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Nucleotide sequence of both reciprocal translocation junction regions in a patient with Ph positive acute lymphoblastic leukaemia, with a breakpoint within the first intron of the *BCR* gene

M.J.M.van der Feltz+*, M.K.K.Shivji, P.B.Allen, N.Heisterkamp[1], J.Groffen[1] and L.M.Wiedemann

Leukaemia Research Fund Centre, Institute of Cancer Research, Chester Beatty Laboratories, 237 Fulham Road, London, SW3 6JB, UK and [1]Childrens Hospital of Los Angeles, 4650 Sunset Boulevard, Los Angeles, CA 90027, USA

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ABSTRACT

Breakpoints on chromosome 22 in the translocation t(9;22) found in Philadelphia positive acute lymphoblastic leukaemia patients fall within two categories. In the first the breakpoint is localized within the breakpoint cluster region of the *BCR* gene, analogous to the chromosome 22 breakpoint in chronic myeloid leukaemia. The second category has a breakpoint 5′ of this area, but still within the *BCR* gene. We have previously shown that these breakpoints occur within the first intron of the *BCR* gene and cloned the $9q^+$ junction from such a patient. We have now determined the sequences around the breakpoints on both translocation partners from this patient as well as the germline regions. The chromosome 9 *ABL* sequence around the breakpoint shows homology to the consensus Alu sequence whereas the chromosome 22 *BCR* sequence does not. At the junction there is a 6 bp duplication of the chromosome 22 sequence which is present both in the $9q^+$ and in the $22q^-$ translocation products. Possible mechanisms for the generation of the translocation are discussed.

INTRODUCTION

The most common chromosomal aberration observed in adult acute lymphoblastic leukaemia (ALL) is the translocation t(9;22)(q34;q11) which is found in 10–20% of these patients (1). Cytogenetically it is detectable by the presence of the $22q^-$ or Philadelphia (Ph) chromosome. The Ph chromosome is also observed in 95% of chronic myeloid leukaemia (CML) patients (2) and in a small percentage of children with ALL (3). In CML and in approximately 50% of adult Ph positive ALL, the breakpoint on chromosome 22 is located in the so-called breakpoint cluster region (bcr) in the 3′ portion of the *BCR* gene** (5,6).

In the other Ph positive ALLs the breakpoints are more 5′ but still in the *BCR* gene at various sites within the first large intron (7–11). In both CML and Ph positive ALL the breakpoints on chromosome 9 are within the *ABL* proto-oncogene, upstream of the common exon (7,12,13). In the hybrid *BCR/ABL* mRNA resulting from the translocation the common *ABL* exon is always spliced onto the various stretches of RNA derived from chromosome 22 depending on the location of the breakpoint (7,13,14). The difference in breakpoints on chromosome 22 is reflected in the size of the hybrid mRNA and the derived protein; an 8.5 kb mRNA and a 210 kD protein result from the breakpoint occurring within the breakpoint cluster region while a 7 kb mRNA and a 190 kD protein are present when the breakpoint is in the first *BCR* intron (7–11, 13–19). Both protein products

**We have adopted the nomenclature recommended in Human Gene Mapping 9 (4) reserving capital letters for the genes *BCR* and *ABL* and retaining lower case bcr as the actual breakpoint cluster region originally described in association with CML (5).

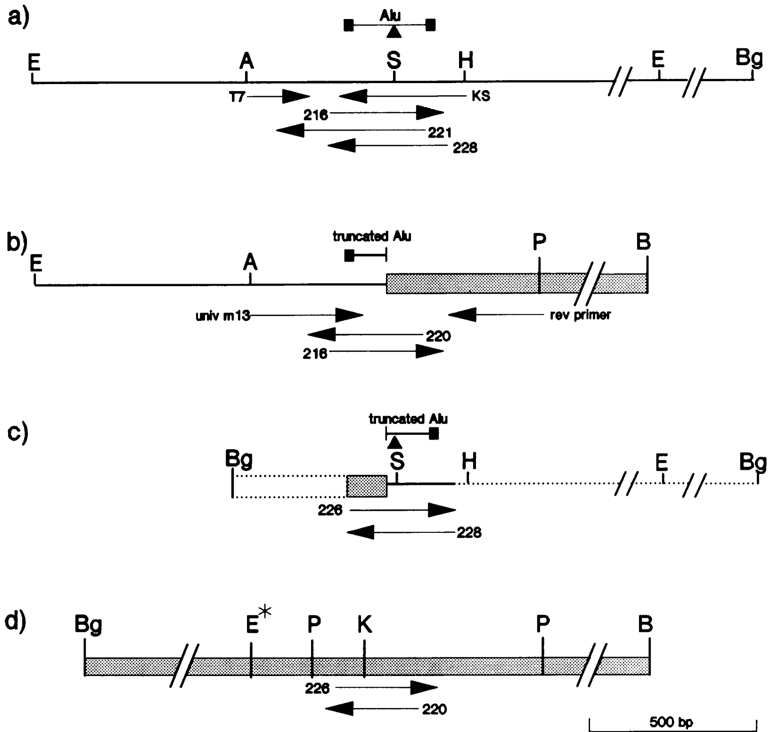


Figure 1. *Restriction maps and sequencing strategy of the translocation junctions of chromosomes 9 and 22 present in the leukaemic cells of patient FY.*
Sequence was obtained from subclones of the regions shown in a) and b) and described in Materials and Methods. Oligomers used as primers for each sequencing reaction are indicated in Figure 2a. Sequences originating from chromosome 9 are indicated as a line while chromosome 22 sequences are shown as rectangular stippled boxes. In c) the PCR fragment cloned is shown in context; the portions indicated by dotted lines and boxes are regions not represented in recombinant clones from the $22q^-$ chromosome. The subclone represented in d) was from a K562 library. Alu homologous regions are indicated; the black triangle denotes the missing 40 bp (see text and Figure 2a). The restriction sites shown are: A, *Acc*I; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; and S, *Sma*I. Not all restriction enzyme sites have been mapped. The *Eco*RI site with an * is contained in a polymorphism present in chromosome 22 (see (20)). Due to this polymorphism this region (1 kb) is not present in the $22q^-$ translocation product described here.

seem to have an enhanced tyrosine kinase activity as compared to the normal 145 kD *ABL* protein when assayed *in vitro*.

Previously we had cloned the $9q^+$ junction fragment from the DNA of a Ph positive p190 ALL patient and localized the breakpoint within the first intron of the *BCR* gene (20). Here we have identified the sequences at the $9q^+$ and 22 q^- junctions to see if the breakpoint is similar or distinct from those occurring in CML junctions (21–23) or other acute leukaemias.

MATERIALS AND METHODS

Cloning

A genomic library of the DNA from a Ph positive p190 ALL patient (FY) was prepared as described (20). From this library the $9q^+$ junction 3.8 kb *Eco*RI/*Bam*HI fragment

[3.8EB/9q+] was subcloned (20) in pUC9 (24). After digestion with *Eco*RI, partial digestion with *Acc*I and filling in of the cohesive ends followed by blunt-end ligation, a subclone was generated containing the 3.1 kb *Acc*I/*Bam*HI fragment [3.1AB/9q+] encompassing the breakpoint junction (Figure 1b). The germline chromosome 9 *ABL* fragment was also isolated from the same library and subcloned as a 1.3 kb *Eco*RI/*Hind*III fragment [1.3EH/9] in Bluescript KS+ (Stratagene) (20). By deleting the *Eco*RI/*Acc*I fragment as above a subclone containing a 0.6 kb insert [0.6AH/9] was generated (Figure 1a). A germline subclone in pUC 19 (25) of chromosome 22 was also obtained as a 5.3 kb *Bam*HI fragment [5.3BB/22) from a K562 library (26) (Figure 1d).

DNA sequencing

Sequencing was done by using the dideoxy chain termination method (27) using Sequenase™ (United States Biochemical Corporation) on double stranded templates (28). The sequencing strategy covering the various regions analyzed is shown in Figure 1. Sequencing was initiated on the *ABL* 0.6AH/9 subclone (Figure 1a) using the T7 primer (Stratagene). To get closer to the junction a second primer was synthesized (primer 216) from the sequence obtained and used to prime both the *ABL* 0.6AH/9 and FY 3.1AB/9q+ subclones (Figure 1a,b), revealing the 9q+ junction sequence. Further primers were synthesized as indicated in Figure 1a,b (primer 220,221) to confirm sequence obtained and to characterize the *BCR* 5.3BB/22 clone (Figure 1d). Only nucleotides obtained by sequencing of both strands are given in Figure 2. Sequencing gels (6% urea-polyacrylamide (20:1), 40 cm) were routinely run for 2, 5 and 8 hours. When ^{35}S-dATP was used the gels were fixed for 1 hour in 10% acetic acid and dried. Exposure was for 1−2 days at room temperature using Kodak XAR-5 film. Computer analyses of sequences were done using the Micro Genie package (Beckman).

Polymerase chain reaction

DNA from patient FY (20) was used to amplify the 22q− junction fragment and a fragment from the normal chromosome 22. As a control a Ph negative myeloid cell line (KG1) (29) was used along with plasmid DNA containing the target sequences for the normal chromosome 22 (5.3BB/22); normal chromosome 9 (0.6AH/9) and chromosome 9q+ (3.1AB/9q+). Oligonucleotide primers were synthesized on an Applied Biosystems 381A DNA synthesizer based on the sequence data generated from the genomic clones described above.

Amplification of the target sequences with Taq polymerase (Cetus/Perkin-Elmer) was done using a modification of the standard DNA amplification procedure (30) but with a higher primer annealing temperature (50°C instead of 40°C). Reaction volumes (100 μl) contained 1 μg genomic DNA (KG1 or FY) or 10 ng plasmid DNA (see above), 2.5 mM each of dATP, dCTP, dGTP and dTTP; 1 μg of each primer (two primers per reaction, Figure 3a) and 10% dimethylsulfoxide (DMSO) in 1× Taq buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$ and 170 μg/ml BSA). Samples were heated at 95°C for 5 minutes and transferred to 50°C for 1 minute. Taq polymerase (2 units in 1× Taq buffer) was added to each sample. The 100 μl reaction volume was overlaid with mineral oil to prevent evaporation. Using a pre-programmed robot arm (Cyber Robotics Ltd., England) 30 cycles were performed of 5 minutes 70°C, 2 minutes 95°C and 1 minute 50°C. Samples (1/10 of each reaction volume) were electrophoresed through a 2% agarose gel (Figure 3b).

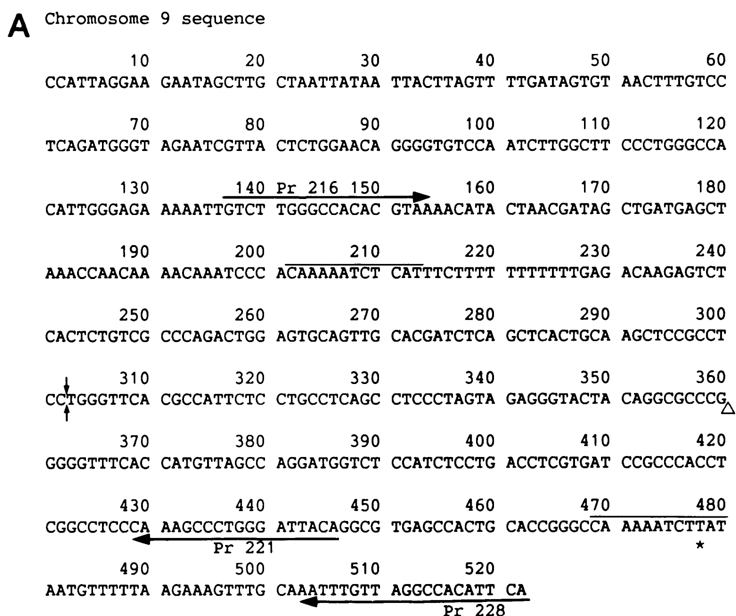
In order to obtain a larger supply of the chromosome 22q− fragment, the above reactions were repeated to further amplify this sequence. The 318 bp fragment was purified using Geneclean™ (BIO 101, Inc.) according to the manufacturer's protocol. The purified

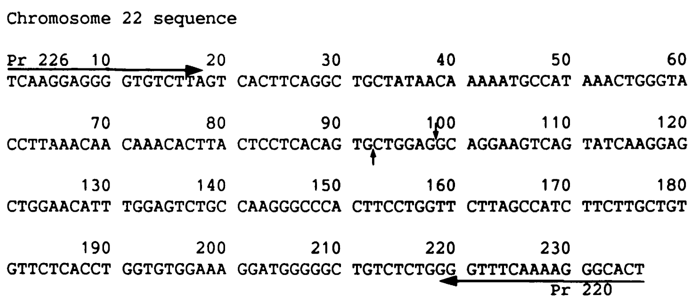
22q⁻ fragment was cloned into the *Sma*I site of Bluescribe (Stratagene) and sequenced as described above from the same primers as used to generate the fragment.

RESULTS

Characterization of the chromosome 9,9q$^+$ and 22 sequences surrounding the junctions.

The breakpoint junction fragments from the DNA of the patient described here have been characterized by cloning and restriction mapping (20). The chromosome 9 breakpoint





B

JUNCTION SEQUENCES

Chromosome 9	GCTCACTGCAAGCTCCGCCTCC------TGGGTTCACGCCATTCTCCTGC
Chromosome 9q+	GCTCACTGCAAGCTCCGCCTCCctggaggcaggaagtcagtatcaaggag
Chromosome 22q-	caaacacttactcctcacagtgctggagTGGGTTCACGCCATTCTCCTGC
Chromosome 22	caaacacttactcctcacagtgctggaggcaggaagtcagtatcaaggag

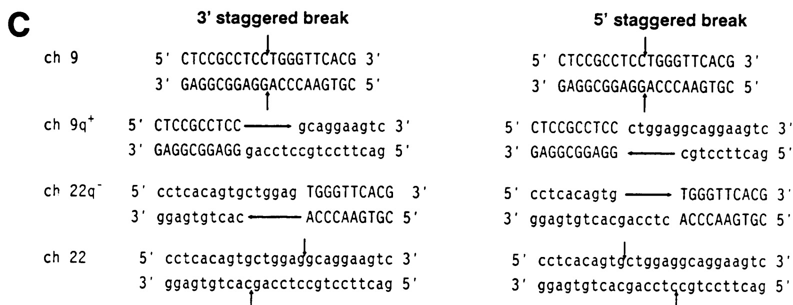


Figure 2.

A. Nucleotide sequence of the chromosome 9 and 22 regions around the breakpoint. Normal chromosome 9 sequences are from patient FY; chromosome 22 sequences are from K562 DNA. The position of the primers (and direction) used in sequencing and PCR is indicated. Predicted breaks are marked by vertical arrows. The Alu flanking repeat in the chromosome 9 sequence is indicated by overline. The single base non-match in the repeat is designated by *. The triangle between nucleotides 360 and 361 denotes the position of the missing 40 bp of Alu family sequence (see text).

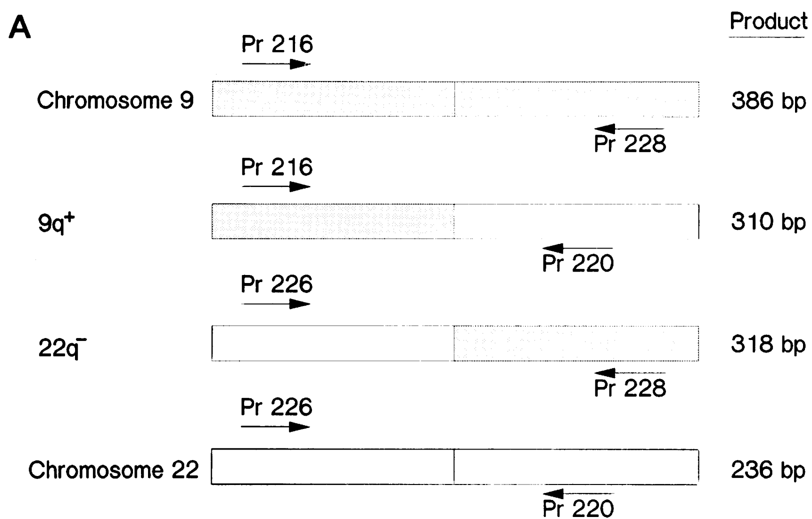
B. Junction sequences in t(9;22) in patient FY. Chromosome 9, $9q^+$ and $22q^-$ sequences are from patient FY; normal chromosome 22 sequences are from K562 DNA. Chromosome 9 derived sequence is indicated by capitals; chromosome 22 derived sequence by lower case. The 6 bp sequence ctggag from chromosome 22 is present in both translocation products.

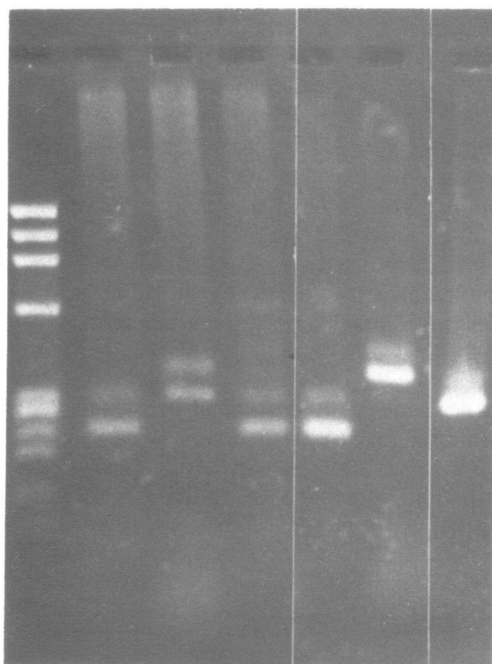
C. A possible mechanism accounting for the t(9;22) in patient FY.

was localized in the intron of *ABL* between exon Ia and the common exon (a2). The breakpoint on chromosome 22 was not in the breakpoint cluster region of the *BCR* gene but was located 5′ in the first intron. In order to further analyze the junctions we sequenced the chromosome 9, $9q^+$ and 22 fragments around the breakpoint. The sequencing strategy is shown in Figure 1 and the sequence itself in Figure 2a,b. In the *ABL* sequence there is a high degree of homology with Alu repetitive sequences (31). Alu flanking repeats can be identified at positions 202–213 and 469–480 (Figure 2a). At nucleotide 360 a 40 bp stretch of Alu is missing. There is no Alu homology in the *BCR* sequence near the breakpoint.

Synthesis by PCR and analysis of the $22q^-$ junction fragment.

The map and size of the *Bgl*II $22q^-$ junction fragment were deduced from the 9, $9q^+$ and 22 maps assuming no large deletions and insertions were present. After *Bgl*II digested DNA from the patient's leukaemic cells was blotted and hybridized with a probe from the *ABL* gene 3′ of the breakpoint, a 6.2 kb rearranged band was observed in addition to the germline 11.2 kb *Bgl*II *ABL* band (20). This was in accordance with the predicted size and therefore confirming the absence of any large insertions or deletions at the $22q^-$ junction. When sequence around the breakpoint was available, oligonucleotide primers were synthesized (indicated in Figure 2a,3a), and used to amplify the $22q^-$junction directly from the patient's DNA by the polymerase chain reaction. We also performed control reactions, both from genomic DNA and from plasmids containing the relevant chromosome 9, $9q^+$ and 22 sequences. When amplified the size of the target DNA fragments would be delineated by the set of primers employed (Figure 3a). In Figure 3b the fragments obtained are shown, after separation by gel electrophoresis. The amplified fragment from chromosome 22 is 236 bp, as predicted in both the genomic (lane 2,4) and the cloned DNA (lane 5) samples. The sizes of the amplified chromosome 9 and $9q^+$ were also as anticipated (lane 6,7). The amplified $22q^-$ fragment was about 320 bp in


A
Product
Pr 216
Chromosome 9
386 bp
Pr 228
Pr 216
9q+
310 bp
Pr 220
Pr 226
22q-
318 bp
Pr 228
Pr 226
Chromosome 22
236 bp
Pr 220


B 1 2 3 4 5 6 7

size (lane 3), supporting the previous interpretation of Southern blot analysis which provided no evidence for major deletions or insertions. This $22q^-$ fragment was cloned and sequenced.

DISCUSSION

Mechanisms responsible for the various types of rearrangement of genomic sequences in normal and neoplastic cells have been an intense area of investigation in a number of laboratories (32). These rearrangements include deletions (33–35), inversions (36,37) and translocations (21–23, 38–40). Some have obvious biological implications, such as those involving the antigen receptor rearrangements (41). In the case of the Ph chromosome, only a limited number of breakpoints, all from patients with CML, have been sequenced and analyzed in detail (21–23). Comparisons between these reveal few obvious conserved features. In each case analyzed, at least one of the two breakpoints occurred within a member of the Alu repeat family. Both the chromosome 9 and the chromosome 22 sequence around one bcr rearranged Ph positive ALL breakpoint analyzed are homologous to Alu sequences (L. Hoefsloot and G. Grosveld, unpublished observations).

Several translocations which occur in B- and T-cell malignancies appear to result from aberrant joining of sequences similar to those present in the immunoglobulin and T-cell receptor gene rearrangements (42). Since the Ph chromosome translocations resulting in the 190 kD *BCR/ABL* protein occur primarily in acute leukaemias with a B-cell precursor phenotype (7,11,17,18,20), it was of interest to determine whether similar antigen receptor sequence features could be identified in association with these rearrangements. In contrast to the junctions observed in the *BCL2* or *MYC* rearrangements (42), there were no obvious features reminiscent of the mechanisms of immunoglobulin rearrangements in the junctions of the reciprocal translocation products in the patient we have analyzed. In particular, no heptamer-nonamer sequence similarity was noted, nor any N region sequences at either junction. It is therefore unlikely that the enzymes which are normally responsible for rearrangement of the immunoglobulin genes are involved in the translocation resulting in this case of Ph positive ALL. A conserved tetranucleotide sequence, GAGG, which has been reported to occur close to the breakpoint in five out of six translocations between *MYC* and the immunoglobulin heavy-chain switch regions in mouse plasmacytomas (43) does occur near the chromosome 22 sequence of the FY breakpoint. Its complement, CCTC, occurs near the chromosome 9 breakpoint. These similarities to the switch region sequence raise the possibility that this translocation may involve enzymes responsible for immunoglobulin switching.

Two clearly distinguishable steps occur in the mechanism of translocation: cleavage and joining. Very little is known about mechanisms of double-stranded cleavage in eukaryotic

Figure 3. *PCR of translocation products.*
A. Cartoon to show the positions of the primers used in the reactions. Various combinations are shown which flank the target breakpoint regions of the patient DNA in this study. The sequence of the primers is indicated in Figure 2a.
B. Ethidium bromide stained gel of the amplified target fragments delineated by the primers described in A. Lanes 2–4 are reactions containing the appropriate genomic DNAs; lanes 5–7 are plasmid controls. Normal chromosome 22 (primer pair 226/220–lane 2: patient FY, lane 4: cell line KG1 and lane 5: 5.3BB/22); normal chromosome 9 (primer pair 216/228–lane 6: 0.6AH/9); chromosome $9q^+$ (primer pair 216/220–lane 7: 3.1AB/$9q^+$) and chromosome $22q^-$ (primer pair 226/228–lane 3 patient FY). Markers (lane 1): *Hae*III digested ΦX174 DNA (1353, 1078,872,603,310,281,271,234,194,118 and 72 bp). Reaction mixture (10 μl) was mixed with 2 μl sample buffer (50% glycerol, 0.5×TBE and 1 mg/ml orange G) and electrophoresed through a 2% agarose gel at 60 mA for 40 minutes.

cells. A stem-loop structure can be drawn in the vicinity of the breaks in both chromosome 9 and 22 sequences described here. Although the free energy values for these do not indicate a particularly stable structure, it is possible that proteins can bind, stabilize and cleave nearby. In addition, Alu binding proteins (whose function is currently unknown) may play a role in cleavage of DNA which may explain the presence of Alu homologous regions in a large number of rearrangement junctions.

In the context of translocations or gene deletions various joining mechanisms have been suggested. In some cases, homologous recombination has been proposed to occur between dispersed repeat families such as Alu. Several of the globin deletions characterized in thalassemias (34), deletions within the low density lipoprotein receptor gene (35), and a translocation between the X and Y chromosome identified in an XX male (40) appear to be the result of an Alu-Alu recombination event. Despite the presence of numerous Alu sequences in and around most Ph chromosome junctions (21–23), including the one presented here, homologous recombination does not appear the general mechanism since an Alu repeat is present at the site of the breaks on only one of the two chromosomal partners in several cases.

Other deletions such as those analyzed in the *aprt* locus (33) often appear to occur between very short direct repeats of 2 to 7 basepairs resulting in a single copy of the repeat in the mutated gene. Recombination between very short regions of homology does not appear to play a role in the translocation presented here.

Another feature of some rearrangements is the loss or duplication of nucleotides at the junction. Roth and colleagues (44) have designed a model system to determine cell-mediated mechanisms of end joining. They find that homology-independent end joining is more common than a homology-dependent process. The model they present can be adapted to the junction products whose sequences are presented in this paper. The sequence junctions indicate that the rearrangement has occurred without any deletions. At the junction, there is a duplication of 6 basepairs originating from chromosome 22, present in both the $9q^+$ and $22q^-$ fragments (Figure 2b). The nature of these sequences suggests that cleavage of the chromosome 9 sequences was the result of a blunt-ended cut, while that on chromosome 22 originated from a staggered cut. It is not possible to predict whether this cut resulted in a 5′ or a 3′ overhang. In either case it is likely that the fragments were joined by single stranded ligation, since 3′ protrusions cannot be filled in and 5′ protrusions rarely are (44). After gap repair and ligation of the other strand both translocation products would contain the 6 basepairs originally from chromosome 22 (Figure 2c). In Roth's model system, both staggered cuts (*Xba*I and *Sac*I) joined to a blunt end (*Sma*I) molecule appear to result in a high proportion ($>60\%$) of junctions without any loss of nucleotides (44). Direct repeats such as those observed in the reciprocal translocation products described here are also reminiscent of retroviral (45) and retroposon (46) insertion sites. Similar mechanisms may play a role there.

It is clear from the above discussion that the Ph chromosome breakpoints so far analyzed represent very diverse features. It may be necessary to expand the number of Ph chromosome junction sequences from both reciprocal products in order to identify true conserved characteristics.

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*To whom correspondence should be addressed

+Present address: Dr Daniel den Hoed Cancer Center, PO Box 5201, 3008 AE Rotterdam, The Netherlands

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