Duplication of the *bcr* and gamma-glutamyl transpeptidase genes

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ABSTRACT

The Philadelphia (Ph') translocation involves rearrangement of the *bcr* gene located on chromosome 22. Hybridization experiments revealed the presence of multiple *bcr* gene-related loci within the human genome. Two of these were molecularly cloned and characterized. Both loci contain exons and introns corresponding to the 3' region of the *bcr* gene. Restriction enzyme and DNA sequence analysis indicate a very high degree of conservation between *bcr* and the two related genomic sequences. Both *bcr*-related loci are located on chromosome 22, one centromeric, the other telomeric, of the *bcr* gene. Within the two *bcr* related genomic sequences, fragments or the complete coding sequences of an unrelated gene were found to be present. This gene was identified; it encodes gamma-glutamyl transferase, an enzyme involved in the glutathione metabolism.

INTRODUCTION

We have previously identified a region on chromosome 22 in which breakpoints occur in a specific chromosomal translocation, the t(9q34; 22q11) or Ph' (Philadelphia) translocation, found in the leukemic cells of over 96% of chronic myelogenous leukemia (CML) patients (1,2). The breakpoints all occur within a limited region of up to 5.8 kb (3) designated the breakpoint cluster region (*bcr*). The *bcr* is a segment of a gene (*bcr* gene) oriented with its 5' end toward the centromere of chromosome 22; breakpoints have been localized between exons 2 and 3 or 3 and 4 of the gene (4; the numbering of exons is arbitrary, as the total number of exons has not yet been defined). On chromosome 9, breakpoints have been localized to an extended region 5' within the human *c-abl* oncogene (5,6); as a result of the translocation, 5' *bcr* gene sequences become fused to 3' *c-abl* sequences on the Ph' chromosome in a head-to-tail fashion. A chimeric mRNA is transcribed (7,8) and translated into a chimeric *bcr/c-abl* fusion protein, P210 (9,10). Since the Ph'-chromosome is specifically found in leukemia, often as the sole chromosomal abnormality, it is likely that the abnormal *bcr/c-abl* fusion protein plays a role in the genesis and/or maintenance of the disease. Although all breakpoints examined to date on the Ph'-chromosome in CML occur consistently within the breakpoint cluster region, we do not know why they occur with such specificity.

Other non-oncogenes, such as the immunoglobulin gene family and the T-cell receptor genes, are also involved in chromosomal translocations; these genes, however,

are rearranged in the course of their development, and it is possible that the translocations are mediated by those enzymes responsible for their normal rearrangement (11). However, using *bcr* gene probes we have examined many human DNAs of different origin to no avail for naturally occurring rearrangements: to date we have only found the gene in "germline configuration".

In the course of our studies on the *bcr* gene, we detected *bcr* gene-related sequences in the human genome. Since the *bcr* gene itself is specifically involved in translocations, the possibility exists that members of its gene family may also be involved in human cancer. As an initial step to investigate this, we undertook to molecularly clone and characterize these sequences. In this study, we report the characterization of two *bcr* gene-related loci, both located on chromosome 22 and the identification within these clones of sequences of the gamma-glutamyl transpeptidase gene.

MATERIALS AND METHODS

Libraries and somatic cell hybrids

The cosmid libraries used in this study include a previously described K562 library, a human lung carcinoma library and a human placental library (12,13). The human placental cDNA library in λ gt11 was purchased from Clontech. PgMe-25 Nu is a human-mouse somatic cell hybrid containing chromosome 22 as its only human component; WESP 2A is a fusion product of mouse WEHI-3B cells with leukocytes of a Ph⁺-positive CML patient, and contains human chromosomes 7, 8, 14 and the Ph⁺-chromosome (5).

Southern blotting and hybridization

High molecular weight DNAs were isolated as described (14). Restriction enzymes were purchased from Bethesda Research Laboratories. DNAs digested with different enzymes were run on agarose gels and blotted according to Southern (15) on nitrocellulose (Schleicher and Schuell, PH 79). Probes were isolated from low melting point agarose gels according to published procedures (14). Nick translation of probes and filter hybridizations were as described (12). Filters were exposed at -70°C to XAR-2 film (Kodak) with DuPont Lightning Plus intensifying screens.

Isolation of clones

The cosmid libraries were screened as described (12). The clones were mapped by digestion of individual restriction enzyme fragments isolated from low melting point agarose gels. The placental cDNA library was screened as described (4) using a combination of three probes. Following hybridization, the phage replica filters were washed at high stringency (0.01 xSSC at 65°C) as a final post-hybridization wash.

RESULTS

The human genome contains many *bcr* gene-related sequences

The *bcr* gene expresses two mRNAs, of approximately 6.0 and 4.5 kb (4,7).

Previously we have isolated a partial cDNA encompassing 2.2 kb of sequences including the complete 3' end of the gene; the approximate positions of the exons, which are distributed over a region of up to 45 kb of genomic DNA, have been determined (4).

Upon hybridization of the cDNA to restriction enzyme-digested human DNA (for example with *Bgl*II, see Figure 1, lane 1) many fragments not belonging to the *bcr* gene locus are detected. Not all subregions of the cDNA detect related sequences equally well: the 5' terminus of the cDNA (a 0.64 kb *Pst*I probe, see Figure 2D) does not clearly detect any *bcr* gene-related fragments (not shown), whereas the middle of the cDNA (0.45 kb *Pst*I probe, Figure 2D) detects two *bcr* gene-related fragments of 12.0 and 6.0 kb (Figure 1, lane 2) in addition to fragments of the *bcr* gene. Finally, 3' probes (0.72 kb *Pst*I probe and a fragment encompassing the 3' untranslated end of the cDNA, Figure 2D) detect numerous fragments, the most prominent being an 8.5 kb *Bgl*II fragment (Figure 1, lanes 3 and 4). The strong hybridization of this fragment may be partially due to the fact that it contains the entire 3' end of the *bcr* gene, including approximately 1 kb of coding and non-coding sequences. However, it is probable that the human genome contains more than one copy of this DNA fragment.

Fig. 1. *bcr* gene-related sequences in human DNA. DNAs digested with *Bgl*II were electrophoresed on an 0.6% agarose gel. A *Hind*III molecular weight marker is shown to the left. Molecular weights are in kilobases. Probes used include the 1.6 kb insert of the *bcr* cDNA, lane 1; the 0.46 kb *Pst*I probe, lane 2; the 0.72 kb *Pst*I probe, lane 3; a 3' *Pst*I probe, lane 4. Probes are shown schematically in Figure 2D.

Cloning and characterization of the VKII-1 and JG-15 loci

To isolate *bcr* gene-related sequences, the entire 2.2 kb cDNA insert was used to screen three human cosmid libraries. Of the numerous positives obtained, those containing sequences of the previously cloned *bcr* gene were excluded from further analysis. Remaining clones could be classified into two categories after restriction enzyme analysis: one set of clones (two cosmids spanning approximately 40 kb) belong to one locus which has been named JG-15; the second set (seven cosmids encompassing more than 65 kb) all originate from a different locus called VKII-1.

A comparison of the restriction enzyme maps of these loci and the *bcr* gene locus (Figure 2, A, B and C) reveals an interesting feature: a number of restriction enzyme sites appear to have been conserved in homologous locations in the 3' end. For example, all three contain the 8.5 kb *Bgl*II fragment with *Kpn*I and *Eco*RI sites immediately 5' to it (Figures 2 and 3). The JG-15 and VKII-1 loci also have sites in common more to the 5' of this *Bgl*II fragment, which are absent from the *bcr* gene locus.

To identify the location of exons, the 2.2 kb *bcr* cDNA insert was hybridized to Southern blotted digests of the cosmids. In contrast to the *bcr* gene locus, for which corresponding exons were found to be spread over a region of 45 kb, hybridizing sequences in the JG-15 and VKII-1 loci were limited to less than 16 kb of DNA. Therefore, we compared "homologous" fragments from the three loci (5.2, 4.5 and 6.8 kb *Bgl*II fragments; see Figure 2A, B and C) by subcloning, mapping and hybridization to the *bcr* cDNA. The fragments contain many homologous restriction enzyme sites (Figure 3A) and three hybridizing regions probably representing exons. All three subclones had an essential identical sequence in the region corresponding to the intron-exon boundary of *bcr* gene exon 6 (4) (Figure 3B). A comparative restriction enzyme analysis of the 8.5 kb *Bgl*II fragments revealed a similar close homology (not shown). These experiments establish that regions in the VKII-1 and JG-15 loci are similar to the 3' end of the *bcr* gene, and that they do not represent processed genes. However, no hybridization could be detected with the 5' end of the *bcr* cDNA even after non-stringent post-hybridization washes. This could indicate that putative 5' coding sequences of these loci have diverged significantly.

Both *bcr* related loci contain fragments of the gene encoding gamma-glutamyl transpeptidase

To identify putative 5' coding sequences of the JG-15 locus, a combination of three repeat-free probes from the 5' end (Figure 2B, cross-hatched boxes) was used to screen a human placental cDNA library; one cDNA with a 1.7 kb insert was isolated. This cDNA hybridized exclusively to the 5' region of cosmid JG-15. Surprisingly in cosmid VKII-1, a region 3' to the *bcr* gene homologous sequences hybridized to the cDNA (Figure 2A, cross-hatched box). Hybridization experiments did not reveal any homology between this cDNA and the *bcr* cDNA. The cDNA was partially sequenced at its ends (not shown); the

Fig. 2. *bcr* gene-related loci VKII-1 and JG-15. Restriction enzyme maps of the VKII-1 locus (A) and the JG-15 locus (B) are compared with that of the *bcr* gene locus (C). Cosmids spanning the region are indicated below the maps. Open boxes above the maps show the location of the homologous 3' 5.2 kb, 4.5 kb, 6.8 kb and 8.5 kb *Bgl*II fragments; these regions contain sequences homologous to the 3' end of the *bcr* cDNA as shown in D with an open bar. 5' *bcr* cDNA sequences are indicated with a solid bar in D; the corresponding 5' exons are shown as solid bars above the *bcr* gene map in C. The location of probes used to isolate the gamma glutamyl transpeptidase cDNA are indicated as cross-hatched boxes above the JG-15 map; the VKII-1 fragment hybridizing to this cDNA is also shown as a cross-hatched box. Restriction enzymes include: B = *Bam*HI; Bg = *Bgl*II; E = *Eco*RI; H = *Hind*III; K = *Kpn*I; P = *Pst*I; PII = *Pvu*II; S = *Sal*I. *E and *Bg denote polymorphic *Eco*RI and *Bgl*II restriction enzyme sites.

Fig. 3. Comparison of subclones of the 4.5, 5.2 and 6.8 kb *Bgl*II fragments.

A restriction map of the three fragments is shown in A; the solid bars in the *bcr* gene fragment indicates the approximate position of *bcr* gene exons 6, 7 and 8. Arrows beneath the maps show regions sequenced; the sequence of the intron-exon boundaries are shown in B. The probe used in Figure 4 (a 0.5 kb *Pst*I fragment) is indicated as a solid bar above the VKII-1 fragment. Restriction enzymes include Bg = *Bgl*II; Bs = *Bst*II; E = *Eco*RI; H = *Hind*III; K = *Kpn*I; P = *Pst*I; PII = *Pvu*II; Ss = *Sst*I; Xb = *Xba*I. Not all *Pvu*II or *Pst*I sites within the 6.8 kb *bcr* gene fragment were mapped.

3' end contains a poly(A) tail, which demonstrates that it represents a transcript of a gene. The deduced amino acid sequence of a small region (225 nucleotides) of cDNA sequence (not shown) was compared with known protein sequences using the FASTP program (4). Sixty of the seventy-five deduced amino acids were identical to those of the recently cloned rat gamma-glutamyl transpeptidase gene. (16) This data strongly indicates that the cloned cDNA encodes human gamma-glutamyl transpeptidase (GGT). The human gamma glutamyl transpeptidase gene appears to be a member of a multigene family; the genomic probes used to isolate the cDNA hybridized to a number of different restriction enzyme fragments in genomic human DNA (not shown).

Fig. 4. Chromosomal localization of the VKII-1 and JG-15 loci. A. Schematic representation of chromosome 22 with the relative location of the VKII-1, *bcr* gene (band q11) and JG-15 loci. B. Southern blot analysis. 15 μ g of DNA was digested with *Bgl*II and run on a 0.6% agarose gel. The blot was hybridized with the 0.5 kb *Pst*I probe shown in Figure 3A. DNAs used include control human, lane 1; somatic cell hybrid PgMe-Nu (chromosome 22), lane 2; somatic cell hybrid WESP 2A (Ph'-chromosome), lane 3; mouse DNA, lane 4; CML cell line K562 DNA, lane 5. The sizes of the hybridizing *Bgl*II fragments (in kb) are indicated to the right of the figure.

Chromosomal localization of the JG-15 and VKII-1 loci

The *bcr* gene-related loci were next characterized with respect to their chromosomal location. A repeat-free probe (a 0.5 kb *Pst*I fragment, see Figure 3A, 1) from the 5.2 kb *Bgl*II fragment of VKII-1 proved suitable for this purpose, even though it cross hybridized to the 6.8 kb *bcr* gene and 4.5 kb JG-15 *Bgl*II fragments: all three fragments could be distinguished easily due to their difference in size (Figure 4B, lane 1). The JG-15 and VKII-1 loci are located on the same chromosome as the *bcr* gene: the 5.2 and 4.5 kb *Bgl*II fragments are present in the DNA of a somatic cell hybrid containing chromosome 22 as its only human component (Figure 4B, lane 2). The 5.2 kb (VKII-1) fragment alone is present in the DNA of a somatic cell hybrid containing the Ph' (22q-) chromosome of a CML patient (Figure 4B, lane 3); thus, the VKII-1 locus must be located 5' to the *bcr* gene on chromosome 22. In concordance with this, VKII-1 sequences are found amplified in the CML cell line K562, which contains amplified remnants of the Ph' chromosome (6,13,17) (Figure 4B, lane 5). The JG-15 locus is non-amplified in K562 (Figure 4B, lane 5) and absent from the Ph'-chromosome (Figure 4B, lane 3); therefore, it must be located 3' to the *bcr* gene locus on chromosome 22. These results are shown schematically in Figure 4A.

Fig. 5. Evolutionary conservation of *bcr*-related loci. DNAs of human (lane 1), gorilla (lane 2), chimpanzee (lane 3) and mouse (lane 4) were digested with *Bgl* II and hybridized to a 3' *bcr* cDNA probe (panel A) or the 0.5 Kb *Pst* I probe (see figure 3 panel B). The 8.5 kb bands visible in panel B represent remaining signals of the 3' *bcr* cDNA probe hybridization.

The results would also imply that the gamma-glutamyl transpeptidase gene is located on chromosome 22. This is in concordance with a recent publication in which the gamma glutamyl transpeptidase gene was assigned to human chromosome 22 (18). Since the 5' end of the gamma glutamyl transpeptidase cDNA does not hybridize to any sequences within the VKII-1 locus, it seems unlikely that this locus represents a functional GGT gene. In contrast, the JG-15 locus does contain more 5' sequences of GGT; we tentatively conclude that the GGT gene is transcribed from the JG-15 locus.

Evolutionary conservation of *bcr* related loci

The close homology at the 3' ends of VKII-1, JG-15 and the *bcr* gene would suggest, that these loci have been duplicated recently in evolution. To examine this, high molecular weight DNA from gorilla, chimpanzee and mouse was digested with *Bgl* II and

hybridized to a *bcr* cDNA 3' probe and subsequently to the 0.5 Kb Pst I probe indicated in figure 3. As shown in figure 5 panel A, the 3' probe detects numerous fragments in gorilla and chimpanzee DNA (lanes 2 and 3), the most prominent being a Bgl II fragment of 8.5 Kb (compared to human DNA, lane 1). A single significant less hybridizing fragment of approximately 25 kb was observed in mouse DNA (lane 4).

Since the 0.5 Kb Pst I fragment (see above) allows the distinction of the VKII-1, JG-15 and *bcr* loci, the DNAs were hybridized to this probe. Surprisingly, the three bands present in human DNA (figure 5 panel B lane 1) are also present in gorilla DNA (lane 2); the band of 5.2 Kb, which represents the VKII-1 locus in human, is absent in chimpanzee DNA, whereas the band representing the JG-15 locus in human appears amplified (figure 5, panel B, lane 3). This probe does not detect any prominent band in mouse DNA (lane 4).

DISCUSSION

In this study we have isolated two different *bcr* gene-related loci from human cosmid libraries. These sequences do not represent the only *bcr*-gene related loci in the human genome: we have recently isolated two additional sets of sequences not originating from loci described here (unpublished results). Moreover, the probe used to screen the libraries encompasses only 2.2 kb of a total of 4.5 kb of *bcr* gene coding sequences. The CML cell line K562 (the source of DNA for one of the libraries) contains amplified remnants of the Ph'-chromosome including the immunoglobulin λ light chain locus (C λ), *bcr* sequences 5' of the breakpoint and *c-abl*, on one marker chromosome; the Ph'-chromosome is no longer visible cytogenetically (6,17). Therefore, it could be argued that any sequences isolated from the amplified region (such as the VKII-1 locus) could contain rearrangements that are specific (e.g., have occurred during amplification) for the K562 cell line and are not present in normal human DNA. However, this does not seem likely for the VKII-1 locus: all six cosmids from the K562 library are overlapping in the same region, and in addition, one cosmid was obtained from a lung carcinoma library, which spans much of the region (see Figure 2A, cosmid VCA 14-1). Two cosmids from different libraries (the K562 and placental DNA libraries) overlap essentially the same region of the JG-15 locus. Therefore, JG-15 and VKII-1 represent authentic loci. In a recent publication of Croce et al. (19) three distinct *bcr* related loci were identified. Small regions of two of these loci (*bcr* 3 and *bcr* 4) were molecularly cloned and analysed by restriction enzyme analysis and chromosomal mapping (19). One of the two *bcr* related loci described in the present study, JG-15, contains a 15 kb region with an identical restriction enzyme map when compared with the 15 kb cloned DNA fragment of *bcr* 3 (19). In addition both JG-15 (present study) and *bcr* 3 could be localized 3' of the *bcr* gene on chromosome 22. (22 q11 - 22 qter). We conclude that JG-15 and *bcr* 3 are identical loci. The other locus described in the present study (VK II-1) has a clearly different restriction enzyme pattern when compared with *bcr* 4 described by Croce et

al. Most probably VKII-1 represents the third *bcr* related locus, not isolated by Croce et al (19). In the latter study, the authors tentatively located this third locus 5' of the *bcr* gene on chromosome 22 which is in concordance with our present studies.

The loci have been characterized by restriction enzyme mapping, by hybridization to a *bcr* gene cDNA and by chromosomal localization. Both loci resemble the *bcr* gene closely but exclusively in its 3' end; many restriction enzyme sites are found in homologous positions, and the intron-exon boundaries corresponding to *bcr* gene exon 6 have an identical nucleotide sequence in all three loci (DNA sequence more 5' in the intron have diverged); the 3' end of the *bcr* cDNA hybridizes strongly to the 3' sequences of JG-15 and VKII-1 even following stringent post-hybridization washes. However, no regions have been detected with homology to *bcr* exon 5, which is separated from exon 6 by a large intron of approximately 15 kb (Figure 2C), or to any other exon 5' of it. This could indicate that 5' exons of VKII-1, JG-15 and the *bcr* gene have diverged. The finding that sequences encoding the gamma-glutamyl transpeptidase gene are present in the 5' region of JG-15, exactly in that region which contains 5' *bcr* exons in the *bcr* gene, makes this explanation less likely although not impossible; in such event, JG-15 5' coding sequences would have to be located between the gamma-glutamyl transpeptidase sequences and the 3' *bcr* gene-homologous region. The JG-15 gene would then be dissimilar to the *bcr* gene, as exons of the latter are distributed over a region of at minimum 100 kb (unpublished results) Moreover, such putative 5' *bcr* gene-related exons would have very little homology with 5' *bcr* exons, as no homology could be detected even when using non-stringent washing conditions.

We have not unequivocally demonstrated that the VKII-1 locus lacks a functional *bcr*-related gene; the restriction enzyme site homology with JG-15 ends approximately halfway in the locus, with 5' regions exhibiting no homology either to the *bcr* gene or to JG-15. This region could therefore possibly contain 5' sequences of the VKII-1 "gene". However, we have failed to isolate any cDNA clones in experiments designed to detect cDNAs that might correspond to the *bcr*-related loci JG-15 and VKII-1. Therefore, the conclusion can be drawn, that these apparently non-functional, isolated 3' ends must have been generated from the intact *bcr* gene by duplication and recombination events on chromosome 22.

Since the sequences of these three loci appear so similar, one would expect that these events would have taken place relatively recently in evolution. However, it appears that both gorilla and chimpanzee have 3' *bcr* gene hybridizing 8.5 Kb Bgl II fragments. Since neither of the two Bgl II sites flanking this fragment are present in the cDNA, there appears to be no reason for this conservation. Similarly, it seems that the 5.2 Kb VKII-1 and 4.5 Kb JG-15 Bgl II fragments are also present in gorilla, although we have not formally proven that these fragments in fact are the homologs of the human fragments. It is possible, however, that these do represent functional genes in gorilla and

chimpanzee. Presently, we have no explanation for the high degree of conservation of these apparently non-functional genes in human.

With regard to the mechanism by which these loci have evolved, the JG-15 locus could have been formed by duplication and transposition of 3' *bcr* gene sequences 3' to the gamma glutamyl transpeptidase gene. It appears, that the latter GGT gene is functional since the cDNA we have isolated corresponds to this locus, based on hybridization and nucleic acid sequence analysis (manuscript in preparation). The VKII-1 locus seems to be composed of three different elements: unique 5' sequences, a 3' *bcr* gene-homologous segment, and sequences homologous to the gamma glutamyl transpeptidase gene 3' to this. The GGT homologous segment is relatively small and encompasses only a region in the 3' end of the gene - it is probably nonfunctional, since it lacks 5' genomic coding sequences. The gamma glutamyl transpeptidase sequences are located 5' of the *bcr* related sequences in JG-15; in contrast, in VKII-1, these sequences are located 3' of the *bcr* related sequences. Consequently it seems unlikely that one of the *bcr* related loci is directly derived from the other through amplification and deletion events. The conclusion seems justified that the *bcr* related sequences present in JG-15 and VKII-1 are the result of independent amplification and recombination events between the *bcr* gene and a gamma glutamyl transpeptidase gene. The mechanism which dictated the specific rearrangements between *bcr* and gamma glutamyl transferase remain obscure for the moment, since we have detected no homology whatsoever between the GGT cDNA and the *bcr* gene. However, it is possible that these genes are located in a rearrangement - prone region of chromosome 22 and that other gamma-glutamyl transpeptidase genes and/or sequences on chromosome 22 have promoted unequal crossing-over events. The presence of multiple, highly conserved 3' *bcr* sequences in the human genome support the hypothesis, that other *bcr* rearrangements, analogous to those found in the t(9;22), have occurred in the recent past. Although such rearrangements have involved regions of the *bcr* gene different from those in the Ph' translocation our hypothesis is supported by the following data: 1. The *bcr* gene appears to be susceptible to different rearrangements events, such as found in chronic myelogenous leukemia and recently, 5' within the gene, in some patients with Ph'-positive acute lymphoblastic leukemia (20-22). 2. the *bcr* and *bcr* related genes are extremely well conserved on a restriction enzyme, gene organization and even nucleotide level. The finding that both the genome of gorilla and chimpanzee (but not mouse) contain these *bcr* related genes would contradict the hypothesis that the rearrangements would have occurred in the recent past. Moreover, to date we have been unsuccessful in the identification of a second gene with which these 3' *bcr* related genes could have recombined to produce a chimeric transcript analogous to the transcripts we have found as the result of the Ph' translocation. Nonetheless the seemingly high level of conservation of these genes in human, chimpanzee and gorilla suggests that the existence of these *bcr* related genes is

not just a genomic accident with no further implications; conservation of this degree indicates a functional importance for which we currently do not have an explanation.

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