Philadelphia-Positive Chronic Myeloid Leukemia With a Chromosome 22 Breakpoint Outside the Breakpoint Cluster Region

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In chronic myelogenous leukemia (CML) the reciprocal translocation resulting in the Philadelphia chromosome (Ph') leads to the formation of a chimeric transcriptional unit carrying both c-abl and bcr genetic information whose transcript is a new fused mRNA of 8.5 kilobases (kb) and whose translational product is a 210-kD phosphoprotein with tyrosine kinase activity implicated in the pathogenesis of CML. Twenty patients affected by Ph'-positive CML were studied by Southern blot analysis with bcr. Unexpectedly, in three Ph'-positive patients, the breakpoint of chromosome 22 was located neither inside the bcr region nor 5' to it. Northern blot analysis of the RNAs of two of these patients showed the absence of a detectable 8.5-kb chimeric mRNA. In the third patient a chimeric mRNA was detected by a c-abl cDNA probe but failed to hybridize with a bcr cDNA probe and showed very low hybridization levels with further 5' bcr cDNA probes. The possibility is raised that in CML a breakpoint outside bcr might either still allow the formation of a chimeric mRNA, possibly through alternative splicing mechanisms, or might prevent the transcription of the chimera. In the latter case different molecular events resulting in the formation of a Ph' chromosome may underlie the same myeloid neoplastic phenotype.

PATIENTS AND METHODS

Patients. Twenty adult patients affected by typical Ph'-positive CML were studied. In all cases leukapheresis procedures as well as bone marrow aspirations were performed after informed and explicit consent of the patient was obtained, as required by our institutional guidelines. Ten patients were in chronic phase, three were in accelerated phase, and seven were in blast crisis (BC). Leukemic cells were obtained by leukapheresis.

Cytogenetic studies. Cytogenetic studies were performed on bone marrow and/or peripheral blood cells after a 24-hour incubation in tissue culture media without mitogen, and standard trypsin Giemsa chromosome preparations were made. The karyotype was expressed by using the recommendations of the International System for Human Cytogenetic Nomenclature.

Southern and Northern blots. Leukocyte-enriched plasma was diluted in RPMI and centrifuged to obtain cell pellets from which nucleic acids were extracted. DNA was isolated according to the technique of Gross-Bellard et al with minor modifications. Control DNA was extracted from leukocytes of two normal blood donors.

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Ten micrograms of each DNA was digested with the restriction endonucleases BamHI, BglII, BamHI together with BglII, EcoRI and HindIII; run in 0.7% agarose gel electrophoresis; and then transferred to a Gene Screen (NEN) membrane with standard Southern blot technique.10

RNA was extracted by following the technique described by Torelli et al.15 Total RNA was then enriched for polyadenylate-containing RNA by one cycle of purification on oligo-dT-cellulose.29 Ten micrograms of poly (A) + RNA were size fractionated by electrophoresis on 0.8% agarose gel containing 7% formaldehyde and then transferred to nitrocellulose filters as described.29

Preparation of DNA probes. To study the rearrangement of bcr we used a subgenomic probe represented by a HindIII-BglIII genomic restriction fragment of 1.2-kb (Pr-1, Oncogene Sciences, Inc, Mineola, NY) that corresponded to an intron region of the gene located in the middle of the bcr region and two contiguous bcr cDNA fragments of 0.6 kb (6.13-0.6) and 0.3 kb (6.13-0.3) representing a coding portion of the gene 5' to the bcr region. The cDNA fragments used to study the expression of the chimeric mRNA were represented by a 0.5-kb human bcr cDNA restriction fragment (Pr-2, Oncogene Sciences) isolated from cDNA Vl-32 and by bcr cDNA probes (6.13 and K31) corresponding together to a coding segment of about 1.4 kb 5' to the sequences represented by the bcr probe Pr-2. Furthermore, a human b-cf cDNA fragment of 1.7 kb (K38) corresponding to the 3' end of the c-abl cDNA was used.

The probes 6.13, K31, and K38 were developed and generously provided by Dr G. Grosved, Erasmus University, Rotterdam, The Netherlands. A c-sis probe corresponding to a 1.7-kb BamHI subgenomic fragment cloned into pBR 322 was used to examine whether a possible breakpoint had occurred in the subbands q12.3 to q13.1. A Cβ probe corresponding to a 0.8-kb EcoRI subgenomic fragment containing the Cβ light-chain gene was used to study the presence of a possible breakpoint in the subband q11.12.

To define as precisely as possible that some of the cases pertained to the myeloid lineage, a probe for Cα was used that corresponded to a 1.2 EcoRI restriction fragment containing a part of the first and all of the second and the third exon of the Ig heavy-chain gene.24 A probe for T cell receptor (TCR) β chain was further used and corresponded to a 0.77-kb insert containing J and C regions cloned in the PstI site of pBR 322.25

All the probes were nick-translated with α32P deoxyctydine triphosphate (dCTP) to a specific activity of about 2 x 108 cpm/μg DNA.

Hybridization and autoradiography. Southern and Northern blot hybridizations were performed according to procedures already described in detail.28 Washing of the Southern blot filters was carried out in 0.3 x SSC at 65°C. Washing of the Northern blot filters was performed in 0.1 x SSC at 65°C. Autoradiography was performed at 80°C with intensifier screens for two to seven days.

RESULTS

All 20 adult patients studied were affected by CML diagnosed according to French-American-British (FAB) morphological and cytochemical criteria.25 Ten patients were in fact in chronic phase, three in accelerated phase, and seven in BC. Cytogenetic analysis of all patients showed the typical t(9;22) translocations, except in one case in which a simple variant translocation of the type t(22;22) was present. Most patients in BC showed numerical chromosomal abnormalities (hyperdiploidy) and/or Ph1 duplication as expected.18

No c-abl rearrangement was detected in any of the cases studied,29 which did not seem surprising because the chromo-

some 9 breakpoints can occur in a region very far upstream to the first c-abl exons. Concerning chromosome 22 breakpoints, restriction data of 17 of 20 patients together with cytogenetic data have already been discussed in detail elsewhere and are consistent with the results already reported in the literature.47

In three cases rather unexpectedly (T.V., R.G., and Q.S., all in chronic phase), no rearranged bands were detectable after digestion of the DNA with the restriction endonucleases BamHI, BglII, HindIII, EcoRI (Fig 1), and BamHI together with BglII (not shown). In fact, with all the aforementioned restriction enzymes in all three cases, the hybridization with the 1.2-kb HindIII-BglII bcr genomic fragment showed only the normal germ line band as in the control DNA from normal leukocytes; they measured, respectively, 3.3 kb in the BamHI-digested DNAs, 5.0 kb in the BglII-digested DNAs, 4.5 kb in the HindIII-digested DNAs, 23 kb in the EcoRI-digested DNAs (Fig 1), and 2.1 kb in the same DNAs digested with BglII together with BamHI. These experiments were performed twice for all three patients with different blood samples collected within an interval of few months. In all three cases both Ig heavy-chain and TCR β chain genes were in a germ line configuration (data not shown), which confirmed, in addition to the

![Fig 1. Southern blot analysis of the DNAs of the three patients (T.V., R.G., and Q.S.) that were digested with EcoRI, HindIII, BglII, and BamHI and hybridized to the 1.2-kb HindIII-BglII bcr genomic fragment (Pr-1). No rearranged bands are present in any of the cases with all the restriction enzymes. Only the normal band, as in control DNA of normal leukocytes (N), is present with all the enzymes in all three cases.](www.bloodjournal.org)
FAB morphological and cytochemical data, that patients' leukemic cells were of the myeloid lineage.

Our findings suggested that in these three patients the breakpoint was located either 5' or 3' to the \( bcr \), but certainly not within it despite the fact that in all three cases a \( t(9;22) \) translocation was present (Fig 2) in two successive cytogenetic analyses carried out within an interval of several months and despite the typical CML phenotype. It must be added that the peripheral cell populations studied were each case.

Because it was suggested that in \( Ph^1 \)-positive ALL cases the breakpoints of chromosome 22 could be located 5' to the \( bcr \) region, we explored the possibility that a similar genomic event could have taken place in our CML patients. Therefore in Southern blot experiments we used the probes 6.13-0.6 (0.6 kb) and 6.13-0.3 (0.3 kb), which represent together 0.9 kb corresponding to the middle portion of the \( bcr \) cDNA sequences 5' upstream the \( bcr \) region (G. Grosveld, personal communication). Again the pattern obtained after digestion with \( BamHI, BglII, \) and \( EcoRI \) was in all three cases identical to that of normal leukaocyte DNA, both with the 0.6 kb probe (data not shown) and with the more 5', 0.3-kb probe (Fig 3).

The DNAs of the three patients were then hybridized to the \( c-sis \) probe after digestion with \( EcoRI \) and \( HindIII \) and to the \( C_\gamma \) probe after digestion with \( EcoRI, BamHI, \) and \( HindIII \). No rearranged bands were found in any case, either with \( c-sis \) or with \( C_\gamma \), with all the restriction enzymes used (data not shown). With the \( c-sis \) probe, however, two of the three patients (T.V. and R.G.) exhibited a polymorphic pattern already described after digestion with \( HindIII \) that gave bands of about 21.7 kb and 14.5 kb (not shown). Furthermore, in one of the three patients (R.G.) a polymorphic restriction pattern, already described, was also observed in the \( EcoRI \)-digested DNA hybridized to the \( C_\gamma \) probe that gave an additional band of about 22 kb (not shown).

As far as the expression of \( bcr \) is concerned, the poly (A) + RNAs of the three described patients together with the poly (A) + RNAs derived from two other classic \( Ph^1 \)-positive CML cases were hybridized on the same filters with the \( bcr \) cDNA probe (Pr-2) representing exons 1 and 2 of the \( bcr \) region and at least two further \( bcr \) exons 5' upstream to them. In all cases two faint bands representing normal \( bcr \) mRNA species were detected (Fig 4). A major 8.5-kb mRNA species was detectable only in the RNAs analyzed as controls and was apparently absent in the RNAs of the three patients already mentioned (Fig 4) despite the fact that the chimeric mRNA, when present, is usually more abundant than the normal \( bcr \) mRNA transcripts.

We then submitted the same RNAs to a further Northern blot analysis using a \( c-abl \) c-DNA probe (K38). This experiment revealed, both in poly (A) + and total RNA of one case (T.V.), the presence, in addition to the normal expected bands, of a faint but still detectable band of about 8.5 kb while confirming the absence of a chimeric mRNA species in the remaining two cases (Fig 5). The results obtained in patient T.V. led us to investigate whether the chimeric mRNA might be detected by \( bcr \) cDNA probes representing coding sequences located more 5' to those represented by Pr-2. In fact Northern blot analysis of the poly (A) + RNA of patient T.V., which was carried out with the \( bcr \) cDNA probes 6.13 and K31 and represented together about 1.4-kb sequences 5' to the Pr-2 sequences, revealed extremely faint bands, one of which apparently corresponds to the chimeric mRNA present in a classic CML case (Fig 6). The same experiment did not show any detectable level of chimeric mRNA in patient Q.S. (Fig 6) or patient R.G. (not shown).

The densitometric analysis did not reveal relevant differences in the amount of RNA present in each lane in all experiments.

DISCUSSION

The analysis of our data led us to the conclusion that in these three patients affected by classic \( Ph^1 \)-positive CML the breakpoint of chromosome 22 was certainly outside the \( bcr \) region. This finding seemed rather unusual, even though in the literature some evidence has already been reported of \( Ph^1 \)-positive CML in which a breakpoint inside \( bcr \) was not found, and it has been suggested that in CML the chromosome 22 breakpoints must be more heterogeneous than previously recognized. Southern blot hybridizations with the \( bcr \) (Pr-1) probe suggested that in these three cases the breakpoint should be located either 5' or 3' to the \( bcr \) region.

Meanwhile, the absence of rearranged bands in the DNAs digested with different enzymes and hybridized with the probes Pr-1, 6.13-0.6, and 6.13-0.3 prevented us from localizing the breakpoint in the coding segments 5' to the \( bcr \) in a region of about 13 kb flanking the 5' site of the \( bcr \).
Consequently, we reasoned that in these three cases the breakpoint should be located either in the far 3' extremity of the bcr gene or outside the gene.

The absence of rearranged bands with both C_\text{4} and c-sis probes made it clear that the breakpoint had occurred neither in the C_\text{4} locus located in the q11.12 subband nor in the c-sis locus harbored in the q12.3-13.1 subbands. At present we cannot explain the apparently high frequency of polymorphism of both these loci in these three cases.

With respect to the Northern blot hybridization data, we did not find a chimeric mRNA of 8.5 kb in two of these three Ph' positive CML patients. We are well aware of the uncertainties related to a quantitative evaluation of mRNA expressed at low level; nevertheless, our results indicate that in these two cases the level of transcription of the 8.5-kb mRNA, if present, must be at least much lower than in common Ph' positive CML. These findings seemed rather unusual because data reported in literature show the presence of this chimeric mRNA in practically all cases of Ph'-positive CML studied thus far with rare exceptions.^{32,33}

So the possibility is raised that a breakpoint of chromosome 22 outside the bcr region might result in a novel genomic structure that does not allow the transcription of a 8.5-kb chimeric mRNA, at least at a detectable level, while leaving unaffected the formation of the mRNA species presumed to be normally transcribed by the bcr and abl genes.\(^2\)

A similar genomic event has been described in several patients affected by ALL with a t(9;22) translocation in which the bcr region was not involved in the chromosomal rearrangement.\(^{11,14,39}\) By analogy with the reports in some Ph'-positive ALL cases,\(^{12,14}\) it would be worthwhile in these two cases to look for the presence of abl-derived kinases different from the chimeric p210 and from the normal p145 while taking in mind that the cells of these two cases are certainly not lymphoid.

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**Fig 3.** Southern blot analysis of the DNAs of the three patients (T.V., R.G., and Q.S.) that were digested with EcoRI, BamHI, and BglII and hybridized to the 0.3-kb 5' bcr cDNA (6.13-0.3) probe. The restriction pattern with all the enzymes in all three cases is identical to that of normal leukocyte DNA (N). A faint hybridization signal of a previous hybridization of the same filter with probe 6.13-0.6 is still detectable in the region of about 8.0 kb in the DNAs digested with BglII.

**Fig 4.** Northern blot analysis of the poly (A) + RNAs of the three patients (R.G., T.V., and Q.S.) hybridized to the bcr cDNA probe (Pr-2). The 8.5-kb chimeric mRNA is clearly detectable only in the two CML populations used as controls (CML 1 and CML 2). The normal bcr mRNA species are present in all the populations.

**Fig 5.** Northern blot analysis of the poly (A) + RNAs of the three patients (R.G., T.V., and Q.S.) hybridized to the c-abl cDNA probe (K38). The 8.5-kb chimeric mRNA is clearly detectable only in the two CML populations used as controls (CML 1 and CML 2) and in one of the three patients (T.V.). The normal abl mRNA species of 8 and 7 kb are present in all the RNA populations.
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