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Genetics

The partial tandem duplication of *ALL1* (*MLL*) is consistently generated by *Alu*-mediated homologous recombination in acute myeloid leukemia

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ABSTRACT **Chromosome abnormalities resulting in gene fusions are commonly associated with acute myeloid leukemia (AML), however, the molecular mechanism(s) responsible for these defects are not well understood. The partial tandem duplication of the *ALL1* (*MLL*) gene is found in patients with AML and trisomy 11 as a sole cytogenetic abnormality and in 11% of patients with AML and normal cytogenetics. This defect results from the genomic fusion of *ALL1* intron 6 or intron 8 to *ALL1* intron 1. Here, we examined the DNA sequence at the genomic fusion in nine cases of AML with a tandem duplication of *ALL1* spanning exons 2–6. Each breakpoint occurred within intron 6 of the *ALL1* breakpoint cluster region and within a discrete 3.8-kb region near the 3′ end of intron 1. In seven cases, a distinct point of fusion of intron 6 with intron 1 could not be identified. Instead, the sequence gradually diverged from an *Alu* element in intron 6 to an *Alu* element in intron 1 through a heteroduplex fusion. Thus, these rearrangements appear to be the result of a recombination event between homologous *Alu* sequences in introns 6 and 1. In two cases, the genomic junction was distinct and involved the fusion of a portion of an *Alu* element in intron 6 with non-*Alu* sequence in intron 1. These data support the hypothesis that a recombination event between homologous *Alu* sequences is responsible for the partial tandem duplication of *ALL1* in the majority of AML cases with this genetic defect. Although *Alu* element-mediated homologous recombination events in germline cells are thought to be responsible for partial gene duplications or deletions in many inherited diseases, this appears to be the first demonstration identifying *Alu* element-mediated recombination as a consistent mechanism for gene rearrangement in somatic tissue.**

In patients with acute leukemia, cytogenetic translocations involving chromosome region 11q23 usually result in the fusion of a gene known as *ALL1* (1, 2), also referred to as *MLL*, *HRX*, and *HTRX1* (3–5), with a variety of different genetic partners (6). In patients with acute myeloid leukemia (AML) but without cytogenetic abnormalities involving 11q23, we discovered a molecular defect involving *ALL1* in which the gene undergoes a partial tandem duplication (PTD) of itself (7, 8). The PTD of *ALL1* is present in the majority of patients with AML and trisomy 11 (+11) as a sole cytogenetic abnormality (9, 10) and in ≈11% of patients with AML and normal cytogenetics (11, 12).

Chromosome abnormalities resulting in gene fusions are commonly associated with AML, however, the molecular mechanism(s) responsible for these defects are not well understood (13). In many of the lymphoid malignancies, the presence of heptamer-nonamer recombination signals adjacent to the genomic breakpoints on both chromosomes has suggested that V(D)J recombinase activity is involved in illegitimate recombination events leading to these translocations (14). A similar mechanism has not been identified in the majority of molecular defects found in AML (13). Chromosome translocations involving *ALL1* often are present in patients who develop secondary leukemia following treatment of a primary malignancy with drugs that inhibit topoisomerase II (15, 16). Although several topoisomerase II DNA binding sites have been identified within the 11q23 breakpoint cluster region (bcr) (17), and *in vitro* treatment of cell lines with topoisomerase II inhibitors induces double-stranded breaks within this region (18), the mechanism of translocation associated with these drugs has not been elucidated.

In two cases of *ALL1* PTD, we and others have provided evidence to suggest that the genomic rearrangement involved a recombination event between *Alu* sequences (19, 20). In this study, we examined the DNA sequence at the genomic fusion in nine additional cases of primary AML, each with an *ALL1* PTD spanning exons 2–6. The results of this study support the hypothesis that a recombination event between homologous *Alu* sequences is responsible for the *ALL1* PTD in the majority of cases with this defect. Although *Alu*-mediated homologous recombination events in germline cells are thought to be responsible for genetic defects in many inherited diseases (21–26), this appears to be the first demonstration identifying *Alu*-mediated homologous recombination as a consistent mechanism for gene rearrangement in somatic tissue.

MATERIALS AND METHODS

Patients. Nine patients with primary AML were studied. The presence of *ALL1* PTD has been described for patients 23 (8), 24 (8), 101 (11), 166 (11), 211 (11), and 300 (11). For patients 316, 340, and 350, the presence of *ALL1* PTD was determined during this study. DNA was extracted from the leukemic specimens, digested with *Eco*RI or *Hin*dIII and probed by Southern blot analysis using the B859 and SAS1 probes (8). By this method, each sample was found to have a rearranged *ALL1* gene (data not shown). By using described amplification conditions (9), further analysis of total cellular RNA from each case by reverse transcription-PCR revealed the presence of an *ALL1* PTD (data not shown). All cases in this study had a tandem duplication of *ALL1* spanning exons 2–6.

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Abbreviations: AML, acute myeloid leukemia; PTD, partial tandem duplication; +11, trisomy 11; bcr, breakpoint cluster region.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF033349).

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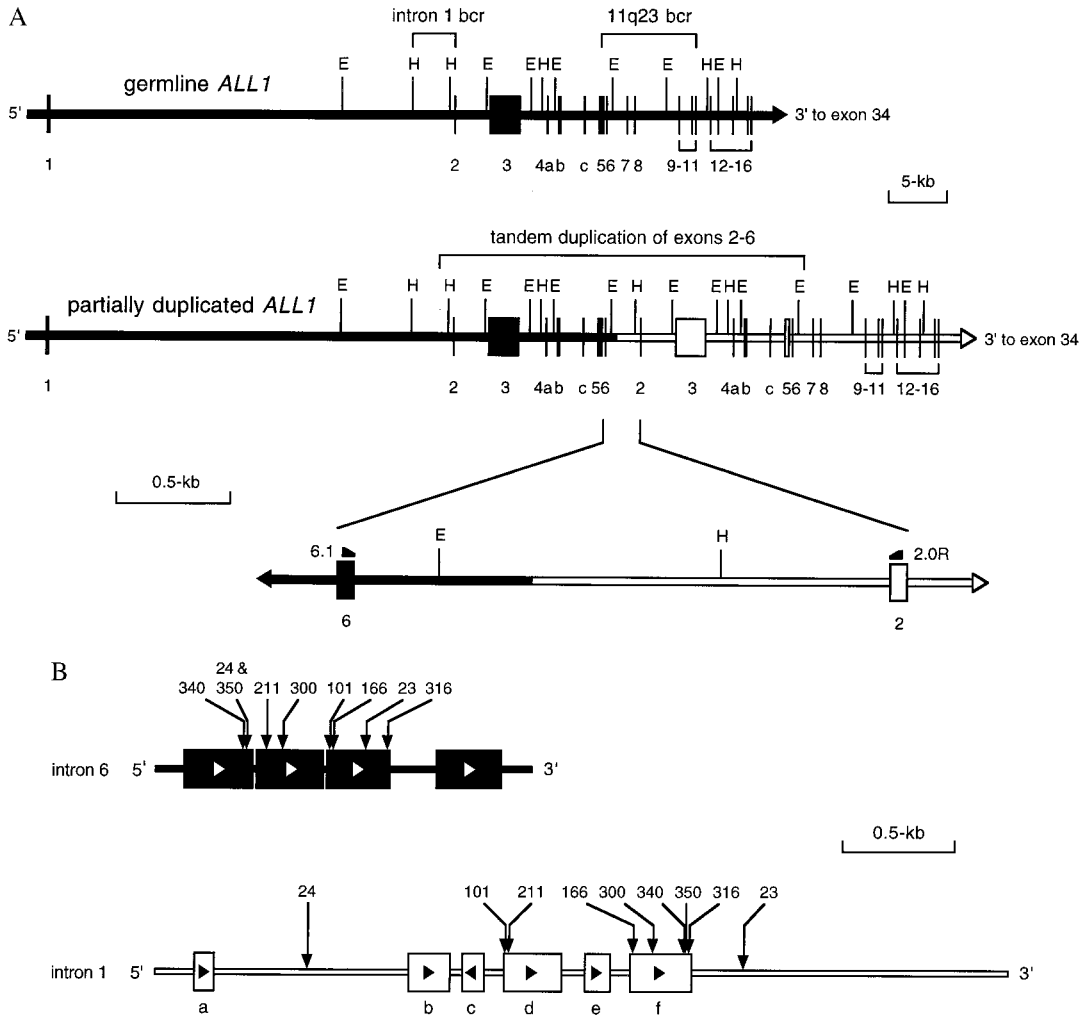

FIG. 1. Cloning the genomic breakpoints involved in the tandem duplication of *ALL1* exons 2–6. (*A*) The wild-type *ALL1* gene is normally composed of 36 exons. For the purposes of this figure, only exons 1–16 are shown. In this study, all breakpoints occurred within intron 6 of the 11q23 bcr and within a discrete region near the 3′ end of intron 1, indicated as intron 1 bcr. Tandem duplication of *ALL1* exons 2–6 results from a fusion of intron 6 with intron 1 in a 5′ to 3′ direction. This is indicated by the fusion of the solid bar with the open bar. The fusion region is enlarged to show the location of PCR primers 6.1 and 2.0R and restriction sites used for cloning the breakpoints. E, *Eco*RI; H, *Hin*dIII. (*B*) *Alu* elements and breakpoints involved in the tandem duplication of *ALL1* exons 2–6. Intron 6 is 1.7 kb long and contains four full-length *Alu* elements, as represented by the solid boxes. Open arrows in the boxes indicate the orientation of the *Alu* elements. The location of the breakpoint for each case is indicated by a vertical arrow and corresponding patient number. The breakpoints of patients 24 and 350 are only one nucleotide apart and are indicated by the same arrow. The intron 1 bcr is 3.8 kb and contains 2 *Alu* elements (boxes d and f), and four shorter portions of *Alu* elements (boxes a, b, c, and e). Solid arrows in the boxes indicate their orientation. The location of the breakpoint for each case is indicated by the vertical arrow and corresponding patient number.

PCR. Genomic DNA was extracted by using a standard isolation procedure (27). PCR was performed by using *Taq* Extender PCR Additive (Stratagene) according to the manufacturer's instructions. The upstream primer was 6.1 from exon 6 and the downstream primer was 2.0R from exon 2 (9). Reactions were carried out for 35 cycles (95°C for 1 min, 60°C for 1 min, and 72°C for 3.5 min), followed by a 10 min extension at 72°C. All PCR products were subsequently analyzed on 0.8% agarose gels stained with ethidium bromide.

Breakpoint Subcloning. PCR amplification products were purified (Qiagen, Chatsworth, CA) and then digested by using restriction enzymes *Eco*RI and *Hin*dIII. In all cases, the presence of an *Eco*RI restriction site in intron 6, 363-bp downstream of exon 6, and a *Hin*dIII restriction site in intron 1, 724-bp upstream of exon 2, allowed for the subcloning of a fragment containing the intronic fusion into pBluescript (Stratagene) (Fig. 1*A*).

DNA Sequencing and Analysis. DNA sequencing of plasmids was performed by using an Applied Biosystems model 373 Stretch DNA Sequencing System. To avoid inclusion of

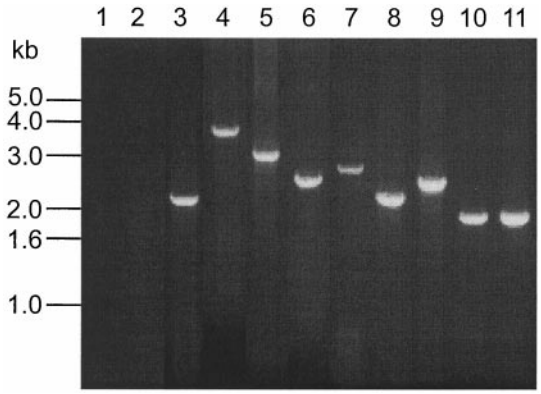

FIG. 2. PCR products amplified from genomic DNA of nine patients with tandem duplication of *ALL1* exons 2–6 by using primers 6.1 and 2.0R. Lanes 1 and 2 are reagent and normal controls, respectively. Lanes 3–11 show the amplified products from patients 23, 24, 101, 166, 211, 300, 316, 340, and 350, respectively. Bands ranged in size from 1.9 to 3.6 kb.

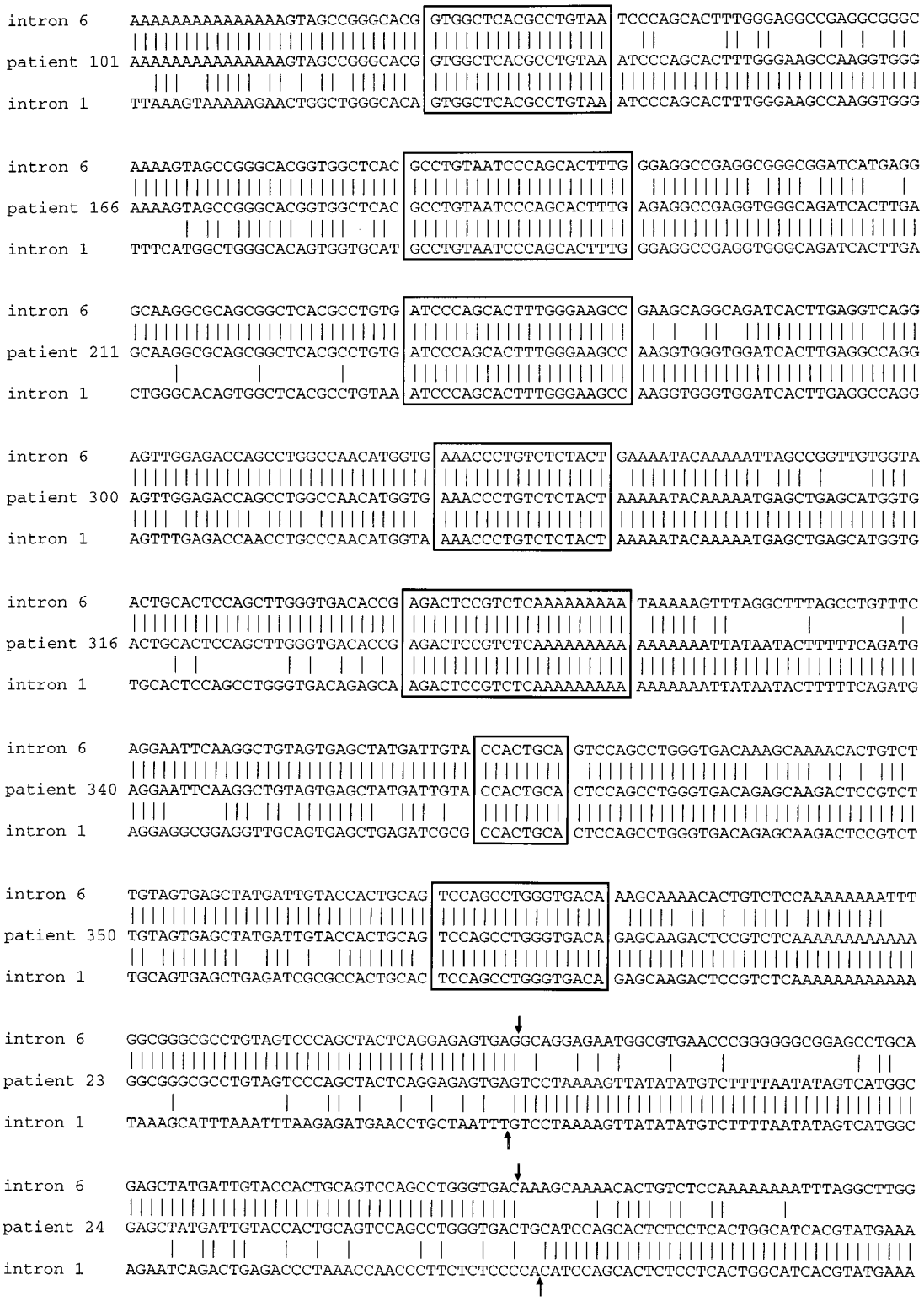

FIG. 3. DNA sequence of genomic fusion from nine cases with tandem duplication of *ALL1* exons 2–6. Fusion sequences are compared with germline intron 6 and intron 1 sequence. Vertical lines indicate sites of nucleotide homology. Patients 101, 166, 211, 316, 340, and 350 showed evidence of recombination between *Alu* elements from introns 6 and 1. Boxed sequences indicate overlapping regions of homology, consistent with heteroduplex formation during homologous recombination. Patients 23 and 24 had fusion of *Alu* sequence from intron 6 to non-*Alu* sequence from intron 1. For these two cases, the arrows indicate the breakpoints within introns 6 and 1. Sequences are 5′ to 3′.

PCR generated point mutations in the sequences, three different subclones from each case were sequenced. All fusion sequences were compared with germline sequences by using the GENEWORKS computer program (IntelliGenetics, Campbell, CA). The *ALL1* intron 6 germline sequence was obtained from GenBank. A genomic DNA fragment containing germline *ALL1* intron 1 was provided by Rolf Marschalek and Iris Nilson (University of Erlangen-Nürnberg, Germany). The intron 1 bcr extends 3.8 kb upstream from exon 2 to a *Hin*dIII site. This region was sequenced and compared against the

GenBank database. *Alu* mapping was performed by using the basic local alignment search tool (BLAST).

RESULTS AND DISCUSSION

The *ALL1* PTD spanning exons 2–6 has two breakpoints within *ALL1*; one within intron 6 and one within intron 1 (8). The result is a fusion of intron 6 with intron 1 in a 5′ to 3′ direction (Fig. 1*A*). To determine the precise location of the intronic breakpoints, fusion sequence was compared with the germline sequences of introns 6 and 1. The sequence of intron 6 has been previously reported and has been shown to contain four *Alu* elements (17). Although intron 1 is ≈35 kb long (28), the intron 1 breakpoints all clustered within a discrete 3.8-kb region near the 3′ end of the intron, which we have designated as the intron 1 bcr (Fig. 1*A*). Sequence analysis of this region identified two *Alu* elements and four shorter portions of *Alu* elements (Fig. 1*B*). Characteristically, *Alu* elements are composed of a tandem repeat of two highly homologous sequences separated by a short A-rich region and are 300 bp long (29). Four distinct segments in the intron 1 bcr do not have these standard *Alu* features, but instead consist of portions of *Alu* sequence ranging from 97 to 186 bp (Fig. 1*B*).

We analyzed the fusion sequence in genomic DNA extracted from the blasts of nine patients with AML and *ALL1* PTD of exons 2–6. In all nine cases, the genomic breakpoints were cloned by using PCR (Fig. 2). All nine cases had the intron 6 breakpoint directly within an *Alu* element. In seven cases (patients 101, 166, 211, 300, 316, 340, and 350) the intron 1 breakpoints were located within one of the two full-length *Alu* elements (Fig. 1*B*). Six of these cases had normal karyotypes, and one case had a karyotype of 46,XY, der(5)t(5;8)(q35;q11.2). The two remaining cases (patients 23 and 24) had intron 1 breakpoints located within non-*Alu* sequence (Fig. 1*B*). Both cases had +11 as a sole cytogenetic abnormality on karyotype analysis (data not shown).

In seven cases (patients 101, 166, 211, 300, 316, 340, and 350), a distinct point of fusion of intron 6 with intron 1 could not be identified. Instead, the sequence gradually diverged through an *Alu* element from intron 6 to intron 1 via an overlapping or heteroduplex fusion region having an average length of 18 bp (range, 8 to 21 bp) (Fig. 3). In each instance, nucleotide sequences were not altered at the site of exchange, and the fusion maintained the natural length and structure of the *Alu* element (Fig. 4). Thus, these rearrangements appear to result from recombination between homologous *Alu* sequences in introns 6 and 1. In two cases (patients 23 and 24), the genomic fusion involved a portion of an *Alu* element in intron 6 with a non-*Alu* sequence in intron 1 (Fig. 3). In both instances, the *Alu* element within intron 6 was disrupted near its 3′ end and resulted in the formation of a truncated *Alu* element (Fig. 4).

Homologous recombination is generally considered to be a natural mechanism for homologous chromosomes in meiotic cells to exchange large segments of DNA (30). Occasionally, however, these events are imperfect and unequal crossing-over between homologous *Alu* elements in meiotic cells has been shown to be the cause of several inherited diseases including predisposition to colon cancer (21), predisposition to breast cancer (22), adenosine deaminase deficiency (23), hypobetalipoproteinemia (24), familial hypercholesterolemia (25), and lipoprotein lipase deficiency (26). In these disorders, *Alu*-mediated homologous recombination events cause either partial deletions or PTDs of a specific gene. In the disorders that result from PTDs, the genetic defects appear to have the same general features as the *ALL1* PTD in that an internal portion of the gene is duplicated in a tandem fashion with an overlap of *Alu* sequence at the genomic fusion. However, while these are inherited germline mutations that occur in meiotic cells, the *ALL1* PTD occurs in somatic cells that only undergo mitosis. Thus, it is reasonable to believe that if the *ALL1* PTD was caused by an unequal cross-over event between homologous chromosomes, then two chromosome 11s would be rearranged; one with a PTD and one with a partial deletion. This does not appear to be the case, however, as Southern blot analysis of *ALL1* PTD consistently identifies a single germline band and a single rearranged band (8–12, 19, 31). Further, in AML cases with +11 or normal cytogenetics that have been studied, only one of the chromosome 11s carry the *ALL1* PTD (32).

A possible mechanism of *ALL1* PTD may therefore involve an intrastrand recombination event. This process, called intrastrand slipped-mispairing, was described by Roth *et al.* (33) to explain tandem duplications found in minisatellite regions

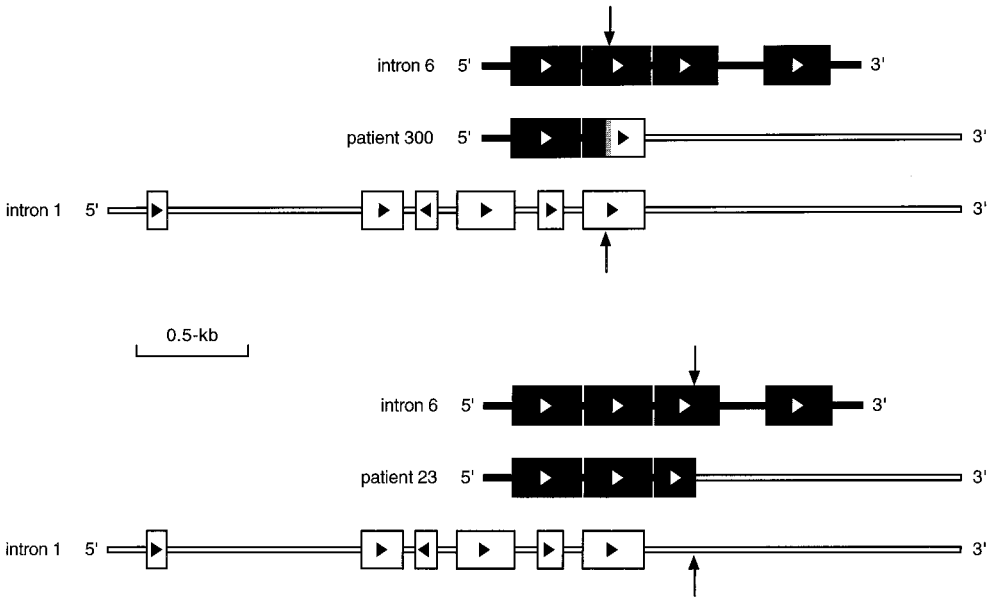

FIG. 4. Genomic structures involved in *ALL* PTD. Patient 300 had breakpoints within *Alu* elements in introns 6 and 1. This fusion resulted in the formation of a full-length *Alu* element. The vertical arrows indicate all breakpoints. Solid and open boxes represent *Alu* elements from introns 6 and 1, respectively. The gray box at the fusion represents the region of overlapping homology. Patient 23 had the intron 6 breakpoint near the 3′ end of an *Alu* element and the intron 1 breakpoint within non-*Alu* sequence. This fusion resulted in the formation of a truncated *Alu* element.

of chromosomes and within the simian virus 40 genome. The intrastrand slipped-mispairing model suggests that upon breakage of a single strand of double-stranded DNA, mispairing can occur between *Alu* elements in a slipped region and homologous *Alu* elements in another region. Following repair synthesis, an additional round of DNA replication will generate a tandem duplication with a short overlap at the point of fusion, and a wild-type allele. Theoretically, a PTD of *ALL1* could be generated by slipped-mispairing if the initial break was within intron 6 of *ALL1*. The slipped region would span exons 2–6 and mispairing would occur between homologous *Alu* sequences from introns 6 and 1. Such a structure could provide a primer for the synthesis of a tandem duplication 3′ of the slipped region (Fig. 5). Alternatively, if the initial break was within intron 1 and mispairing occurred between homologous *Alu* sequences from introns 1 and 6, exons 2 though 6 could be synthesized immediately 5′ and tandem to the trapped structure (data not shown). The cause of the initial single strand DNA breaks in this model is unknown.

Two cases (patients 23 and 24) presented here did not have a heteroduplex at the intronic fusion, and did not appear to result from intrastrand recombination between *Alu* elements. These were the only two cases in this series with +11 as a sole cytogenetic abnormality. Interestingly, one other case of AML with +11 and the PTD of *ALL1* spanning exons 2–6 has been shown to fuse a portion of an *Alu* element in intron 6 with a non-*Alu* sequence in intron 1 (10). Further analysis of the upstream and downstream sequences flanking the breakpoints in both cases reported here revealed the presence of *Alu* elements in introns 6 and 1 that were aligned. An example of this can be seen for patient 23 in the lower portion of Fig. 4 where, although the breakpoint occurred within the third *Alu* element in intron 6, the second *Alu* element aligns with one of the full-length *Alu* elements from intron 1. Although neither of the PTDs in these cases appear to directly result from homologous recombination, it is plausible that the alignment of *Alu* elements may help to stabilize a putative recombination complex. The significance of these findings only in cases of AML with the PTD of *ALL1* and +11 is currently unclear.

In summary, the results of this study support the hypothesis that the *ALL1* PTD spanning exons 2–6 in AML is caused by a homologous recombination event between *Alu* sequences

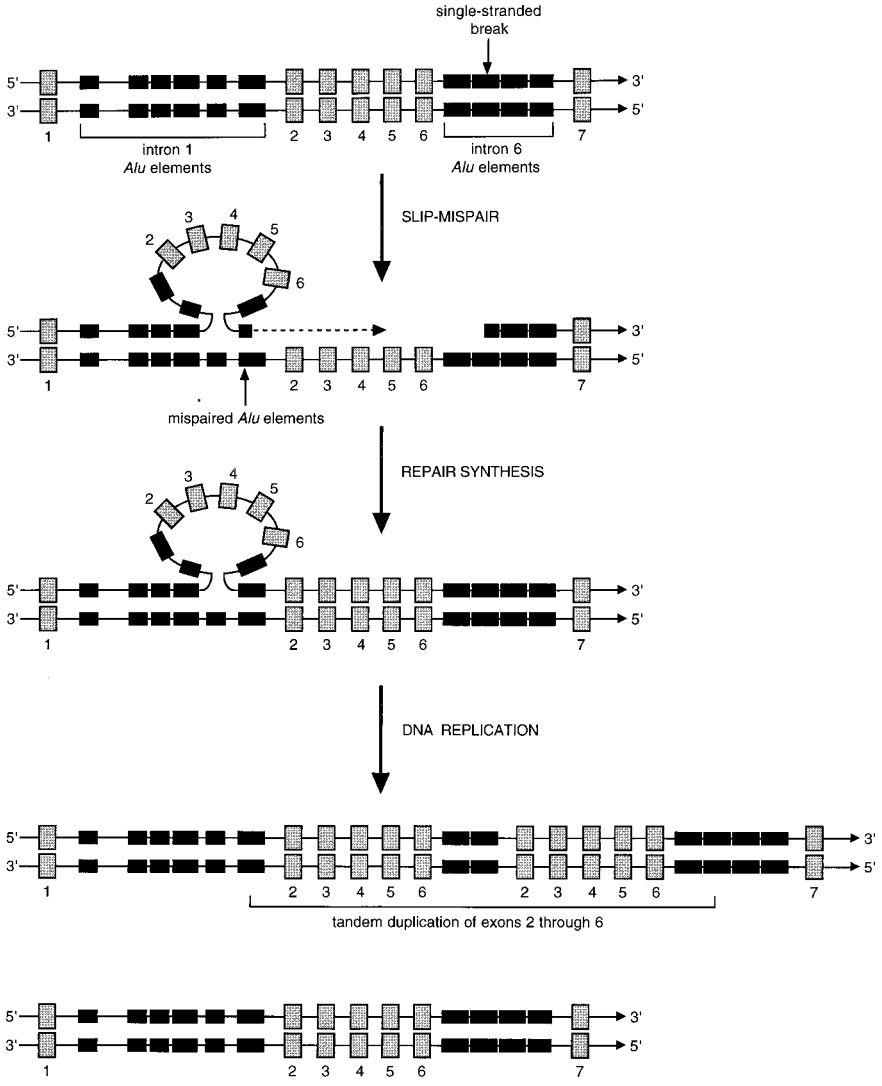

FIG. 5. The intrastrand slipped-mispairing model as applied to the *ALL1* PTD. Shown at the top of the figure is a segment of the wild-type *ALL1* gene as double-stranded DNA. Gray numbered boxes represent exons. Solid boxes represent *Alu* elements from introns 1 and 6. In the model, slipped mispairing of a single strand of double-stranded DNA generates a structure that could prime the synthesis of the slipped region, resulting in a tandem duplication. In this example, the break in a single strand occurs in the middle of the second *Alu* element in intron 6 (vertical arrow). The resultant slipped region mispairs with an *Alu* element in intron 1. Repair synthesis produces a single strand with a tandem duplication of exons 2–6. A subsequent round of DNA replication produces and one chromosome with the partial tandem duplication of *ALL1* and one chromosome with a wild-type *ALL1*. Adapted from Roth *et al.* (33).

within introns 6 and 1. This appears to be the first demonstration identifying *Alu*-mediated homologous recombination as a consistent mechanism for gene rearrangement in somatic tissue.

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