Long-Range Mapping of the Philadelphia Chromosome by Pulsed-Field Gel Electrophoresis

By Carol A. Westbrook, Charles M. Rubin, John J. Carrino, Michelle M. Le Beau, André Bernards, and Janet D. Rowley

The Philadelphia chromosome (Ph1) of chronic myelogenous leukemia (CML) contains sequences from chromosome 9, including the ABL protooncogene, that have been translocated to the breakpoint cluster region (bcr) of chromosome 22, giving rise to a bcr-ABL fusion gene, whose product has been implicated in the genesis of CML. Although chromosome 22 translocation breakpoints in CML virtually always occur within the 5.8-kilobase (kb) bcr, chromosome 9 breakpoints have been identified within the known limits of ABL in only a few instances. For a better understanding of the variability of the breakpoints on chromosome 9, we studied the CML cell line BV173. Using pulsed-field gel electrophoresis (PFGE), large-scale maps of the t(9;22) junctions were constructed. The chromosome 9 breakpoint was shown to have occurred within an ABL intron, 160 kb upstream of the v-abl homologous sequences, but still 36 kb downstream of the 5'-most ABL exon. bcr-ABL and ABL-bcr fusion genes were demonstrated on the Ph1 and the 9q+ chromosomes, respectively; both of these genes are expressed. These results suggest that the 9:22 translocation breakpoints in CML consistently occur within the limits of the large ABL gene. RNA splicing, sometimes of very large regions, appears to compensate for the variability in breakpoint location. These studies show that PFGE is a powerful new tool for the analysis of chromosomal translocations in human malignancies.

MATERIALS AND METHODS

Cell lines. BV173 was established from the malignant cells of a patient with CML in blast crisis.7 Other lymphoid cell lines used as controls are Molt 16,11 RCH-ACV,12 and SUP-B13.13

Cytogenetic analysis. Cytogenetic analysis with a trypsin-Giemsa banding technique was done on metaphase cells of the BV173 cell line in logarithmic phase growth. Chromosome abnormalities are described according to the International System for Human Cytogenetic Nomenclature.

From the Joint Section of Hematology/Oncology, Department of Medicine, The University of Chicago; and The Whitehead Institute for Biomedical Research, Cambridge, MA.

Submitted July 21, 1987; accepted October 27, 1987.

Supported in part by US Department of Energy contract DE-FGO2-86ER60408, US Public Health Service Grant No. CA-16910 (J.D.R., C.A.W.), American Cancer Society-Illinois Division Grant No. 86-44, the Louis Block Foundation, and the Otho S.A. Sprague Memorial Institute (C.A.W.). C.A.W. is the Stratton-Jaffé Fellow of the American Society of Hematology, C.M.R. and J.J.C. are supported by US Public Health Service Grants Nos. GM-07190 and CA-09273, respectively. M.M.L. is a Special Fellow of the Leukemia Society of America, and A.B. is supported by the European Molecular Biology Organization.

Address reprint requests to Carol A. Westbrook, PhD, University of Chicago, Department of Medicine, Box 420, 5841 S Maryland Ave, Chicago, IL 60637.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1988 by Grune & Stratton, Inc.
Southern hybridization and DNA probes. Southern blots were prepared according to standard methods and hybridized under high-stringency conditions. DNA probes were labeled with 32P by the random-primer method of Feinberg and Vogelstein. The following ABL probes were used for these studies: pHab15, a 0.85-kb BamHI/HindIII fragment containing sequences of exon 1b; T39-1-2, a 1.0-kb EcoRI fragment containing chromosome 9 sequences immediately 3' to the translocation breakpoint in the CML cell line K562; T28-2-2, a 0.6-kb BamHI fragment from an intron in the v-abl homologous region; and ABL-SS, a 0.8-kb SstI fragment cloned from a t(9;22) breakpoint in a patient with CML. The bcr exon 4 probe is a 1.3-kb HindIII/EcoRI genomic probe containing sequences homologous to this exon. The 3'-ber probe is a 1.2-kb HindIII/BglII fragment of bcr (Oncogene Science, Mineola, NY), whereas the 5'-ber probe is a 0.6-kb HindIII/EcoRI genomic fragment that lies 2 kb upstream of the 3' probe. The probe for the ABL common exons is a 0.65-kb MspI fragment prepared from an ABL cDNA clone. The location of the genomic probes is shown in Fig 1A and B.

PFGE. DNA of very high mol wt was prepared and digested in agarose plugs as previously described. The plugs were placed in 1% agarose gel, alongside mol wt markers of bacteriophage λ multimers and intact yeast chromosomes. Orthogonal-field electrophoresis was carried out according to Carle and Olson's modification of the system of Schwartz and Cantor, as previously described. Inversion field electrophoresis was performed as described by Carle et al., in a 1% agarose gel (dimensions 10 × 20 × 0.5 cm), and run for 24 hours at 12°C using a 200-V inverting field. The switching cycle changed linearly on a progressively increasing ramp, such that the forward migration intervals were nine and 60 seconds at the start and end of the electrophoresis, respectively. The reverse intervals were one-third of the forward intervals throughout the electrophoresis. After orthogonal- or inversion-field electrophoresis, the gel was treated with 0.25 mol/L HCl for 20 minutes, 4 mol/L NaOH/6 mol/L NaCl for 90 minutes and 1.5 mol/L NaCl/5 mol/L Tris HCl for 60 minutes. The DNA was then transferred to GeneScreen Plus membrane (NEN Bioproducts, Boston) according to the manufacturer's recommendations. Hybridization and autoradiography was performed by previously described methods. The filters were washed and sequentially rehybridized according to the manufacturer's recommendations.

Northern analysis. Poly-A-selected RNA was prepared from 1.5 × 106 cells of each cell line studied. Cells were lysed in 10 mmol/L Tris, pH 7.4, 1.5 mmol/L MgCl2, 10 mmol/L NaCl, 1% sodium dodecyl sulfate (SDS), 5% betamercaptoethanol, 350 μg/μl Proteinase K, and 10 mmol/L Vanadyl ribonucleoside complex (BRL, Gaithersburg, MD). Lysates were adjusted to 0.5 mol/L LiCl, and polyadenylated RNA was selected by chromatography on oligo(dT)25 cellulose. The RNA was denatured with formaldehyde, electrophoresed through 1% agarose, and transferred to nitrocellulose. Hybridization conditions were the same as those used for the Southern blots.

All recombinant DNA research was conducted according to the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules.

RESULTS

Cytogenetic analysis. Cytogenetic studies at the time of our molecular analysis demonstrated the typical 9q+ chromosome and three copies of the Ph1. No normal homologue of chromosome 9 was present; instead, there were two copies of a derivative of chromosome 9, which resulted from a translocation between the long arms of chromosomes 8 and 9, and involved bands q11 and q13, respectively. Because the reciprocal product of the t(8;9) was not present, the effect of this translocation was a loss of chromosome 9 material distal to band q13, including the site of the normal ABL locus at band q34. The complete karyotype of BV173 was 48, X, -Y, -9, der(1)(pter→q42::1q32→q42::1q32→qter), del(6)(q13q21), i(8q), inv(12) (p13q13), +der(9)(t8;9)(q11q13), +der(9)(t9;9)(q11q13), t(12;22)(q34;q11), +der(22)(t9;22)(q34;q11), +der(22)(t9;22)(q34;q11), +der(22)(t9;22)(q34;q11), +der(22)(t9;22)(q34;q11).

Identification of the translocation breakpoint within bcr. Hybridization of the 3'-ber probe to BglII and BamHI digests revealed germline (5.0 and 3.3 kb, respectively) and rearranged (9.5 and 6.6 kb, respectively) bands in each, whereas only a germline band was identified in a HindIII digest. Based on a published restriction map of bcr, these data indicated that chromosome 22 breakpoint in this cell line was -1.0 kb 5' of the sequences recognized by the probe (Fig 1B). Because sequences distal (3') to the breakpoint are translocated to chromosome 9, restriction fragments that hybridize to the 3'-ber probe must originate from either the 9q+ or the normal chromosome 22.

Analysis of the 9q+ chromosome. Probes from the 5' and 3' sides of the breakpoint within bcr (Fig 1B) were used to identify restriction fragments originating from the Ph1 and the 9q+ chromosomes, respectively. DNA from BV173 was digested with the restriction enzymes NotI and SfiI, and fractioned by orthogonal-field alternation gel electrophoresis. Hybridization of NotI digests to the 3'-ber probe revealed a 350-kb rearranged band that also hybridized to the pHab15 and T39-1-2 probes (Fig 2A), which are derived from the extreme 5' end of ABL. Similarly, in SfiI digests, a rearranged band of -35 kb hybridized to both 3'-ber and T39-1-2 probes (Fig 2B). Therefore, these rearranged bands contain the translocation junction of the 9q+ chromosome. As expected, neither of these rearranged bands hybridized to probes from within the ABL common exons (not shown), which had been translocated to the Ph1. The location of the chromosome 9 breakpoint was determined by reference to a restriction map of bcr that includes SfiI sites. Because SfiI sites occur 3 kb 3' and 3 kb 5' of the bcr breakpoint, the translocation breakpoint on chromosome 9 in BV173 lies just -35 kb downstream of exon 1b, or 160 kb upstream of exon II, within the ABL first intron. Thus, exon 1b is separated from all other known coding regions of ABL (Fig 1C).

Analysis of the 22q- chromosome. The Ph1 (22q-) chromosome was mapped by hybridization of NotI digests of BV173 DNA to probes from the 3' end of ABL, the 3' and 5' sides of bcr, and IGLC. The 3' ABL probe (T28-2-2) recognizes sequences distal to the translocation breakpoint on chromosome 9, and the 5'-ber probe recognizes sequences proximal to the translocation breakpoint on chromosome 22; therefore, each is expected to hybridize to DNA from the 22q-chromosome. The Hu-λ-C2 also was used for this study because IGLC lies proximal to bcr on the q-arm of chromosome 22, (Fig 1B) and invariably remains on the Ph1 chromosome in CML. Field-inversion gel electrophoresis was used to obtain better resolution of the extremely high mol wt bands produced by NotI. As shown in Fig 3, the
Hu-λ-C2 probe hybridized to two NotI bands of high mol wt in the lane containing BV173 DNA, but to only a single band within the same size range in DNA from a cell line that did not have a t(9;22). The variation in mobility of the germline bands which hybridize to Hu-λ-C2 is an artifact of inversion-field electrophoresis, and is due to variations in the amount of DNA in a lane; the germline band in BV173 appears to be the same size as that seen in at least six other cell lines when examined by other gel systems (C.A. Westbrook and C.M. Rubin, unpublished observations). These bands are at ~1,200 and 1,600 kb, as determined by the relative migration of yeast chromosomes of known size.21 Both the 3'-bcr and the 5'-bcr probes, but not the ABL probe T28-2-2, hybridized to the single band in the control and to the larger of the BV173 bands, identifying these as germline bands. The faster migrating band in BV173, however, hybridized to Hu-λ-C2, 5'-bcr, and T28-2-2, but not to 3'-bcr, demonstrat-
ing physical linkage of IGLC to the bcr-ABL fusion gene on the Ph\(^1\) chromosome. In SfiI digests, a rearranged band of ~200 kb was seen with T28-2-2 and the 5′-bcr probes (not shown), thereby defining the breakpoint location as being ~150 kb upstream of exon Ia (Fig 1D), consistent with the location shown in Fig 1C.

Fig 3. PFGE analysis of the 22q− (Ph\(^1\)) chromosome in BV173. NotI-digested DNA from the lymphoid cell line RCH-ACV (lanes 1), and BV173 (lanes 2), fractionated by field-inversion gel electrophoresis and sequentially hybridized to IGLC, bcr, and ABL probes. Panels are labeled according to specific probe used. Mol wts of the hybridizing fragments, estimated from the relative migration distances of bacteriophage λ multimers and intact yeast chromosomes, are given in kilobases to the left of each autoradiogram. Arrowheads indicate positions of two yeast chromosomes of known molecular size. Germline and rearranged bands are indicated with lines or arrows, respectively.

Unexpectedly, there was no cleavage of the NotI site in exon Ia (Fig 4). A 12.1-kb KpnI fragment containing this site was of normal size, indicating the absence of a detectable deletion in this region. Double digestion with KpnI and NotI further confirmed that the NotI site was not cleavable in BV173 DNA. A polymorphism of this site has not been seen in cell lines with normal chromosomes. Loss of this restriction site may be due to methylation, since the NotI recognition sequence (GCGGCCGC) has two CpG dinucleotides that are potential methylation targets. We observed that this NotI site also does not cleave in the CML cell line K562\(^2\) and in cells of a patient with CML (C.A. Westbrook, and C.M. Rubin, unpublished observations). It is tempting to speculate that methylation of this particular NotI site may be a consequence of its translocation to the Ph\(^1\). Moreover, methylation of CpGs within the promoter regions of some genes has been associated with decreased gene expression\(^26,27\); therefore, it may influence transcription of the 6.0-kb message in BV173, which begins at exon Ia.

**Fig 4.** Loss of NotI cleavage within ABL exon Ia in BV173. (A) Lanes 1 and 4 contain DNA from the BV173 cell line, and lanes 2 and 3 contain DNA from the MolT-16 cell line. DNA was digested with Kpnl only (lanes 1 and 2) or with Kpnl followed by NotI (lanes 3 and 4). The digests were fractionated, transferred, and hybridized to the ABL-SS probe. This probe recognizes 12.1-kb and 9.0-kb Kpnl restriction fragments; the larger fragment is cleaved by NotI to produce a smaller fragment of ~9.0 kb. (B) The ABL genome in the vicinity of the ABL-SS probe. The restriction sites are: B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; K, KpnI; N, NotI; X, XbaI. Location of the ABL-SS probe is indicated by hatched box.

**Transcription of ABL in BV173.** The effects of the t(9;22) on ABL transcription were investigated by Northern analysis. Interpretation of the results was facilitated by loss of the normal ABL allele in BV173, which had been suggested by cytogenetic studies and was confirmed by the absence of germline ABL fragments on PFGE analysis (Figs 2 and 3). Thus, all ABL transcription in BV173 occurs from either the 9q+ or the Ph\(^1\) chromosome. Transcription of a complete 7.0-kb message containing exon Ib is impossible since the normal ABL locus is absent and since exon Ib cannot be spliced to the common exons, which have been translocated. On the other hand, the type Ia transcriptional unit is intact on the Ph\(^1\); therefore, complete 6.0-kb type Ia mRNA can be produced if a functional promoter is present.

Northern blots were prepared from poly-A–selected RNA of BV173 cells and from the lymphoid cell line RCH-ACV used as a control. Hybridization to the ABL cDNA probe containing portions of the ABL common exons revealed the expected 6.0-kb type Ia and 7.0-kb type Ib ABL messages in the control cell line. In BV173, the 6.0-kb type Ia transcript and the characteristic 8.5-kb bcr-ABL transcript were seen (Fig 5). Although the amount of the 6.0-kb transcript in BV173 cells appears to be roughly comparable to that seen in the control cell line, it is much less abundant than the larger, 8.5-kb message that originates upstream. Our previously reported immunoprecipitation analysis of BV173\(^19\) indicated that these cells produced an ABL p145 protein of normal size and abundance, which presumably originates from this 6.0-kb mRNA.

Rehybridization of the Northern blot to the exon Ib probe pHab15′ confirmed that the normal 7.0-kb ABL message was
common exons. Thus, each Philadelphia translocation has clearly still within the PH'MAPPING chromosome. The genomic structure of the t(9;22) in BVI 73 determine whether the 5.2-kb message also hybridized to the definitively until the ABL message, even if the breakpoint is distant from the consistent target CML. with is probably typical of that seen seen with the t(9;22). That is because ~8% of patients with CML have complex variant Ph' translocations, in which the chromosome 9 derivative contains sequences from a chromosome other than 22 and therefore cannot contain a 5'ABL-3' bcr fusion gene such as that found in BV173.

In this study, we showed that PFGE is useful for analysis of human chromosomal translocations. Rearrangements within the 230-kb ABL gene were easily demonstrated with this method, whereas conventional Southern blot analysis of a region of this size would have been virtually impossible. PFGE will undoubtedly prove useful for the analysis of other neoplasms that are characterized by specific chromosomal rearrangements, including those postulated to involve the ABL gene, such as Ph'-positive acute lymphoblastic leukemia and rare cases of Ph'-negative CML.

ACKNOWLEDGMENT

We thank David Baltimore and Manuel O. Diaz for invaluable scientific criticism, John Groffen for the probes T28-2-2 and T39-1-2, David Leibowitz for the bcr, exon 4, S' bcr, and ABL-SS probes, Owen N. Witte for the ABL cDNA probes, and Philip Leder for the Hu-A-C probe. We also thank Mei-Lu Bian for assisting in karyotyping BV173, and Timothy A. Geiger and Michelle M. Steele for expert technical assistance.

REFERENCES

11. McKeithan TW, Shima EA, Le Beau MM, Minowada J, Rowley JD, Diaz MO: Molecular cloning of the breakpoint junctions of a human chromosomal 8;14 translocation involving the T-cell receptor-α chain gene and sequences on the 3' side of MYC. Proc Natl Acad Sci USA 83:6636, 1986
25. Emanuel BS, Selden J, Wang E, Nowell PC, Croce CM: In situ hybridization and translocation breakpoint mapping I: Non-identical 22q11 breakpoint for the t(9;22) of CML and the t(8;22) of Burkitt's lymphoma. Cyogenet Cell Genet 38:127, 1984
29. Clark SS, McLaughlin J, Crist WM, Champlin R, Witte ON: Unique forms of the abl tyrosine kinase distinguish Ph'-positive CML from Ph'-positive ALL. Science 235:85, 1987
Long-range mapping of the Philadelphia chromosome by pulsed-field gel electrophoresis

CA Westbrook, CM Rubin, JJ Carrino, MM Le Beau, A Bernards and JD Rowley