 in patients JA and MC. The distributions were constructed from 32 and 29 cells, respectively.



9



22

Table 5: Summary of oncogene mobility results obtained in ten untreated leukemia patients using the probes abl and sis or bcr.

Patient	Leukemia	Karyotype	abl mobility	sis or bcr mobility
NI	CML	46,XY	present	absent
BJ	CML	46,XY	present	
WR	CML	46,XY	present	
FRA	CML	46,XY	present	absent
NIS	CML	46,XY	present	absent
LA	CML	46,XY,t(9;22)(q34;q11)	present	absent
LAD	CGL	46,XY,9q-,11q-,14q+,22q+	present	absent
MUR	ALL	46,XY	present	absent
HA	aplastic anemia	46,XY,-7,+G	present	present
JA	CML	46,XY	absent	absent
MC	ALL	46,XY,-16,-Y,+2	absent	absent

4.0 DISCUSSION

4.1 Chromosomal Rearrangements in 72 Leukemia Patients

The rearrangements described in Table 1 represent acquired chromosomal abnormalities resulting from the leukemic process. Since the majority of patients are diagnosed as CML, both standard and variant Ph' translocations are present.

The first type of variant Ph' translocation is complex because it involves chromosomes 9, 22 and at least one other chromosome such as 2, 5, 7 or 15. Complex Ph' translocations found in this investigation include: 46,XX,del(2)(p13p23)t[dup9(q13q34);22(q34;q11)]; 46,XX,t(5;9;22)(q31;q34;q11); 46,XX,del(7)(p12,p15),t(9;22)(q34;q11); 46,XX,t(9;15;22)(q34;q15;q11). Other complex Ph' translocations have been previously reported (Oshimura et al., 1982; Sandberg, 1980; Borgstrom, 1981).

The second type of variant Ph' translocation is "simple" because it involves chromosome 22 and another chromosome other than 9. From the data, 41 CML patients and 2 CGL patients were characterized with deletions in chromosome 22 at band q11 (Table 1). These patients probably represent "simple" Ph' translocations. They were not fully characterized for several reasons. The first reason is the difficulty involved in the preparation and banding of cancer chromosomes (Hossfeld and Weh, 1984). Secondly, these karyotypes were completed without the use of the methotrexate technique to elongate the chromosomes and allow more accurate analysis. It is now known that complete analysis on complicated leukemia karyotypes can be accomplished through in situ

hybridization (Hagemeijer et al., 1984) or restriction-enzyme chromosomal banding (Babu et al., 1987).

The CML cytogenetic literature strongly suggests that all variant Ph' translocations of the "simple" type are actually undetected complex ones, all involving region 9q34 (Lewis et al., 1983; Hagemeijer et al., 1984). Many so-called "simple" translocation variants including del(22)(q11) variants previously reported in early CML literature (Hagemeijer et al., 1980), have been found to be undetected complex variants of the Ph' chromosome (Lewis et al., 1983; Hagemeijer et al., 1984). Another striking observation in favor of this hypothesis is the place of breakpoints on chromosomes other than 9 and 22, as reviewed by Sandberg (1980) in 85 variant Ph' translocations. In 44 of the 85 variants, the breakpoints were found to be dispersed along the length of the third chromosome, but never in the terminal bands. In the remaining 41 cases, the breakpoints were invariably in the terminal band of the chromosome recipient for the deletion in 22. The explanation for this observation is that a translocation between the terminal band of the recipient chromosome and 9q34 is at the limit of detection by standard cytogenetic methods, whereas translocation of a substantial part of a chromosome to chromosome 9 results in a 9q+ derivative. Therefore, chromosome region 9q34 seems to be involved in the so-called "simple" Ph' translocations, complex Ph' translocations and the standard Ph' translocations. Band 9q34 may prove to be a crucial chromosomal region implicated in the etiology of CML.

The relationship between cancer-specific breakpoints and fragile sites is seen in chromosomes 1, 2, 5, 7, 8, 9, 11, 16, 17 and 22 in this

investigation (Figure 2). The highly significant association between cancer-specific breakpoints and fragile sites has been previously demonstrated statistically (Hecht and Sutherland, 1984; Hecht and Glover, 1984). "Exact" association between breakpoints and fragile sites is seen at 1q32, 2p31, 5q31, 7p12, 11q23, 16q22 and 17p12. "Close" association between breakpoints and fragile sties is seen at 8q21, 9q34 and 22q11.

The most interesting relationship in the data occurs between breakpoints and oncogenes (Figure 2). A "close" association between the two events is found in chromosomes 1, 2, 5, 7, 8, 17 and 22. But "exact" map concordance between the two events was seen in only 2 chromosomes, 9 and 11. In chromosome 9, 16 CML patients and 4 ALL patients had the breakpoint at 9q34 which corresponds exactly to oncogene abl. In chromosome 11, two AML patients and one CML patient had the breakpoint at 11q23, which corresponds exactly to oncogene ets-1.

It is clear that there is a relationship between breakpoints, fragile sites and oncogenes. The "close" association of breakpoints to the corresponding fragile sites and oncogenes is seen in chromosomes 1, 2, 5, 7, 8, 9, 17 and 22 in this investigation. The identification of cancer-specific breakpoints that are highly associated with fragile sites may lead to clues regarding the map location of important oncogenes in particular leukemias.

Two precise breakpoints, 22q11 and 9q34, characterize a reciprocal translocation, the most common anomaly in CML. One of the breakpoints may represent the site of a proto-oncogene which becomes activated as a result of chromosomal rearrangement (Yunis, 1983; Yunis, 1984a). The

breakpoint at 22q11 is "closely" associated to the sis oncogene. This gene has not been implicated in the genesis of CML (Gale and Canaani, 1984). Since both standard and variant Ph' translocations all involve region 9q34, the abl oncogene may be the activated gene. What activates this proto-oncogene may be its general proximity, following translocation, to a regulatory sequence normally used by an active gene(s) of a differentiated cell (Croce et al., 1984). Translocation of a proto-oncogene may alter both the structure and function of the gene in ways that mimic those found in the transduced and oncogenic version of the same gene (Davis et al., 1985). The cytogenetic data in this investigation indicate that the abl oncogene located at cancer-specific breakpoint 9q34 is a predominant factor in CML. Fragile site 9q32 located near this region may aid in the rearrangement of the abl oncogene. Moreover, the abl oncogene may be involved in the submicroscopic oncogene rearrangements present in Ph'-negative CML patients (see section 4.3.2).

4.2 Fragile Sites

The common fragile sites, induced by aphidicolin, are present in the normal cells of leukemic patients but not the cancer cells. From this investigation, it was found that the frequency of common fragile sites was statistically increased ($p < 10^{-6}$) as compared with non-leukemic individuals. Increased expression of common fragile sites has been previously demonstrated in patients with AML and in patients with follicular lymphoma (Yunis and Soreng, 1984).

The expression of fragile sites in the normal cells were found not to correspond with the breakpoints of translocations in the cancer

cells. For example, patient MU with AML did not show an increase in the expression of the rare fragile site in chromosome 16 although it had breakpoints at 16q22 and 16q24 as depicted in Table 2. The reason for this lack of expression could be that the 16q22 fragile site is distamycin-A inducible and could not be expressed with aphidicolin. Some reports that used distamycin-A noted an unusually high frequency of fragile site 16q22 in AML-M4 patients with the inversion (Yunis, 1983; Le Beau and Rowley, 1984) whereas others did not detect it in three similar patients (Glover et al., 1986). Patient MA with ALL had a translocation t(11;19) but the breakpoints could not be determined. Therefore, a comparison of breakpoints with fragile sites is not possible in this patient.

The expression of the fragile sites in eight leukemia patients demonstrated that four main chromosomes were involved. Highly expressed fragile sites, depicted in Figure 6, clustered on chromosomes 1, 2, 3 and 5. Furthermore, the map location of highly expressed fragile sites corresponds with the map location of the oncogenes described in Figure 6. The map concordance between fragile sites and oncogenes has been previously demonstrated in Figure 2 and by other investigators (Hecht and Sutherland, 1984; Le Beau and Rowley, 1984; Hecht and Hecht, 1984). There seems to be a restricted number of chromosomal regions highly susceptible to fragility in the cancer karyotype. This hypothesis has been suggested by Mitelman, 1984. Indeed, fragile sites that are close to oncogenes could serve as markers in the search for and analysis of oncogenes (Yunis and Soreng, 1984).

The highly expressed fragile sites on chromosomes 1, 2, 3 and 5

also correspond to particular cancer-specific breakpoints. For example, fragile site 1p22 corresponds to the breakpoint in the translocation characteristic of malignant melanoma (Balaban et al., 1984). Fragile sites 2p11 and 2p13 correspond to the breakpoints of t(2;8)(p11 or p13;q24) in ALL (Berger et al., 1982a). Fragile sites 3p14 and 3p24 correspond to the breakpoints in del(3)(p14p24) in carcinoma of the lung (Whang-Peng et al., 1982a; Whang-Peng et al., 1982b). Fragile site 5q31 corresponds to interstitial deletions of varying lengths present in ANLL, preleukemia and secondary leukemias (Rowley and Potter, 1976; Rowley et al., 1977; Fourth International Workshop on Chromosomes in Leukemia, 1984). The relationship between fragile sites and cancer-specific breakpoints has been suggested by many investigators (Yunis, 1983; Yunis, 1984; Hecht and Sutherland, 1984; de la Chapelle and Berger, 1984). As a result of this relationship, fragile sites could serve as markers for cancer-specific breakpoints.

There is clearly a need for further study of fragile sites in cancer. This research should include studies of cancer in fragile site families and, conversely, studies of fragile sites in cancer families. A number of issues surround the role of fragile sites in oncogenesis. Numerous questions can be asked. Are people with fragile sites predisposed to cancer? Which fragile sites predispose to cancer? What level of cancer risk does each fragile site carry? If fragile sites do prove to carry an increased risk of cancer, can the utilization of fragile sites aid in the detection, prevention or treatment of cancer? Perhaps fragile sites could explain why leukemias with a translocation appear to arise "spontaneously" (Yunis, 1984a). At the present time,

more data is required before any definitive statement regarding cause and effect can be made. It will be necessary to demonstrate on the DNA level that a particular fragile site, breakpoint and oncogene are at the same point in the genome.

The cytogenetic data from the investigations of chromosome rearrangements and fragile sites confirm their relationship with each other and with oncogenes. The increase in frequency of fragile sites in normal cells from leukemia patients suggest that they are constitutive abnormalities that may serve as "weakpoints" in the genome (Yunis and Soreng, 1984). Some weakpoints also may provide the structural basis for somatic recombination, a mechanism proposed to operate in neoplasia (Cavenee et al., 1983; Naylor et al., 1984; Koufos et al., 1984). Indeed, patients NI, BJ, WR and NIS showed both increased expression of fragile sites and submicroscopic abl mobility. Since fragile sites appear when cells are deprived of folic acid and thymidine, this suggests that they may have a unique DNA structure which becomes prone to rearrangement as a result of deprivation of DNA precursors (Yunis and Soreng, 1984). Such rearrangements may help explain the microscopic and submicroscopic oncogene rearrangements characteristic of leukemia.

4.3 Oncogene Mobility

4.3.1 Non-Leukemic Individuals

Approximately 5% of Mendelian mutations associated with karyotypic abnormalities display neoplastic tendencies (for review see Dallapiccola, 1987). For example, ataxia-telangiectasia, Fanconi's anemia and Bloom's syndrome are genetic syndromes resulting in increased

susceptibility to cancer (Dallapiccola, 1987). In this investigation, chromosomal abnormalities resulting in oncogene rearrangement or amplification were studied in non-leukemic individuals. These individuals may be at increased risk for neoplasia.

Patient RUS is characterized by a translocation, 46,XX,t(9;11)(q22;p15). In situ hybridization with abl has demonstrated that this oncogene has been displaced onto the translocation chromosome, t(9;11) (Figure 11b). Hybridization to both chromosome 9 and t(9;11) is expected although significant hybridization only to t(9;11) was obtained. Due to suboptimal banding in this patient, hybridization to chromosome 9 may have occurred but was misinterpreted as hybridization to another C-group chromosome. The translocation chromosome, t(9;11), could be identified in all cases because of its distinctive morphology. The breakpoints of the translocation include the abl oncogene but do not correspond precisely with the oncogene's map location. As a result of oncogene displacement, there may be an increased tendency for neoplasia. For instance, this translocation, t(9;11), characterizes both AML-M4 and AML-M5 (Hagemeijer et al., 1982; Fourth International Workshop on Chromosomes in Leukemia, 1984; Michalski et al., 1983).

Patient RUM has the unbalanced translocation, 46,XX,-14,t(12;14)(p11;p11). The breakpoints of this translocation, as with RUS, do not correspond exactly with an oncogene. Instead, the translocated region includes the K-ras-2 oncogene along with other chromosomal material. The results from in situ hybridization demonstrated a 19% hybridization efficiency to the translocation chromosome and an 11% hybridization efficiency to chromosome 12 (Figure

12a). The composite hybridization efficiency to 12p is 30%. Although a high hybridization efficiency to 12p was expected because of complete 12p trisomy, a 30% hybridization was obtained possibly as a result of suboptimal hybridization conditions. A predisposition to cancer may be indicated in this patient because of oncogene displacement and complete 12p trisomy. It is known that unbalanced chromosome rearrangements involving 12 and 14 are highly associated with neoplasia. For example, anomalies of chromosome 12 are highly associated with non-Burkitt's lymphoma (de la Chapelle and Berger, 1984) and anomalies of chromosome 14 are associated with ALL and Burkitt's lymphoma (de la Chapelle and Berger, 1984).

Patient GO is characterized by an inversion, 46,XX,inv(9)(q32q34). The breakpoint of the inversion corresponds precisely with the 9q34 breakpoint in CML. In situ hybridization results suggest that the abl oncogene becomes more proximal to the centromere as a result of this aberration in the inverted homologue of chromosome 9 as seen by the 19% hybridization efficiency (Figure 13b). The minor peak at 9q34 is not statistically significant in the inverted homologue. The normal homologue of chromosome 9 demonstrated a 10% abl hybridization efficiency at 9q34. A predisposition for neoplasia may be associated with oncogene displacement because the breakpoint at 9q34 is highly associated with CML, ALL and AML (Berger et al., 1982a; de la Chapelle and Berger, 1984). Furthermore, members of the family carrying an inversion in chromosome 9, ins(9)(q22.1q34.3q34.1), have been found to have high frequencies of various malignancies including CML

(P. Allderdice, Personal Communication).

Patient WO has an isochromosome for the short arm of chromosome 12, 47,XY,i(12p). This chromosomal region contains the K-ras-2 oncogene. As a result of this isochromosome, WO has partial 12p tetrasomy. In situ hybridization results (Figure 14a) demonstrate a 70% hybridization efficiency to 12p12 that is highly significant ($p < 10^{-6}$). Sixty-one of the 154 grains present on 12p, were located on the isochromosome. Fourty-three of the isochromosome grains were mainly present as double grains on one terminus whereas nine grains were present on both arms of the isochromosome. Similar grains distribution on an isochromosome was documented by Mattei et al., 1985 for the isochromosome, i(18p). A high hybridization efficiency, namely, 71%, was also shown by this investigator. The high hybridization efficiency in WO resulted from double silver grains being located on the isochromosome along with single grains on chromosome 12. Since the relationship between grain formation and gene reiteration number is proportional (Henderson, 1982), it can be used in gene amplification studies (Wolman et al., 1985). Because in situ hybridization resulted in the consistent formation of double grains on the isochromosome, and occasionally, triple grain formation, an increase in the copy number of K-ras-2 in patient WO is suggested. Therefore, the results from in situ hybridization confirm the karyotype in WO as partial 12p tetrasomy. Moreover, this patient has the clinical symptoms associated with Pallister-Killian syndrome resulting from excess 12p chromosomal material. Recently, a molecular investigation of Pallister Killian Syndrome confirmed tetrasomy 12p by quantitative Southern blot hydridizations using a K-ras-2 probe

(Peltomaki et al., 1987).

Predisposition to neoplasia due to amplification of K-ras-2 is questionable in WO. Many tumors are associated with increased oncogene copy number (for review see Bishop, 1985). In contrast, proto-oncogenes such as H-ras-1 undergo up to 4-fold amplification during the life span of normal human fibroblasts (Srivastava et al., 1985).

Patient RH has a duplication of the terminus of chromosome 9, namely, 46,XX,dup(9)(q33q34). The breakpoint in this duplication corresponds with the 9q34 breakpoint in CML. Hybridization efficiencies of 13% and 45% were obtained for the normal and duplicated homologues of chromosome 9, respectively. In situ hybridization with the abl oncogene demonstrated a combined hybridization efficiency of 58% to the terminus of chromosome 9 from band q33 to q34 from both the duplicated and normal homologues of chromosome 9 (Figure 15b). The homologues of chromosome 9 were difficult to distinguish; therefore, the presence of double hybridization grains was used as an indicator of the duplicated homologue of chromosome 9. The hybridization efficiency was very high (58%) as seen in patient WO. These results suggest that the abl oncogene is amplified as a result of the chromosomal duplication of 9q33;9q34. Since the grains on the duplicated homologue of chromosome 9 are mainly of unequal size, one complete and one incomplete form of the abl oncogene may be present, resulting in one strong signal and another weak signal. The in situ hybridization results and the cytogenetic anomaly confirm that RH has partial 9q trisomy for the segment 9q33;9q34. This extra chromosomal region in RH is also implicated in the symptoms associated with 9q34 syndrome.

The issue of whether or not amplification of abl predisposes to malignancy is questionable. The literature on 9q34 syndrome does not suggest a predisposition factor. However, the relationship between a duplication syndrome and a tendency for neoplasia has been demonstrated in Beckwith Wiedemann syndrome (Sotelo-Avila et al., 1980). These patients have a duplication of 11p15 which exactly corresponds to the H-ras oncogene. The association between Beckwith-Wiedemann syndrome and neoplasia is well known (Sotelo-Avila and Gooch, 1976). For instance, predisposition to intra-abdominal malignancy (Sotelo-Avila and Gooch, 1976) and Wilms tumor (Turleau et al., 1984) is common in this syndrome.

The non-leukemic individuals in this investigation are characterized by translocations or inversions that displace an oncogene or by duplications or isochromosomes that amplify oncogenes. A greater number of patients must be studied over a longer period of time in order to associate particular mutations with a predisposition for neoplasia.

4.3.2 Leukemic Individuals

The eleven leukemia patients used in this investigation do not have a standard Ph' translocation. These Ph'-negative patients can be divided into two groups based on oncogene mobility.

The first group includes patient JA with CML and patient MC with ALL. These patients are not characterized by mobility of the oncogenes abl or sis (Figure 26). Similar molecular results have been documented in one CML patient (Bartram, 1985a). Oncogene mobility has never been demonstrated in ALL although 20% of patients have a Ph' chromosome (Hossfeld, 1987).

The second group is characterized by the genomic movement of the

abl oncogene from chromosome 9 to chromosome 22 by a mechanism other than chromosomal translocation in a 400-band karyotype (Figures 16a, 17a, 18a, 19a and 20a). Five of the nine patients in this group have been diagnosed with CML. These are patients NI, BJ, WR, FRA and NIS. Their karyotypes are normal. The movement of abl was not accompanied by the movement of sis or bcr as in Philadelphia-positive CML (Groffen et al., 1983a; de Klein et al., 1982). This pattern of abl mobility to chromosome 22 in the absence of sis mobility was confirmed by the presence of two hybridization grains on chromosome 22 in patient NIS after simultaneous hybridization with abl and sis. Furthermore, the hybridization of abl to chromosome 22 occurs without significant hybridization to chromosome 9 (Figures 16a, 17a, 18a, 19a and 20a). The hybridization distributions of the abl oncogene seem to indicate homozygosity for the homologue of chromosome 9 in which abl translocation to chromosome 22 has occurred. This may have resulted from loss of the homologue of chromosome 9 with the abl gene and duplication of the remaining homologue. In order to establish homozygosity of chromosome 9, a number of polymorphic DNA markers for this chromosome can be used. Furthermore, abl dosage in normal and cancerous tissue can be investigated with quantitative Southern blot analysis. The hypothesis for chromosome allele loss in cancerous tissue has been substantiated in the case of retinoblastoma, Wilms tumor, acoustic neuroma and in colorectal carcinoma (Solomon et al., 1987).

The displacement of abl into a normal chromosome 22 without chromosomal translocation was previously demonstrated by Morris et al., 1986. Five CML patients undergoing treatment were used by this research

group. They found that two of five CML patients with a normal karyotype revealed mobility of the abl oncogene in the absence of either sis or bcr movement. Furthermore, new restriction fragments in the DNA from these two patients suggested the presence of a break in the bcr gene. Such a break separates the 5' and 3' portions of bcr and could allow insertion of abl adjacent to the 5' portion of bcr. The absence of sis mobility in a single CML patient with a normal karyotype was also demonstrated by Bartram et al. (1984). Rearrangement of bcr in a CML patient with normal chromosomes was also previously reported by Ganesan et al. (1986). In contrast to these reports, Bartram (1985a) demonstrated bcr rearrangement and the subsequent transcription of a chimeric 8.5 kb bcr/abl RNA species without translocation of abl. The demonstration of heterogeneity among Ph'-negative CML patients detected by abl, sis and bcr sequence may contribute to a novel subclassification of this poorly defined group of leukemias. Indeed, the five CML patients in this investigation along with the patients reported by Morris et al. (1986), Bartram et al. (1984) and Ganesan et al. (1986) indicate that a subtype of Ph'-negative CML demonstrates abl mobility previously attributed only to Ph'-positive CML.

Genomic rearrangement of the abl oncogene was also demonstrated in three non-CML patients. The first patient is LAD. This patient was diagnosed with CGL. LAD has an abnormal karyotype, 46,XY,9q-,11q-,14q+,22q+. In this patient, abl was shown to be present on chromosome 14 (Figure 22a). Since the clinical course of Ph'-negative CGL and Ph'-positive CML is similar (Shaw, 1982), movement of abl could be an analogous molecular event. The second patient is

MUR. This patient has been diagnosed with ALL. MUR has a normal karyotype although abl is shown to be located on chromosome 22 (Figure 23a). At the molecular level, a similar bcr-abl chimeric RNA species is translated into an 190K aberrant protein. Recent molecular data indicate that in Ph'-positive ALL, abl is translocated into the 5' region of the bcr gene (Fainstein et al., 1987). The mobility data in this investigation suggest that this phenomena is also present in Ph'-negative ALL. The final patient is HA with aplastic anemia. This patient's karyotype is abnormal, 46,XY,-7,+G. The abl oncogene was shown to move to chromosome 22 (Figure 23a) while the sis oncogene moved to chromosome 9 (Figure 24a). Since aplastic anemia may represent a pre-leukemic state, oncogene mobility may be required for progression of the neoplastic process. Rearrangement of abl by in situ hybridization to chromosome 22 for CGL, ALL and aplastic anemia has not been previously documented. The presence of abl hybridization to only chromosome 22 rather than to both 9 and 22 is also a feature of the CGL and ALL patient. A mechanism similar to that proposed in CML resulting in homozygosity of the homologue of chromosome 9 without the abl gene may be operating in these patients.

The final patient that showed abl rearrangement is CML patient LA. This patient seems to have a standard Ph' translocation, t(9;22), at the microscopic level. In situ hybridization revealed that abl was present on the normal homologue of chromosome 22 rather than the abnormal homologue, namely, the Ph' chromosome (Figure 21a). These results suggest that the karyotype is not a standard Ph' translocation but a variant Ph' translocation involving 9,22 and another chromosome.

In the literature, some patients with variant Ph' translocations have shown molecular results similar to LA. For example, a patient with the translocation, t(9;12) showed mobility of the abl oncogene (Bartram et al., 1985), whereas a patient with translocation, t(9;11;22), showed no mobility of the sis oncogene. In contrast, patients with karyotypes, t(8;9;22) and t(12;9;22), revealed mobility of bcr onto chromosome 8 and 12, respectively, along with bcr rearrangement. Rearrangement of bcr was also reported by Ganesan et al. (1986) and Ohyashiki et al. (1987) in patients with the translocations, t(4;9;22) and t(5;22;9), respectively. Similar bcr rearrangement is used in patients with a standard Ph' translocation in order to diagnose CML with an efficiency of almost 100% (Benn et al., 1987). Mobility of the abl oncogene, as described in this investigation, along with bcr rearrangement, may serve to classify certain variant Ph' translocations as a subtype of Ph'-positive CML at the molecular level.

In summary, mobility of abl in Ph'-negative CML is apparent in five patients, namely, NI, BJ, WR, FRA, NIS and in one CML patient with a variant Ph' translocation, LA. This phenomena was also present in a Ph'-negative CGL patient, LAD. The clinical course of Ph'-negative CGL and Ph'-positive CML is very similar (Shaw, 1982). For this reason, Ph'-negative CGL is currently thought to be a "nonentity" in the literature (Travis et al., 1986). Therefore, if either the abl or sis oncogene is involved in the development of CML, abl appears to be the more significant one because of its insertion into chromosome 22 in these patients. The significance of the abl oncogene in the ALL patient, MUR, and aplastic anemia patient, HA, cannot be implied from

the data. A greater number of ALL and preleukemia patients is required.

The greater significance of the role of abl in comparison to sis has been suggested in the literature. This hypothesis is evident from reports involving interferon therapy in CML. Reduced abl transcriptional expression after treatment with interferon may lead to regression of CML while additional changes in the abl oncogene may lead to transformation, clonal evolution, and termination in blast crisis (Brodsky et al., 1987). Also, the maintenance of leukocytosis in CML patients requires abl transcriptional expression in some or all blood cells (Brodsky et al., 1987). The abl oncogene shows excessive transcriptional expression in the chronic phase of CML (Brodsky et al., 1987). Reports of higher abl oncogene expression during the chronic phase appear to be associated with purification of mononuclear cells (Gillespie et al., 1984). These reports are consistent with the idea that only some blood cells possess elevated levels of abl mRNA during the chronic phase and these cells increase in number in CML (Brodsky et al., 1987). In contrast, Gale and Canaani (1984) were unable to demonstrate sis transcription in leukemic cells from CML patients. Transcripts of sis have not been detected in many other malignancies of the hematopoietic system (Westin et al., 1982). It has been generally accepted that sis oncogene is not important in the pathophysiology of CML (Gale and Canaani, 1984; Bartram et al., 1984).

Mobility of the abl oncogene in leukemia can lead to a unique subclassification system based on morphological, clinical and molecular characteristics. The heterogeneous array of Ph'-negative patients may

be subdivided by *in situ* hybridization and other molecular investigations. For example, Ph'-negative patients with *abl* mobility in the absence of *bcr* or *sis* mobility have been shown to demonstrate the morphological and clinical features of Ph'-positive CML (Morris et al., 1986). Indeed, *in situ* hybridization studies may serve to identify some cases of Ph'-negative CML with a clinical course similar to Ph'-positive CML. Cytogeneticists have described the clonal evolution in some cases of Ph'-negative CML to be identical to that of Ph'-positive CML (Mintz et al., 1979). Moreover, oncologists have demonstrated a bimodal distribution in the survival of Ph'-negative CML patients showing that the average, median survival time of a subgroup of Ph'-negative CML is the same as Ph'-positive CML (Ezdinli et al., 1970). The mobility of *abl* in Ph'-negative CML may serve as an indicator of both prognosis and diagnosis. It may even prove possible to diagnose Ph'-positive CML before the actual development of the Ph'-chromosome.

5.0 SUMMARY

The cytogenetic and molecular studies on chromosomes from untreated patients with Ph'-negative leukemia, including CML, revealed the following:

Chromosomal Rearrangements

1. Ninety-eight of the 108 breakpoints either "exactly" map or "closely" map to oncogenes in the 72 leukemia patients. Similarly, 99 of the 108 breakpoints either "exactly" map or "closely" map to fragile sites (Table 1 and Figure 2).

2. All variant Ph' translocations are characterized by the 9q34 breakpoint.

3. Exact chromosomal band concordance between the breakpoint, 9q34, and the oncogene, abl, occurred most frequently in these patients.

4. Band 9q34 may be a crucial chromosomal region implicated in the etiology of CMLs including cases without a Ph' chromosome.

Fragile Sites

1. Aphidicolin-induced common fragile sites occur with a statistically increased frequency ($p < 10^{-6}$) in the normal cells of eight leukemia patients as compared to the normal cells of non-leukemia patients (Table 3).

2. The expression of fragile sites in the normal cells of one patient did not correspond with the breakpoints of the translocation in the cancer cells (Table 2).

3. The highly expressed fragile sites in all patients clustered to four main chromosomes, namely, 1, 2, 3 and 5 (Figure 6).

4. The map location of highly expressed fragile sites corresponds to the map location of cancer-specific breakpoints and oncogenes reported in the literature.

5. Four patients, NI, BJ, WR and NIS, demonstrated both increased expression of fragile sites and submicroscopic abl mobility. In contrast, one patient (MC) demonstrated increased expression of fragile sites without abl mobility (Table 3 and 5).

6. Since fragile sites are found in normal cells, they are constitutional and not acquired by the cancer cells. They may predispose patients to microscopic and submicroscopic chromosomal rearrangements.

Oncogene Mobility

Non-Leukemic Individuals

1. Patient RUS demonstrates mobility of abl (Figure 11b) as a result of the chromosomal translocation, t(9;11)(q22;p15).

2. Patient RUM demonstrates K-ras-2 mobility (Figure 12a) resulting from the chromosomal translocation, -14,+t(12;14)(p11;p11).

3. Patient GO demonstrates mobility of abl (Figure 13b) as a result of the inversion, inv(9)(q32q34).

4. Patients WO and RH demonstrate amplification of K-ras-2 and abl, respectively, as a result of chromosomal duplication (Figure 14a and 15b).

5. The issue of whether or not these patients may be at increased risk for neoplasia is questionable.

Leukemic Individuals

1. Mobility of the oncogenes abl and sis was not apparent in one patient with Ph'-negative CML (JA) and one patient with Ph'-negative ALL (MC) (Figure 26).

2. Mobility of abl to chromosome 22 (Figure 24a) and mobility of sis to chromosome 9 (Figure 25a) was apparent in one Philadelphia-negative aplastic anemia patient (HA). Mobility of abl and sis in aplastic anemia has not been previously documented in the literature.

3. Mobility of abl to chromosome 22 was demonstrated in one patient with Ph'-negative CGL (LAD) and in one patient with Ph'-negative ALL (MUR) (Figures 22a and 23a). Similar abl mobility data has not been previously reported in the literature for CGL and ALL.

4. Mobility of abl to chromosome 22 was demonstrated in five Ph'-negative CML patients (NI, BJ, WR, NIS, FRA) and in one CML patient with a variant Ph' translocation (LA) (Figures 16a to 21a). Similar oncogene mobility data has only been demonstrated in leukemia patients undergoing treatment (Morris et al., 1986; Bartram et al., 1984; Ganesan et al., 1986).

5. Confirmation of the mobility of abl to chromosome 22 in the absence of sis mobility was demonstrated through the consistent formation of both abl and sis hybridization grains on chromosome 22 in patient NIS (Figure 20a).

6.0 CONCLUSION

In situ hybridization studies have provided evidence for mobility of the *abl* oncogene in eight cases of Ph'-negative leukemia, including five cases of CML. This approach may serve to identify some cases of Ph'-negative CML as Ph'-positive CML based on the mobility of the *abl* oncogene. In this way, mobility of this gene in Ph'-negative CML may serve as a prognostic indicator. Future molecular studies into the genetic events at 9q34 should prove very exciting.

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8.0 APPENDIX

The FAB Classification of ALL

L1: A relatively homogeneous cell population with 75% or more small cells with scanty cytoplasm, finely dispersed chromatin, and regular nuclear shape. Nucleoli are inconspicuous in more than 75% of the cells.

L2: A heterogeneous cell population as regards size, chromatin pattern, and nuclear shape. The cells are usually large with the cytoplasm occupying 20% or more of the surface area of the cell. Nucleoli are frequently large in 25% or more of the cells.

L3: A large and relatively homogeneous cell population with regular nuclei and a fine chromatin pattern. Nucleoli are prominent. Cytoplasm is moderately abundant with vacuolization and deep basophilia. Lymphoblasts resemble those seen in Burkitt's lymphoma.

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The FAB Classification of AML

M1: Myeloblastic leukemia without maturation. Blasts show minimal evidence of myeloid differentiation with more than 3% of the blasts myeloperoxidase-positive and/or containing azurophilic granules, Auer rods, or both. No evidence of maturation is present.

M2: Myeloblastic leukemia with maturation. Some maturation of the granulocytic series is evident with more than 50% of the nucleated bone marrow cells consisting of myeloblasts and promyelocytes. In some cases, maturation may proceed beyond the promyelocyte stage, frequently

with abnormal morphologic forms.

M3: Hypergranular promyelocytic leukemia. The predominant cells are abnormal promyelocytes packed with dense granulation and multiple Auer rods.

M3 variant: "Microgranular" promyelocytic leukemia. An atypical form with minimal granulation in most cells. Nuclei of cells in blood are bilobed, multilobed, or reinform. Occasional typical cells are present.

M4: Myelomonocytic leukemia. Evidence of both granulocytic and monocytic differentiation is present in varying proportions. More than 20% of the nucleated cells in the blood and/or bone marrow are promonocytes and monocytes and at least 20% of the nucleated marrow cells are myeloblasts and promyelocytes.

M5: Monocytic leukemia

$M5_A$: Poorly differentiated (monoblastic) leukemia. Large monoblasts with abundant cytoplasm frequently exhibiting pseudopodia or budding. Promonocytes may be present but are uncommon.

$M5_B$: Well-differentiated monocytic leukemia. Monoblasts, promonocytes, and monocytes are all found. The predominant cell in the bone marrow is the promonocyte.

M6: Erythroleukemia. Erythroblasts exceed 50% in the bone marrow, and bizarre morphologic variants are found. Many erythroid precursors are strongly periodic acid-Schiff positive. Auer rods may be seen in the myeloblasts.

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Cytochemical Profiles in AML

Cytochemical Reaction	Morphologic Subtypes According to FAB Criteria					
	M1	M2	M3	M4	M5	M6
Peroxidase or Sudan black	+(≥3%)	+ to +++	+++	++ to +++	0 to ++	+ to +++
Naphthol ASD acetate esterase	+	++	++	+ to +++	+++	++
Napththol ASD acetate esterase-fluoride	+	++	++	+ to ++	0 to ±	++
Periodic acid-Schiff	0 to ±	0 to +	0 to +	0 to ++	0 to ++	± to +++*

Legend: 0 = negative
± = equivocal
\+ = slight positivity
++ = moderate positivity
+++ = strong positivity

*Periodic acid-Schiff positivity may be found in the cancerous erythroid precursors.

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Cytogenetic Abberations in AML Subgroups

FAB Subroup(s)	Chromosome Defect(s)
AML-M2	t(6;9), t(8;21)
AML-M1, AML-M3	t(9;22), t(15;17)
AML-M1	t(8;21), t(6;9)
AML-M2, AML-M4	inv(16)
AML-M2, AML-M4, AML-M5	t(9;11), del(5q)
AML-M2, AML-M4, AML-M5, AML-M6	del(5q), del(7q), +8

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