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Genetics

Short, direct repeats at the breakpoints of deletions of the retinoblastoma gene

(oncogene/mutation/chromosome 13)

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ABSTRACT **We found deletions involving the retinoblastoma gene in 12 of 49 tumors from patients with retinoblastoma or osteosarcoma. After mapping the deletion breakpoints, we found that no two breakpoints coincided. Thus, our data do not support the conclusions of others regarding the existence of a "hotspot" for deletion breakpoints in this gene. In 4 of the tumors, we sequenced 200 base pairs surrounding each deletion breakpoint. Three deletions had termini within pairs of short, direct repeats ranging in size from 4 to 7 base pairs. These results indicate that the "slipped mispairing" mechanism may predominate in the generation of deletions at this locus. Our review of deletion breakpoints at other genetic loci reveals that the nature of the sequences present at deletion breakpoints (short, direct repeats versus middle repetitive elements) varies according to the genetic locus under study.**

The retinoblastoma gene serves as a model for the study of recessive oncogenes. Mutations at the retinoblastoma locus play a role in the development of retinoblastoma, a malignant tumor arising in the eyes of young children, and possibly in the development of other human cancers, such as osteosarcoma (1–4), small cell lung carcinoma (5), and breast carcinoma (6, 7). The recent cloning (1, 2, 8) and restriction and exon mapping (9) of the retinoblastoma locus have allowed for a detailed analysis of the molecular basis of mutations that cause inactivation of the retinoblastoma gene and subsequent oncogenic transformation.

Mutations of the retinoblastoma gene occur at a rate much higher than for most other human genes (10). Approximately 10–40% of the mutations are deletions detectable by Southern blotting (1–3, 11). The data we present here address two questions concerning deletions that inactivate the retinoblastoma gene. We would like to know whether deletions occur preferentially at hotspots within the gene and whether specific DNA sequences within the gene predispose to them. To study these issues we mapped the deletion breakpoints in 12 tumors and sequenced the breakpoints in 4 of them. We present here the results of this study and discuss their relevance to the possible mechanisms for the generation of deletions of this gene.

MATERIALS AND METHODS

Southern Blot Analysis. DNA was purified from 40 primary retinoblastoma or osteosarcoma tumor fragments and from 9 tumor cell lines. In certain cases, leukocyte DNA was also purified from the patients and their parents. DNA from the retinoblastoma tumor cell line RB#47 (RBLA-12) was provided by E. Bogenmann (University of Southern California) (12). Aliquots of DNA were digested with various restriction enzymes (New England Biolabs) and subjected to electrophoresis in 0.8% agarose gels. The DNA was transferred to nitrocellulose filters and hybridized with single-copy, genomic probes previously isolated from the retinoblastoma gene (13, 14). The probes were radiolabeled with ^{32}P (New England Nuclear) by a modification of the random-primer technique that uses the Klenow fragment of DNA polymerase I.

Genomic Cloning. In DNA from tumors RB#1, RB#47, RB#49, and OS-15, Southern blot analysis revealed *Xba* I restriction fragments of abnormal size. We isolated these deletion junction fragments from bacteriophage libraries constructed from the tumor DNA. Genomic DNA from each of these tumors was digested to completion with *Xba* I and ligated into λong C bacteriophage arms (Stratagene). The ligation mixtures were packaged with Gigapack Plus or Gold (Stratagene) and titered on *Escherichia coli* strain P2392. About 500,000 independent plaques from each library were screened with the appropriate probe by the technique of Benton and Davis (15). Positive phage colonies were plaque-purified and amplified. We constructed a restriction map of the *Xba* I junction fragments and subcloned small DNA fragments bridging the deletion junction into the plasmid vector Bluescribe (Stratagene). In addition, we subcloned the corresponding sequences at the 5′ and 3′ breakpoints from a set of overlapping recombinant bacteriophage spanning the retinoblastoma gene that had been previously isolated in our laboratory (14). The normal 3′ breakpoint of RB#1 was cloned by screening a human genomic library, provided by S. Orkin (Harvard Medical School), with a single-copy probe 3′ to the deletion breakpoint that was isolated from the *Xba* I junction fragment.

DNA Sequencing. The junction fragments and corresponding normal fragments were sequenced using the dideoxy chain-termination method by using Sequenase (United States Biochemical). For each deletion, we sequenced both strands of at least 100 base pairs (bp) on each side of each breakpoint. For sequencing primers, we synthesized oligonucleotides with a Pharmacia Gene Assembler DNA synthesizer.

RESULTS

Ascertainment of Tumors with Deletions. We analyzed DNA from 49 tumors (38 retinoblastomas, 3 second tumors from patients with previous retinoblastomas, and 8 osteosarcomas) with cDNA and genomic probes from the retinoblastoma locus. We found 11 retinoblastomas and 1 osteosarcoma with deletions of the retinoblastoma gene. No two tumors had identical deletion breakpoints within the retinoblastoma locus. In every deletion that we identified, except for the one in retinoblastoma cell line WERI-1, the deletion breakpoints were either within the retinoblastoma gene or beyond the 3′ polyadenylylation site. Only WERI-1, with a homozygous deletion encompassing the entire transcriptional unit, had a

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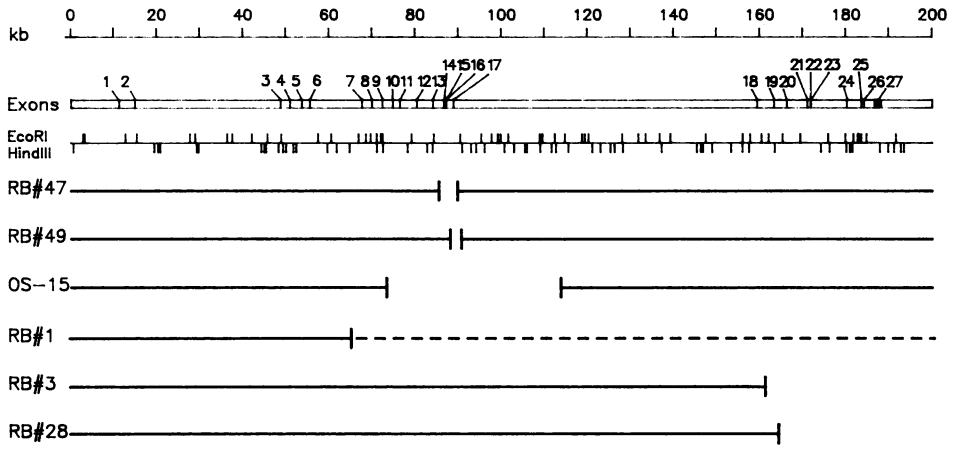


FIG. 1. Restriction and exon map of the retinoblastoma gene showing the location of the breakpoints of deletions of the gene in five retinoblastomas and one osteosarcoma. The dotted line in RB#1 indicates that the deletion in this tumor is heterozygous. The other five deletions are homozygous. The deletions in tumors RB#47, RB#49, and OS-15 have both breakpoints within the gene. Tumors RB#1, RB#3, and RB#28 have one breakpoint within the gene and one breakpoint, not shown, that is beyond the 3′ polyadenylylation site of the gene.

deletion breakpoint beyond the 5′ end of the gene. We precisely mapped the deletion breakpoints in six deletions, selected on the basis of their easily identifiable junction fragments with single-copy probes. Fig. 1 shows a map of the retinoblastoma gene with the breakpoints of these six deletions. In three cases, both deletion breakpoints lie within the retinoblastoma gene. The other three deletions have 5′ breakpoints within the gene, but their 3′ breakpoints are beyond the 3′ polyadenylylation site.

Genetic Origin of Deletions. Based on family histories, clinical presentations, and analysis of leukocyte DNA from the patients and their parents, we were able to determine which of the deletions in Fig. 1 were germ-line mutations and which were somatic. For example, RB#1 is from a patient with bilateral retinoblastoma who has no family history of retinoblastoma. The deletion in RB#1 was detected in DNA from the patient's leukocytes but was not seen in leukocyte DNA from either parent; therefore, this deletion is a new germ-line mutation. By similar reasoning, we concluded that the deletions present in RB#28 and RB#49 are also germ-line mutations. On the other hand, the deletions present in tumors RB#3, RB#47, and OS-15 are somatic mutations.

Sequence Analysis of Deletion Breakpoints. *Retinoblastoma RB#1.* Fig. 2 shows the restriction maps of the *Xba* I junction fragment from RB#1 and the corresponding normal fragment from the retinoblastoma gene. RB#1 has a heterozygous deletion of at least 135 kilobases (kb) that includes exons 7–27 (see Fig. 1). Assuming a stable mRNA transcript, deletion of these exons would cause the loss of 726 of the 928 amino acids of the retinoblastoma protein.

We sequenced the DNA surrounding both deletion breakpoints in the junction fragment and in the corresponding normal fragment (Fig. 2). Sequence analysis revealed the presence of an imperfect 7-bp direct repeat (TTTAWAC; W = A or T) at the 5′ and 3′ breakpoints. One copy of the repeat is deleted and one copy is retained in the mutant allele.

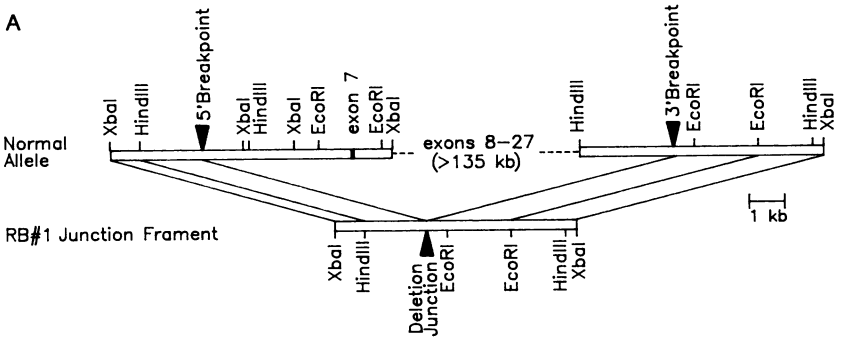
Retinoblastoma RB#47. The restriction maps of the cloned junction fragment from tumor RB#47 and the corresponding fragment from the retinoblastoma gene are shown in Fig. 3. The restriction map shows that this tumor has a homozygous deletion of 4.2 kb that eliminates exons 14–17. These exons contain a total of 363 bases of coding sequence, and an mRNA transcript with this deletion would code for a protein missing 121 amino acids.

Examination of the sequence across the deletion breakpoints of RB#47 reveals that there are four bases of homology (GCCA) at the 5′ and 3′ breakpoints. As in tumor RB#1, one copy of the sequence is deleted and one is retained in the tumor. The 3′ breakpoint of the RB#47 deletion lies within a *Kpn* I repetitive DNA sequence in intron 17 (16).

Osteosarcoma OS-15. Fig. 4 shows the restriction map of the junction fragment from OS-15 and the sequence of the breakpoints. OS-15 has a homozygous deletion of 40 kb that eliminates exons 10–17. This deletion corresponds to the loss of codons 314–565 with no change in the translation reading frame, resulting in the loss of 262 amino acids from the retinoblastoma protein.

We sequenced the DNA surrounding both deletion breakpoints (Fig. 4). We found that there is a 6-bp region of homology (AGAAG) at the 5′ and 3′ deletion breakpoints. One copy of the repeat sequence is retained, and the other is partially deleted.

Retinoblastoma RB#49. RB#49 has a homozygous deletion that eliminates exon 17, as shown in Fig. 5. Exon 17 contains 197 bases of coding sequence, and its deletion corresponds to the loss of 66 codons, as well as a change in



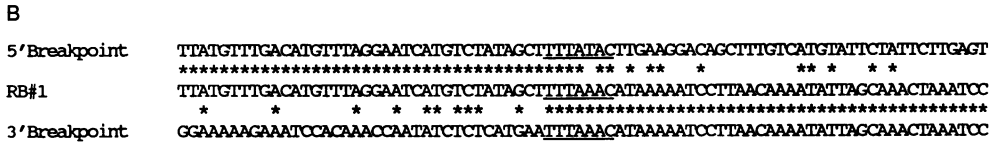


FIG. 2. (*A*) Restriction maps of the RB#1 junction fragment and the fragments containing the 5′ and 3′ deletion breakpoints. The 3′ breakpoint is beyond the 3′ polyadenylylation site of the gene and is at least 135 kb downstream from the 5′ breakpoint. (*B*) Nucleotide sequence of the deletion junction and the 5′ and 3′ breakpoints. A 7-bp region of homology at the breakpoints and at the deletion junction is underlined. ★, Identity.

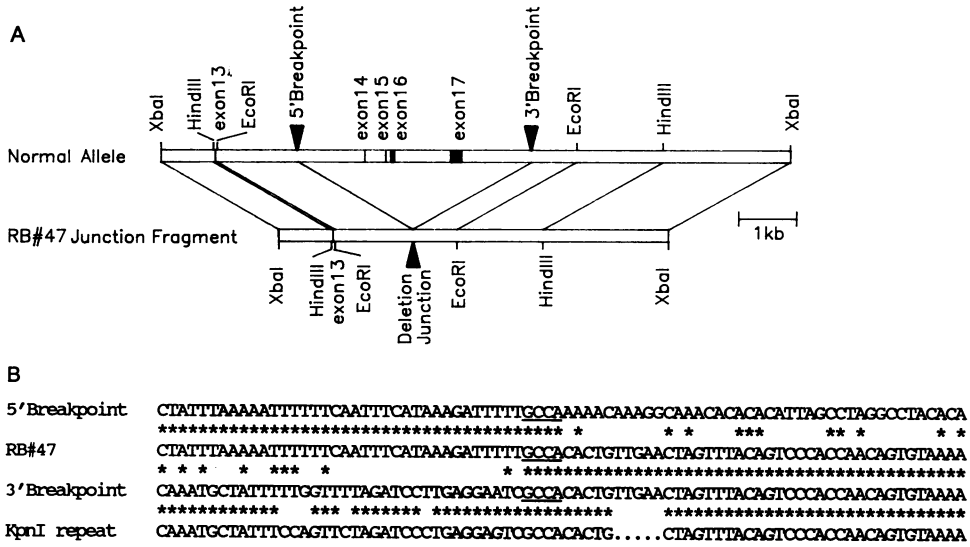


FIG. 3. (*A*) Restriction maps of the RB#47 junction fragment and the fragment containing the 5′ and 3′ deletion breakpoints. (*B*) Sequence of the RB#47 deletion junction compared with the 5′ and 3′ breakpoints. A 4-bp direct repeat that is present at both breakpoints and at the deletion junction is underlined. The 3′ breakpoint is compared with a *Kpn* I interspersed repeat (16). The dots in the *Kpn* I repeat sequence denote an 18-bp sequence that is not homologous to the 3′ breakpoint. ★, Identity.

the translation reading frame. The fourth codon downstream in the new frame is a premature stop codon. Therefore, this deletion, at a minimum, would result in the loss of 424 of the 928 amino acids present in the retinoblastoma protein.

By sequencing the deletion breakpoints, we found that 2.1 kb were deleted and replaced with an 80-bp sequence (Fig. 5). This DNA, which is extremely A+T rich (87%), creates 14-bp inverted repeats when inserted at the deletion junction. It also contains two tandem copies of a 16-bp sequence. Finally, the 3′ end of the insertion contains an 11-base sequence that is homologous (10/11 bases are identical) to the adjacent normal sequence. Comparison of the inserted sequence with the sequences in the GenBank Database† revealed homology of 61 bases of the inserted sequence with a sequence involved in a t(2;8) chromosomal translocation in a Burkitt lymphoma cell line (17).

DISCUSSION

Bookstein *et al.* (18) recently reported that three separate tumors had similar and possibly identical heterozygous deletions, indicating the presence of a possible deletion hotspot in intron 1. We mapped the breakpoints of 12 oncogenic deletions of the retinoblastoma gene and found that no two deletion breakpoints within the gene were identical. Of the 12 tumors that we studied, only Y-79, one of the three tumors that Bookstein *et al.* described, had a deletion in intron 1. Our results argue against a precise DNA sequence within the retinoblastoma gene as a common hotspot for deletion breakpoints. However, 2 of 12 deletions have both breakpoints within a 7.8-kb *Hin*dIII fragment that contains exons 13–17, and 7 of 12 deletions include this region. Others (2) have also found that this *Hin*dIII fragment is frequently involved in deletions of the retinoblastoma gene. This region may be an example of a breakpoint cluster region. Such regions appear to be frequently involved in translocations in malignancies such as chronic myelogenous leukemia (19), as well as in deletions in the human α-globin gene cluster (20) and in the hamster APRT locus (21). It is not known why certain regions are prone to deletions, but they may contain sequences such as dyad symmetries, short direct repeats, and interspersed repetitive DNA sequences that render them particularly susceptible to recombinational events. This situation does not appear to be operative here, because during our work we sequenced 3.8 kb of this region without finding a preponderance of such sequences. Therefore, the reason for the frequent inclusion of exons 13–17 in deletions of the retinoblastoma gene remains unclear. The result may simply be due to an ascertainment bias caused by the easy detection of deletions of this region by the cDNA probes currently used.

To better understand the possible mechanisms for the formation of deletions causing retinoblastoma, we cloned and sequenced the deletion breakpoints in four tumors. Only one of the eight deletion breakpoints was within a middle repetitive genetic element. However, we found that short, direct repeats were present at the deletion breakpoints in three of the four tumors—RB#1, RB#47, and OS-15. Two of these deletions were somatic and one was germ line. In the fourth tumor, RB#49, there was no homology at the deletion breakpoints. However, at the deletion junction there was an 80-bp insertion; the inserted sequence contained tandem 16-bp direct repeats and was flanked by 14-bp inverted repeats. Part of the 3′ inverted repeat also had 10/11 bases of homology with the 3′ deletion breakpoint. The inserted sequence is homologous to a region involved in a t(2;8) translocation in a Burkitt lymphoma cell line (17). The large amount of homology within the insert and with the surrounding DNA, as well as the homology with a region involved in a chromosomal translocation, indicate that the deletion in RB#47 may have been caused by a complex rearrangement. Further analysis of the sequences surrounding the deletion breakpoints in these four tumors revealed no additional homologies.

Our finding of short regions of homology at six of the eight sequenced deletion termini is similar to what has been seen in studies of deletions of the *E. coli lac*I gene (22, 23), the mouse immunoglobulin gene (24), and the human β-globin gene (25–34). At the human β-globin locus, for example, there are short, direct repeats in 15 of 20 deletions in which the sequence of both breakpoints is known (25–34). In contrast, *Alu* sequences at both breakpoints appear to be a common but not invariable feature at other loci. For example, at the low-density lipoprotein receptor locus, evidence for *Alu-Alu* recombination was found in four of five deletions in which both breakpoints were sequenced (35–39). Other human genes in which *Alu-Alu* recombination has been thought to be involved in deletion formation include the adenosine deaminase gene (40) and the β-hexosaminidase gene (41). It remains to be seen whether genetic loci can be categorized according to their propensity to develop deletions with either short, direct repeats or middle repetitive elements at the breakpoints. If such a categorization is borne out, it implies that the mechanisms responsible for the formation of dele-

†EMBL/GenBank Genetic Sequence Database (1988) GenBank (Intelligenetics, Mountain View, CA), Tape Release 57.

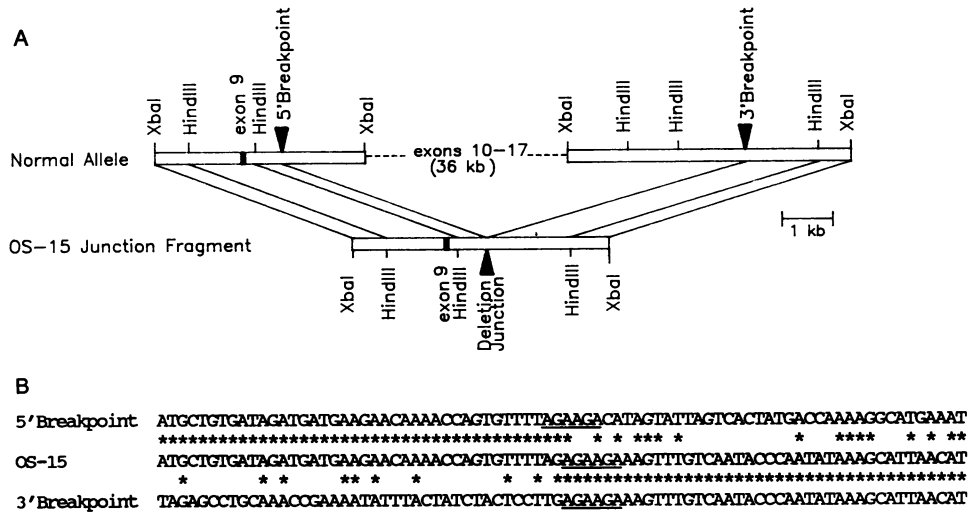


FIG. 4. (*A*) Restriction maps of the OS-15 junction fragment and the fragments containing the 5′ and 3′ deletion junctions. (*B*) Sequence of the deletion junction and the deletion breakpoints. A 6-bp region of homology at the breakpoints and at the deletion junction is underlined. ★, Identity.

tions vary according to local features inherent in different chromosomal regions.

Our findings indicate that a knowledge of the mechanism of deletion formation involving short, direct repeats has relevance to an understanding of oncogenesis in humans. However, the molecular mechanism of such deletions is not well understood. There is evidence for a role for short, direct repeats in the generation of deletions in both prokaryotic and eukaryotic cells. Efstradiadis *et al.* (25) have argued that the repeats found at the breakpoints of deletions of the β-globin locus are not long enough to mediate unequal crossing over via homologous recombination. His group and others have supported a model of deletion formation by "slipped mispairing" during DNA replication (22, 23, 42). In this model, initially proposed by Streisinger *et al.* (43) to explain the generation of frameshift mutations, the repeated sequences mispair during DNA replication, leading to the formation and excision of a single-stranded loop between the repeats. One repeat present in the loop is then deleted along with the segment between the repeats, and one repeat remains in the mutant DNA. Roth *et al.* (44) have analyzed many eukaryotic deletions and found that short, direct repeats of at least 2 bp occurred more frequently than would be expected from random breakage and reunion. The three deletions that we analyzed that involve short, direct repeats range in size from 2.1 kb to >135 kb. As Vanin *et al.* (27) have noted, for slipped mispairing to occur the two breakpoints must be physically close to each other in the nucleus—although they may be far apart on a linear map of the gene. These workers have studied four large deletions of the β-globin gene and argued that the deletions occurred through the loss of chromatin loops during replication. Similarly, it is possible that the 5′ and 3′ breakpoints in tumors RB#1, RB#47, and OS-15 are physically close in the nucleus, having been brought together by anchorage to the nuclear matrix.

This study leaves unanswered a number of questions about deletions of the retinoblastoma gene. (*i*) We have found that most deletions are internal or extend off the 3′ end of the gene yet we are unable to explain why. (*ii*) We have also noted that the 7.8-kb *Hin*dIII fragment containing exons 13–17 is frequently included in deletions but have found no evidence that explains why this region may be highly susceptible to deletion. (*iii*) We have found no evidence for a correlation between the genetic origin of a deletion (somatic versus germ

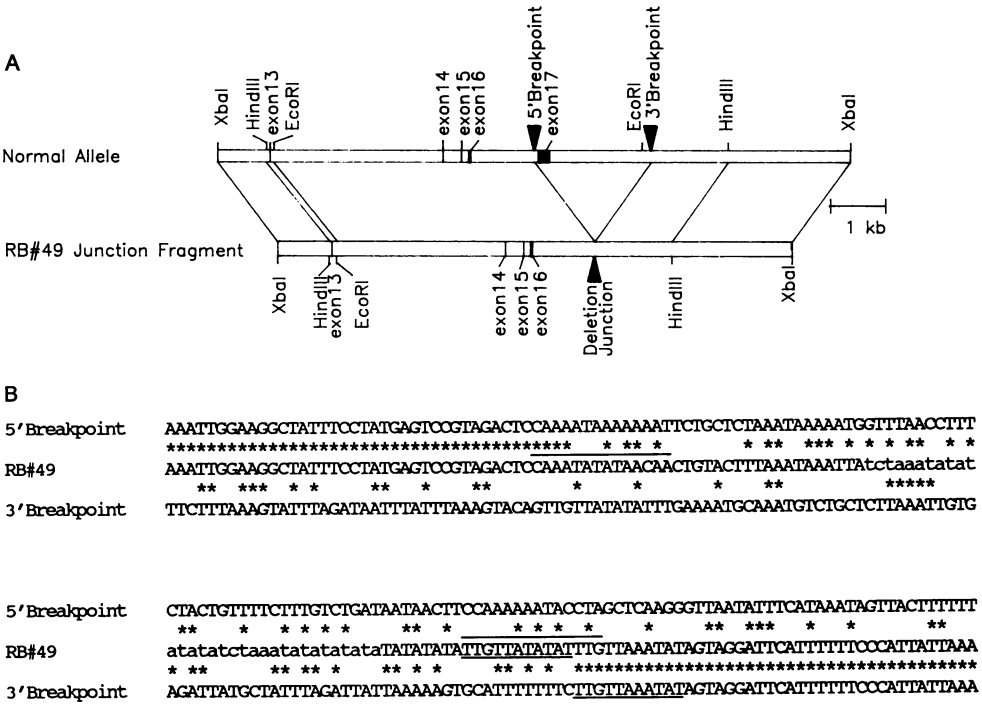


FIG. 5. (*A*) Restriction maps of the junction fragment of RB#49 and the fragment containing the 5′ and 3′ deletion breakpoints. (*B*) Nucleotide sequences of the deletion junction and the corresponding 5′ and 3′ breakpoints. An 80-bp sequence replaces the deleted region. A tandem 16-bp direct repeat in the inserted sequence is indicated by lowercase letters. A 14-bp inverted repeat is indicated by lines above the sequences. An 11-bp sequence and adjacent homologous normal sequence are underlined. ★, Identity.

line) and the sequences at the breakpoints, although considering the small number of tumors we examined, such a relationship could still exist.

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