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Molecular analysis of both translocation products of a Philadelphia-positive CML patient

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ABSTRACT

The breakpoint regions of both translocation products of the (9;22) Philadelphia translocation of CML patient 83-H84 and their normal chromosome 9 and 22 counterparts have been cloned and analysed. Southern blotting with bcr probes and DNA sequencing revealed that the breaks on chromosome 22 occurred 3' of bcr exon b3 and that the 88 nucleotides between the breakpoints in the chromosome 22 bcr region were deleted. Besides this small deletion of chromosome 22 sequences a large deletion of chromosome 9 sequences (>70kb) was observed. The chromosome 9 sequences remaining on the 9q+ chromosome (9q+ breakpoint) are located at least 100 kb upstream of the v-abl homologous c-abl exons whereas the translocated chromosome 9 sequences (22q- breakpoint) could be mapped 30 kb upstream of these c-abl sequences. The breakpoints were situated in Alu-repetitive sequences either on chromosome 22 or on chromosome 9, strengthening the hypothesis that Alu-repetitive sequences can be hot spots for recombination.

INTRODUCTION

Chronic myelocytic leukemia (CML), a pluripotent stem cell disease is characterized by the presence of a Philadelphia (Ph^1) chromosome in the leukemic cells of more than 90% of all CML patients (1). This Ph^1 chromosome is the result of a reciprocal translocation between chromosomes 9 and 22 (2,3). Previous studies indicated that in all Ph^1 (+) CML patients a human oncogene c-abl, normally located on 9q34, was translocated to a specific, limited area on chromosome 22, the breakpoint cluster region (bcr)(4,5.) The 5.0 kb bcr contains 4 small coding regions (6) and is an internal part of a large 'bcr' gene. Thusfar all chromosome 22 breakpoints (>30) map within two introns of this bcr. However, breakpoints on chromosome 9 are scattered over a very large area, which may vary from 14 kb (7) up to more than 100 kb (8) upstream of the v-abl

homologous sequences of the c-abl gene. As a result of the translocation, the c-abl sequences are linked in a head-to-tail fashion to the 5´ bcr sequences on the Ph^1 chromosome. Recent studies indicated, (8,9,10) by demonstration of the presence of a chimeric bcr/c-abl mRNA, that this region is transcriptionally active. The 5´ bcr and c-abl coding sequences are linked by RNA splicing, apparently independent from the distance between the two genes on the Ph^1 chromosome. The detection of an abnormally sized c-abl protein (11) supports this hypothesis and is the presumable translation product of this chimeric mRNA found in the leukemic cells of Ph^1(+) CML patients and CML-derived cell lines. Virtually nothing is known about the mechanism of chromosomal translocation in CML. Sequence data from the breakpoints of two CML patients suggest that Alu-repetitive sequences may play a role (6,12). Here we report the cloning of the breakpoint regions of both translocation products of a CML patient with a t(9;22). Mapping and sequencing of the chromosomal breakpoint regions and their normal counterparts revealed that the translocation did not occur in a conservative manner: both chromosome 9 and 22 sequences were deleted. Furthermore Alu-repetitive sequences were located near or at the breakpoint in this CML patient.

MATERIALS AND METHODS

CML patient and cell lines

CML patient 83-H84 is an 18-year old male. In this study leukophoresis material from the chronic phase of CML (karyotype t(9;22)(q34;q11) prior to treatment was obtained. The cell line K562 (13) and Hela cells were used as controls.

Southern blotting and hybridization

High-molecular weight DNA´s, isolated as described (14) were digested with restriction enzymes, electrophoresed on 0.7% Agarose gels and blotted according to Southern (15). Isolated DNA fragments (8) were labeled with ^{32}P as described by Feinberg and Vogelstein (16). Probes containing repetitive sequences were preincubated with sonicated human DNA to Cot=1 (10 mg/ml, 0.6M sodium phosphate buffer pH7, 3 hr at 65°C). Hybridization and washing conditions were as described in previous

publications (3,5).

Genomic cloning

To isolate the breakpoint regions a genomic library of patient 83-H84 DNA was constructed in λ-EMBL-3 (17). Normal chromosome 9 counterpart of phage EMBL-3a (9q+) was isolated from another CML-EMBL-3 library. The normal chromosome 22 or chromosome 9 sequences were previously cloned from human cosmid or phage libraries (5,7,8).

DNA sequencing

Subcloned fragments of the breakpoint areas in pUC9 were sequenced according the methods of Maxam and Gilbert (18). In those cases where no suitable sites were available, a series of Bal 31 deleted subclones were generated and sequenced using end labeled sites of the pUC9 poly linker.

RESULTS

Identification and cloning of the chimeric DNA fragments

To identify the chimeric DNA fragments, BglII digested DNA of CML 83-H84 and control cell lines were hybridized to bcr probes. The 5′ bcr probe (2.2 kb Bg-H, Fig. 1C) detects an abnormal fragment of 5.7 kb (Fig. 2D) besides the normal 5.0 kb BglII fragment. This 5.7 kb fragment is not present in Hela or K562 DNA and represents the 22q- breakpoint BglII fragment of CML 83-H84. In a similar manner we identified the 9q+ BglII fragment of 2.4 kb (Fig. 2A) using the 3′ bcr probe (1.2 kb H-Bg, Fig. 1C). To analyse the aberrant fragments in more detail, a λ-EMBL-3 library was constructed of size-fractionated, partially MboI digested CML 83-H84 DNA. This library ($1x10^6$ ph) was screened with both the 5′ and 3′ bcr probe. Positive phages, hybridizing only to either the 5′ or the 3′ bcr probe, were isolated and mapped by double digestion of individually isolated restriction enzyme fragments from low-melting point agarose gels (Fig. 1B, 1D). The phages hybridizing to the 5′ or 3′ bcr probe contained the expected abnormal BglII fragments of 5.7 and 2.4 kb resp. The 5′ end of the 5.7 kb fragment was co-linear with bcr sequences 5′ of the 0.7 BamHI - HindIII fragment (Fig.1C), whereas the 3′ end of the 5.7 kb fragment was identical to previously cloned chromosome 9 sequences (Fig. 1D,

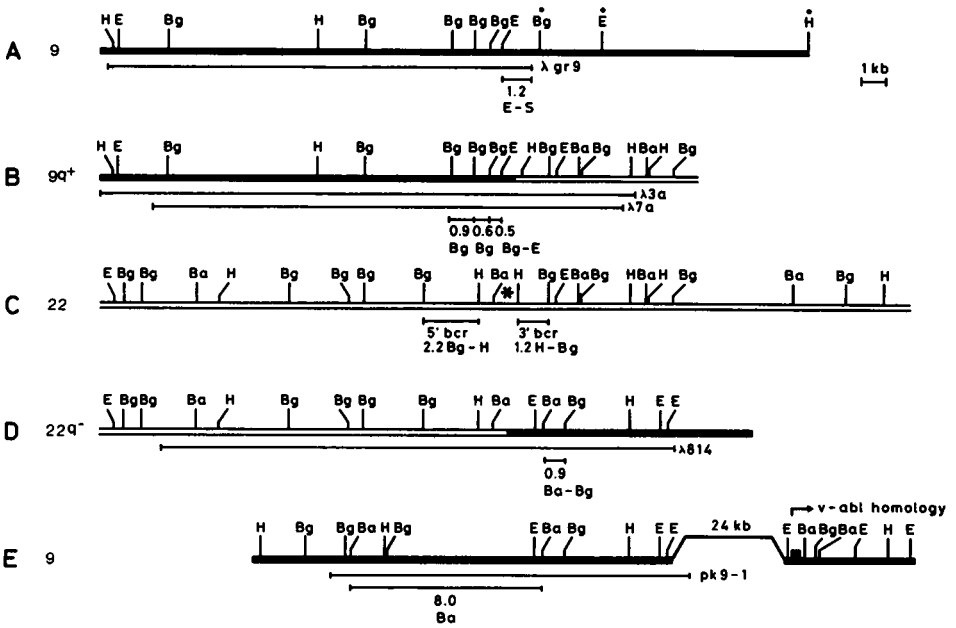

Fig. 1. Restriction enzyme map of the 9q+ (B) and 22q- (D) breakpoint regions and their normal chromosome 9 (A and E) and chromosome 22 (C) counterparts. Solid bars represent chromosome 9 sequences, whereas the open bars indicate sequences originating from chromosome 22 DNA fragments cloned in phage and probes used in the study are indicated below the relevant maps. A * marks the 0.7 kb Ba-H fragments in map C in which both chromosome 22 breakpoints are located. The restriction enzyme sites on the 3′ end of map A (indicated by a dot) are deduced from Southern blots. Ba = BamHI, Bg = BglII, E = EcoRI, H = HindIII, S = SalI.

1E). These sequences, and as a consequence the 22q- breakpoint on chromosome 9, were located 30 kb at the 5′ side of the known c-abl exons (3,7). The 3′ end of the 2.4 kb chimeric fragment was colinear with the 3′ bcr sequences, confirming a breakpoint in the 0.7 kb BamHI-HindIII segment of the bcr. The chromosome 9 origin of the 5′ end of this 2.4 kb fragment was demonstrated using somatic cell hybrids containing either chromosome 9 or 22 (data not shown). We used two small BglII fragments of 0.9 and 0.6 kb as probes (Fig. 1B) to isolate recombinant phages that contained the normal chromosome 9 counterpart of this chimeric fragment. Although the CML 83-H84 EMBL-3 library contained several positive phages, restriction enzyme analysis revealed

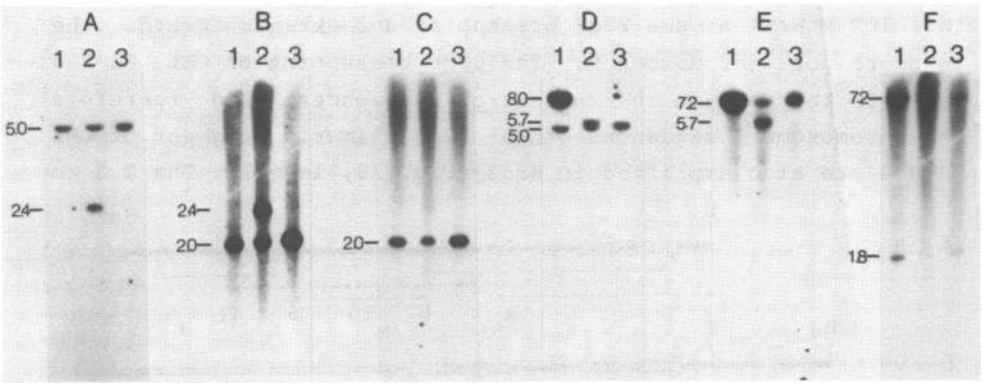

Fig. 2. Southern blots of BglII digested DNA of K562 (lane 1), CML 83-H84 (lane 2) and Hela (lane 3) hybridized with bcr probes (A,D); chromosome 9 probes (B,C,E,F). A: 1.2 kb HindIII - BglII (3´ bcr, 1C), B: 0.5 kb BglII - EcoRI (from 3a), C: 1.2 kb EcoRI - SalI (from gr.9), D: 2.2 kb BglII - HindIII (5´ bcr, 1C), E: 0.9 kb BamHI BglII (from λ814) and F: 8 kb BamHI(from pK9-1). The used probes are indicated in figure 1 below the relevant restriction enzyme maps.

that these did not cover the 9q+ breakpoint. Therefore a positive phage (λgr.9) extending 0.6 kb 3´ of the 9q+ breakpoint was isolated from a CML-EMBL-3 library of another patient (gr)(Fig. 1A). To exclude the possibility of cloning artefacts we made chromosome 9 probes from the chimeric fragments and hybridized them to Southern blots of CML 83-H84 DNA and control cell lines. As shown in Fig. 2B the 0.5 kb, Bg-E probe (Fig. 1B) hybridizes to a normal 2.0 kb BglII fragment in all DNAs and only in the CML 83-H84 DNA the 2.4 kb 9q+ chimeric fragment is present. A 0.9 kb Ba-Bg probe (Fig. 1D) detects the 5.7 kb 22q- chimeric BglII fragment in the CML 83-H84 DNA (Fig. 2E). The normal 7.2 kb BglII fragment present in Hela and CML 83-H84 DNA is amplified at least 4 fold in K562 DNA.

Minimal distance between the two chromosome 9 breakpoints

In a recent report (8) we have demonstrated that in K562 the 22q- breakpoint is located at a distance of at least 100 kb upstream of the known c-abl sequences. This entire region, including the 5´ bcr (Fig. 2D, 8.0 kb BglII fragment), the more than 100 kb of chromosome 9 sequences upstream of the known c-abl sequences and c-abl sequences, is amplified at least four fold in K562. The amplification of the chromosome 9 sequences

in K562 starts at the 22q- breakpoint and extends towards the telomere of chromosome 9. The 22q- breakpoint of CML 83-H84 maps 30 kb upstream the known c-abl sequences, and therefore the chromosome 9 sequences of the 22q- chimeric fragment of CML 83-H84 are also amplified in K562 (Fig. 2E, lane 1). The 2.0 kb

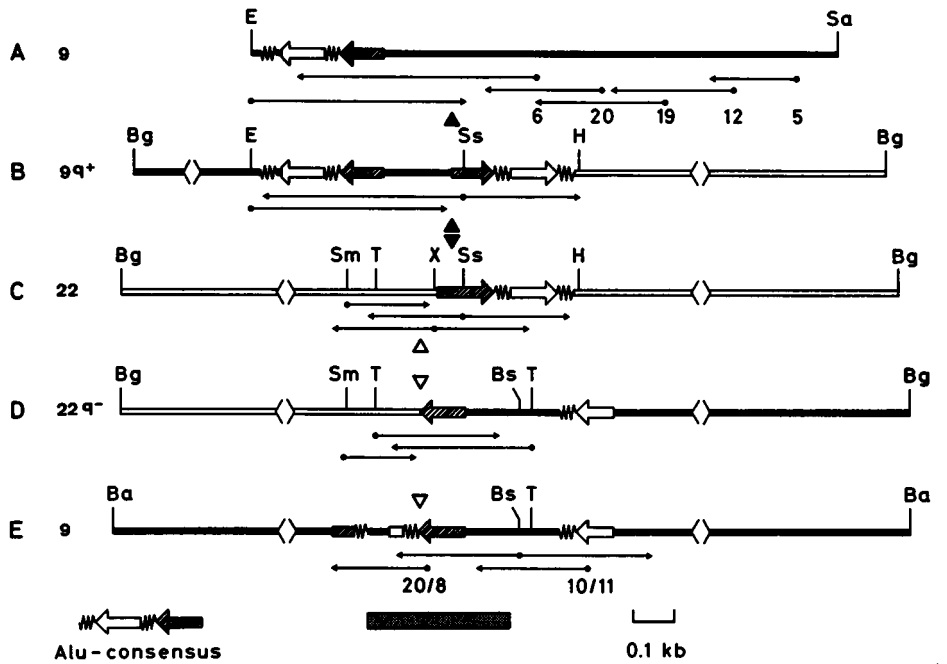

Fig. 3. Restriction enzyme maps of the subcloned breakpoint (B,D) and corresponding normal chromosome 9 (A,E) and 22 (C) fragments. Solid bars indicate chromosome 9 sequences and open bars indicate chromosome 22 sequences.The arrows below the relevant maps indicate the sequence strategies. All sequences were done by the method of Maxam and Gilbert (18) and dots indicate the position of the end label. Only the restriction enzym maps and Alu-repetitive sequences around the breakpoints are shown (——< >—— indicates known DNA ommitted from the figure). The hatched bar below the figure indicates the position of the 300 bases shown in figure 4.
The position and orientation of Alu-repetitive sequences are indicated in the maps using the Alu-consenses symbol shown at the bottom. The 300 bp Alu-consensus (19) consist of two nearly homologous halves (arrows) each followed by an A-rich tract (zigzags)(12). The used subclone (see also figure 1): A: 1.2 kb EcoRI - SalI, B: 2.4 kb BglII, C:5.0 kb BglII, D: 5.7 kb BglII, E: 8.0 kb BamHI, restriction enzymes: Ba = BamHI, Bg = BglII, Bs = BstII, E = EcoRI, H = HindIII, Sa = SalI, Sm = SmaI, Ss = SstI, T = TaqI, X = XhoI.

normal BglII fragment containing the 9q+ breakpoint of CML 83-H84 is not amplified in K562 and therefore must map to the 5′ side of the K562 Ph^1-breakpoint (Fig. 2B, lane 1) and thus is located at a distance of more than 100 kb upstream of the known c-abl sequences. This indicates that the distance between the two chromosome 9 sequences is at least 70 kb.

We were not able to detect any additional hybridizing BglII fragments using either a 1.2 kb E-S probe (Fig. 1A) spanning the 9q+ breakpoint or a 8.0 kb Ba probe (Fig. 1E) spanning the 22q- breakpoint (Fig. 2C and 2F). The previously identified 9q+ and 22q- breakpoint BglII fragments of 2.4 kb and 5.7 kb, resp. hybridize only very faintly to these probes because these parts of the probes contained several repetitive sequences which where competed out with human cot-1 DNA during hybridization. However the remaining parts of the 1.2 kb E-S or 8.0 kb Ba probes, which were able to detect the normal hybridizing 2.0 and 7.2 kb BglII fragments did not detect any additional hybridizing fragments. Probably the chromosome 9 sequences between the two breakpoints are deleted although we cannot exclude the possibility that these sequences are present somewhere else in the genome.

DNA sequences of breakpoints and normal counterparts

In order to determine the exact recombination site, subcloned breakpoint fragments (Fig. 3B, 3D) and corresponding normal chromosome 9 (Fig. 3A, 3E) and 22 (Fig. 3C) fragments were sequenced (18) as designated in Figure 3. The comparison of the breakpoint sequences with their normal counterparts is shown in Figure 4. At the breakpoints no nucleotides are inserted, but the boxed chromosome 22 sequences between the 22q- and 9q+ breakpoint (position 105, 193, resp.) are deleted. Comparison of the sequences with a human Alu repeat consensus sequence (19) revealed that both the breakpoints occurred in a Alu-repetitive sequence: the 9q+ breakpoint in a chromosome 22 Alu repeat and the 22q- breakpoint in a chromosome 9 Alu repeat. The orientation and exact localization of the Alu repeats and other Alu related sequences in the vicinity of the breakpoints is shown in Figure 3.

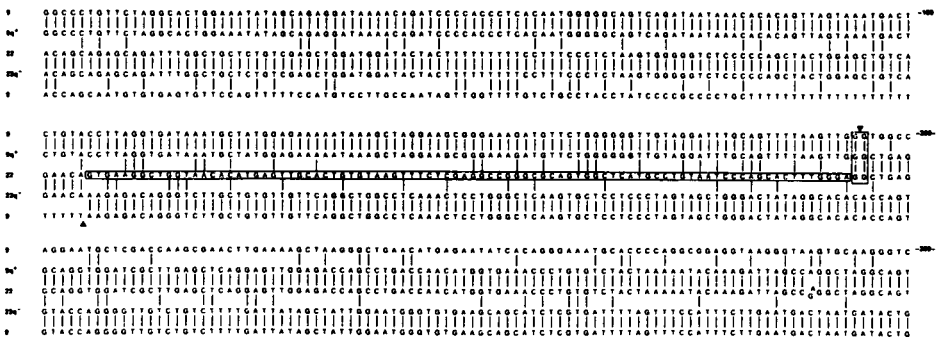

Fig. 4. DNA sequence analysis of the breakpoint subclones and their normal chromosome 9 and 22 counterparts. Sequence strategy and methods are indicated in figure 3. Only 300 nucleotides (indicated in figure 3) of the determined sequence is shown in this figure (other seqences are available on request from publisher). Sequence 9 represents the sequence of subclone A in figure 3, $9q^+$; the sequence of subclone B etc. Homology is indicated by vertical bars and the boxed sequences in the 22 sequence are the deleted chromosome 22 bcr sequences. ▲ : $9q^+$ breakpoint, △ : 22q- breakpoint of CML 83-H84.

DISCUSSION

Using a 5′ and 3′ bcr probe, we were able to identify the 22q- and 9q+ breakpoint fragments of a Ph^1-positive CML patient (CML 83-H84). The same bcr probes were used to screen a CML 83-H84 EMBL-3 phage library and positive phages containing the aberrant fragments were isolated and analysed. The chromosome 9 sequences present in the 22q- subclone were identical to previously cloned chromosome 9 sequences and map 30 kb upstream of c-abl exon a2 (8)(the first v-abl homologous exon of c-abl). These sequences were amplified in K562, a CML-derived cell line. The chromosome 9 sequences of the 9q+ breakpoint subclone were not amplified in K562. The amplification of chromosome 9 sequences in K562 starts with the chromosome 9 sequences located at the 22q- breakpoint of K562 and this whole area, including the 5′ part of the bcr gene and the c-abl oncogene is amplified. The 22q- breakpoint of this cell line maps at a distance of more than 100 kb 5′ of the c-abl exon a2 (8). Since the chromosome 9 sequences in the 9q+ breakpoint segment of CML 83-H84 are not amplified in K562, these must be located at the 5′ side of the K562 breakpoint on chromosome 9, at a distance

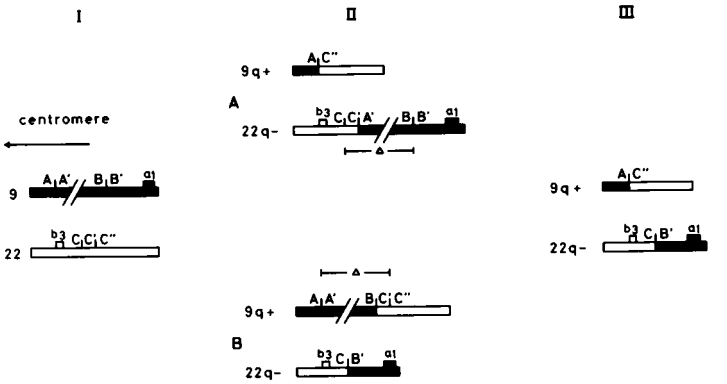

Fig. 5. Hypothetical model of the Ph^1 translocation and secondary recombination in CML patient 83-H854.
I. The normal chromosome 9 and (closed bars) chromosome 22 (open bars).
II. The resulting translocation products of the initial Ph^1 translocation and
III. The result of either a recombination of the 22q- chromosome (IIA) or the 9q+ chromosome (IIB) A,A´,B,B´are chromosome 9 sequences; C, C´,C´´ are chromosome 22 sequences, A/C´´ represents the 9q+ breakpoint whereas C/B´ represents the 22q- breakpoints shown in figure 1B and 1D resp. ———//——— indicates a gap of at least 70 kb. b3 = bcr exon b3 (6) a1 c-abl exon a1 (8). The distances are not in scale.

of >70 kb (>100-30) 5´ of the 22q- breakpoint of CML 83-H84. The chromosome 9 sequences inbetween these two breakpoints are probably deleted since no extra aberrant fragments were detectable when we used chimeric fragments as probes in a Southern blot of CML 83-H84 DNA and control lines DNA. However we cannot exclude the possibility that these sequences are present somewhere else in the genome. Sequencing confirmed that the breakpoints in the bcr of chromosome 22 were located 3´ of bcr exon b3 (6), but not at an identical site. The 88 nucleotides of bcr between the two chromosome 22 breakpoints are also deleted. Probably these deletions are the results of secondary recombination events, occurring at either the original Ph^1 or 9q+ chromosome (Fig. 5). Although we have no direct proof we favor the explanation that the second recombination event took place at the Ph^1 chromosome (Fig. 5, IIA) This would result in a shorter Ph^1 chromosome on which the 5´ bcr exon b3 is located 30 kb 5´ of c-abl exon a1, whereas on the original Ph^1 chromo-

some the distance between these two coding regions is more than 100 kb. This second recombination could either be necessary to remove chromosome 9 inhibitory or regulatory sequences in order to allow transcription of the chimeric bcr/c-abl mRNA, or it provides the resulting leukemic cell with growth advantages so that it has replaced the normal stem cells and original Ph^1 positive leukemic cells. In other CML's, such as CML 0319129 (6) where the recombination took place at chromosome 9 sequences located 14 kb 5' of c-abl exon a1, no such secondary recombinations are necessary. The breakpoint sequences of this patient (CML 0319129) showed a perfect conservative break without loss of chromosome 9 or 22 sequences. Comparison of the DNA sequences at the breakpoint regions of CML 83-H84, K562 (20) and and two other CML patients (6) suggests that homologous recombination is unlikely since there is no apparent homology between chromosome 9 and 22 breakpoint sequences. Nor is there any evidence for crossover within an homologous oligonucleotide. Similar results have been obtained from sequence analysis of the t(8;14) translocations in Burkitt lymphoma, in which no obvious homology was detectable between the recombined sequences on chromosome 8 or 14 (21,22). However, in CML there is some evidence that Alu-repetitive sequences are involved. In CML 83-H84 both the 22q- and the 9q+ breakpoint occur within an Alu-repetitive sequence. Similar homology to Alu-repetitive sequences are present at the breakpoints of two other CML patients (6,12) the cell line K562 (20) and the 22q- breakpoint of a Ph^1 positive Acute Lymphoblastic Leukemia (ALL) patient (23). Illegitimate recombination within Alu sequences has also been reported in non-CML related recombination events (24,25). Therefore it is well conceivable that Alu-repetitive sequences are hot spots for recombination and as such play a role in the juxtaposition of the 5' bcr and c-abl sequences. The apparent transcription of a chimeric bcr/c-abl mRNA (9,26) could be facilitated by secondary recombination events, bringing the bcr exons in closer proximity to the c-abl coding regions. But the question remains open whether the translocation product of this mRNA, the 210 kD abnormal c-abl protein is the cause or merely a consequence of transformation in CML.

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