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Human retinoblastoma susceptibility gene: Genomic organization and analysis of heterozygous intragenic deletion mutants

(cancer genetics/DNA polymorphism/mutation)

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ABSTRACT A gene in chromosome region 13q14 has been identified as the human retinoblastoma susceptibility (*RB*) gene on the basis of altered gene expression found in virtually all retinoblastomas. In order to further characterize the *RB* gene and its structural alterations, we examined genomic clones of the *RB* gene isolated from both a normal human genomic library and a library made from DNA of the retinoblastoma cell line Y79. First, a restriction and exon map of the *RB* gene was constructed by aligning overlapping genomic clones, yielding three contiguous regions ("contigs") of 150 kilobases total length separated by two gaps. At least 20 exons were identified in genomic clones, and these were provisionally numbered. Second, two overlapping genomic clones that demonstrated a DNA deletion of exons 2 through 6 from one *RB* allele were isolated from the Y79 library. To confirm and extend this result, a unique sequence probe from intron 1 was used to detect similar and possibly identical heterozygous deletions in genomic DNA from three retinoblastoma cell lines, thereby explaining the origins of their shortened *RB* mRNA transcripts. The same probe detected genomic rearrangements in fibroblasts from two hereditary retinoblastoma patients, indicating that intron 1 includes a frequent site for mutations conferring predisposition to retinoblastoma. Third, this probe also detected a polymorphic site for *Bam*HI with allele frequencies near 0.5/0.5. Identification of commonly mutated regions will contribute significantly to genetic diagnosis in retinoblastoma patients and families.

Retinoblastoma is an intraocular cancer of early childhood that arises from the developing retina. In a substantial proportion of cases, susceptibility to retinoblastoma can be inherited from a parent who was previously cured of the tumor (1, 2). In such families, the inheritance pattern (about 50% of the offspring are affected) indicates transmission of a single dominant autosomal gene. This gene [the retinoblastoma susceptibility (*RB*) gene] was localized to chromosome band 13q14 by linkage analysis of retinoblastoma pedigrees and examination of cases having cytogenetic deletions in somatic cells (3, 4). Based on a statistical analysis of clinical data, Knudson (5) inferred that retinoblastoma could result from as few as two mutational events. Cavenee *et al.* (6) demonstrated specific loss of heterozygous chromosome 13 markers in retinoblastomas compared to somatic cells from the same patients and suggested that the tumor might be caused by inactivation of both alleles of a single gene on chromosome 13. At the molecular level, Dryja *et al.* (7) found two retinoblastomas with small homozygous deletions limited to region 13q14. These studies implied that a mutant *RB* allele is "recessive" to its normal counterpart within a cell and that the latter essentially functions to prevent tumor formation during retinal development. A class of such "cancer suppressor" genes has been postulated to explain other forms of heritable cancer predisposition and chromosomal abnormalities analogous to those seen in retinoblastoma (8, 9).

Recently, a gene in band 13q14 encoding an mRNA of 4.7 kilobases (kb) was identified as the *RB* gene based on tumor-specific alterations in expression and its apparent recessive nature at the cellular level (10). cDNA segments representing the *RB* gene transcript have been cloned (10–12) and sequenced (10). All hereditary and nonhereditary retinoblastomas examined to date have demonstrated altered *RB* gene expression: RB mRNA transcripts are either markedly reduced in quantity or are abnormal in length (10–12). About 40% of retinoblastomas show DNA deletions detectable by RB cDNA probes. Antibodies generated against the *RB* gene product, $pp110^{RB}$, show complete absence of this nucleophosphoprotein in five out of five retinoblastomas, whereas it was easily detected in all normal or neoplastic cells containing a normal RB mRNA transcript (13). These data further strengthen the hypothesis that the absence of functional RB protein is potentially oncogenic.

Given the different patterns of genetic alteration observed in retinoblastomas, it is evident that the *RB* gene is subject to a variety of mutational mechanisms, the details of which are as yet unknown. In order to further characterize the *RB* gene and some of its mutations, we constructed a restriction and exon map of the intact *RB* gene based on analysis of genomic DNA clones. The derived map identifies most, if not all, of the exons present in the full-length cDNA sequence (4757 nucleotides). A genomic library made from retinoblastoma cell line Y79 yielded two clones containing an intragenic deletion junction that was not previously detected by cDNA probes for the *RB* gene. Using a single-copy probe, p6NR0.5, similar heterozygous deletions were demonstrated in genomic DNA from three different retinoblastoma cell lines (Y79, RB355, and WERI-27), suggesting the presence of a common mutational site in intron 1. Finally, probe p6NR0.5 also detected a restriction fragment length polymorphism with endonuclease *Bam*HI. Two common alleles have frequencies of approximately 0.5/0.5, which is optimal for use in genetic diagnosis.

MATERIALS AND METHODS

Origin of DNA Probes. cDNA probes RB1 and RB5, obtained as described (10), detect 4.7-kb mRNA transcripts of the *RB* gene in all normal tissues and many nonretinoblastoma tumors. Restriction and sequence analysis demonstrated that these cloned fragments overlap and, when aligned, represent an mRNA transcript of 4523 nucleotides (10). From the above clones the following probes were generated, as diagrammed in Fig. 1. RB1 was cleaved by *Eco*RI into 0.9-kb (RB0.9) and 0.7-kb (RB0.7) fragments. RB5 was cleaved by *Bgl* II into three fragments called RB710 (0.7 kb, *Eco*RI–*Bgl* II), RB1.8 (1.8 kb, *Bgl* II–*Bgl* II), and RB1.0 (1.0 kb, *Bgl* II–*Eco*RI). A cDNA library made from

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FIG. 1. Overview of the exon map of the *RB* gene and alignment to the cDNA sequence. Three regions of contiguous, overlapping genomic clones ("contigs") separated by gaps are diagrammed along the top line. Exons (vertical bars) are numbered as explained in *Results*. Oligonucleotides a–k, synthesized according to the revised cDNA sequence of the *RB* gene (13), are matched to exons with which they hybridize. cDNA clones and derived probes are shown below the genomic clones. Restriction sites (as predicted from the cDNA sequence) are indicated as follows: A, *Acc* I; B, *Bgl* II; D, *Dde* I; E, *Eco*RV; H, *Hin*dIII; I, *Hpa* I; N, *Nco* I; P, *Pst* I; R, *Eco*RI; S, *Stu* I. bp, Base pairs.

retinoblastoma cell line Y79 (14) was screened with RB1 as a probe and yielded another cDNA clone, Y79RB, of 3.8 kb (unpublished results). Sequence analysis showed that Y79-RB contained the 5′ end of the normal sequence and in fact extended an additional 242 base pairs in the 5′ direction (13). The additional 5′ portion of Y79RB was separated by *Dde* I digestion and preparative PAGE and was designated Y79RB200 (Fig. 1). All cDNA probes were labeled with [α-^{32}P]dATP by the random primer method (15).

Probe p6NR0.5 was prepared by subcloning a 0.5-kb *Eco*RI fragment of phage clone N6 (Fig. 2*A*) into plasmid pGEM-1 (Pharmacia). This fragment was shown to contain only single-copy DNA by lack of hybridization to total human genomic DNA labeled with [α-^{32}P]dATP by nick-translation (16).

Selected deoxyoligonucleotides were chemically synthesized (Gene Assembler, Pharmacia) by the phosphotriester method (17) according to the cDNA sequence (13). 5′-Terminal labeling with [γ-^{32}P]ATP was accomplished with T4 kinase (16).

Preparation of Genomic Libraries. Genomic DNA was obtained from retinoblastoma cell line Y79 (14) and normal peripheral blood leukocytes from one of us (W.-H.L.), and mutant and normal genomic libraries, respectively, were constructed by standard techniques (16) in λEMBL3 or λ Dash vectors (Stratagene, La Jolla, CA). An additional normal library was constructed from a hybrid human–Chinese hamster ovary cell line containing human chromosome 13 (18).

Isolation and Characterization of Phage Clones. Standard plaque-screening techniques were employed as described (16). The majority of phage clones (Fig. 2) were isolated by separately screening both normal and mutant genomic libraries with cDNA probes (RB0.9, RB0.7, RB710, RB1.8, and RB1.0) representing most of the normal RB mRNA transcript (Fig. 1). Clones Y1 and Y4 were obtained as described

FIG. 2. Detailed restriction and exon map of the *RB* gene. Contigs A, B, and C are as in Fig. 1. Only nonredundant genomic clones, represented by bracketed lines, are shown; clone names starting with Y, N, or C indicate isolation from Y79, normal, or chromosome 13-specific human libraries, respectively. The derived restriction map is exhaustive for *Eco*RI (R), *Hin*dIII (H), and *Sal* I sites; only selected *Bam*HI (B) and *Nco* I sites are shown. Restriction sites are illustrated to ±0.25 kb accuracy. Exons (solid boxes) are not intended to be to scale except for exon 20. *, Polymorphic *Bam*HI site. ***, Small (≈1 kb) gap (T. Dryja, personal communication). Clones Y2.3 and Y7.3 depart from the map in regions indicated by broken lines.

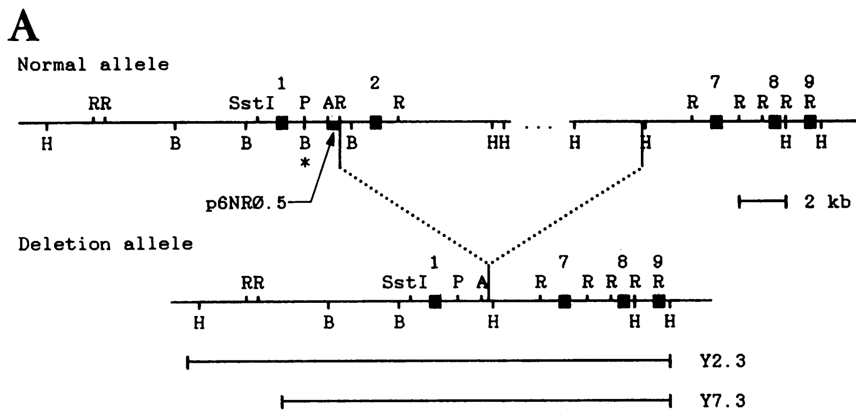
previously (10), clone N6.2 was isolated by screening the normal library with a 3.0-kb *Bam*HI fragment of clone Y2.3, and Y1.4 was similarly obtained using an *Nco* I fragment of Y4 as a probe. Cloned genomic DNA was characterized by digestion with selected restriction enzymes followed by DNA blotting and hybridization (16) with appropriate cDNA or oligonucleotide probes. Many restriction sites could be mapped by observation of restriction fragment patterns in overlapping clones; others were assigned by double-enzyme digests or *cos* mapping (19).

RESULTS

Genomic Organization of the *RB* Gene. Genomic clones, obtained as described in *Materials and Methods*, were assorted into three overlapping groups (contigs) based on shared restriction fragments and shared exons (Fig. 2). These were oriented with respect to the cDNA sequence by hybridization with cDNA or oligonucleotide probes. Exons were initially identified as minimal-length *Eco*RI and/or *Hin*dIII restriction fragments containing sequences hybridizing to RB cDNA clones; we provisionally numbered 17 such fragments. The endpoints of some clones served to further localize particular exons (e.g., exons 12 and 13). Oligonucleotides a through k were located in the exon map by hybridizing to DNA blots of genomic clones (Fig. 1). Exons 13 and 14 were distinguished by double-stranded DNA sequencing (20) of clone C12 using oligonucleotide g as primer, by which a potential intron/exon junction was identified.

We established the location of exon 1 as follows. Y79RB200, representing the normal cDNA 5′ end, contained a G+C-rich region that rendered it unusable as a probe for either genomic DNA blots or phage screening. Instead, we obtained clone N6.2 as described in *Materials and Methods*; its overlap with N6 and N8 was confirmed by specific hybridization with single-copy genomic probe p6NR0.5, located in intron 1 (Fig. 2*A*). Probe Y79RB200 hybridized strongly to a 2.3-kb *Bam*HI restriction fragment of clone N6.2. Oligonucleotide a, complementary to nucleotides 35–52 of the revised normal cDNA sequence (13), hybridized to the same 2.3-kb *Bam*HI fragment. The coincidence of cDNA length (4757 bp) and mRNA size (4.7 kb), as well as primer extension studies and double-stranded genomic sequencing (data not shown), indicated that our cDNA sequence was essentially complete and that the first exon was correctly located. Restriction and sequence analysis demonstrated that the last exon, exon 20, was 1.9 kb in length and included the translation stop codon (nucleotides 2924–2926). Thus the remaining 19 (or more) exons averaged about 150 nucleotides each.

Although exon-containing restriction fragments might include more than one actual exon separated by small introns, we were more concerned that no additional exons remained in the uncloned gaps between contigs. By sequencing genomic clones, we verified that only one splice site separated oligonucleotides b and c in the cDNA sequence and, similarly, that only one splice site separated oligonucleotides g and h (Fig. 1; unpublished results). Thus additional exons were excluded from the two gap regions, which consequently contained only intron sequences.



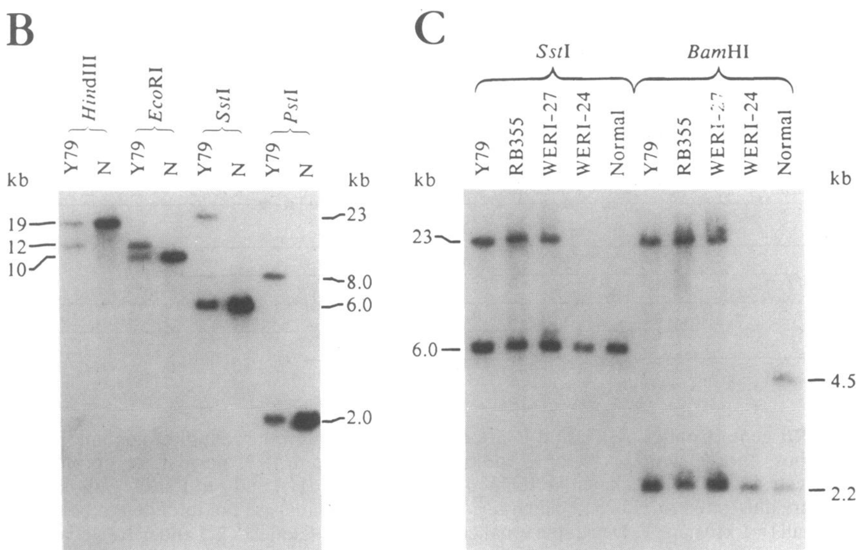


FIG. 3. Heterozygous, intragenic deletion common to Y79, RB355, and WERI-27. (*A*) Deletion allele of Y79 compared to a normal allele. Deletion junction clones Y2.3 and Y7.3 from Y79 are shown. Exons and restriction sites are indicated as in Fig. 2. P, *Pst* I; A, *Acc* I. *, Polymorphic *Bam*HI site. (*B*) DNA blot of genomic DNA extracted from retinoblastoma cell line Y79 and normal peripheral blood leukocytes. Genomic DNA (5 μg per lane) was digested with restriction endonuclease *Hin*dIII, *Eco*RI, *Sst* I, or *Pst* I, electrophoretically separated, and transferred to nitrocellulose as described (16). Filters were hybridized with probe p6NR0.5 in 40% formamide/6× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0)/5× Denhardt's solution (1× Denhardt's = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin)/0.1% $NaDodSO_4$/denatured salmon sperm DNA (100 μg/ml)/100 mM phosphate buffer (pH 7.0) at 42°C for 24 hr. Filters were washed twice in 2× SSC/0.1% $NaDodSO_4$ at room temperature for 15 min and once in 0.2× SSC/0.1% $NaDodSO_4$ at 60°C for 30 min. Autoradiography was done with Kodak XAR-5 film at −70°C for 2 days with an intensifying screen. (*C*) DNA blot of genomic DNA extracted from retinoblastoma cell lines Y79, RB355, WERI-27, and WERI-24 and normal peripheral blood leukocytes. The DNA was digested with restriction endonucleases *Sst* I and *Bam*HI and was processed exactly as described in *B*.

Characterization of Deletion Mutants. Overlapping clones Y2.3 and Y7.3, which represented part of the region around exons 7 and 8 (Fig. 3*A*), were isolated from the Y79 library. However, the other portion of these clones apparently departed from the map. Although suggestive of deletion junctions, these clones were initially viewed with caution since (*i*) clones from the presumptive deleted region had been isolated from the same library, and (*ii*) genomic DNA from Y79 appeared normal by DNA blotting when probed with RB cDNA (10, 12). Since the identity of the extraneous DNA was not known, DNA insertion or translocation was also possible. The issue was resolved by identifying exon 1 in clones Y2.3 and Y7.3 as well as in N6.2 from the normal library, as described above. Therefore, we inferred that Y2.3 and Y7.3 demonstrate a deletion removing exons 2–6 from one of the *RB* alleles in Y79 (Fig. 3*A*).

Further evidence for this deletion was obtained when probe p6NR0.5, located in intron 1, was hybridized with a DNA blot of Y79 and normal genomic DNA digested by restriction endonucleases *Hin*dIII, *Eco*RI, *Sst* I, and *Pst* I (Fig. 3*B*). In normal DNA, the probe detected *Eco*RI and *Hin*dIII fragments of 10 and 19 kb, respectively. In Y79 DNA, altered restriction fragments in addition to normal fragments were apparent for all restriction enzymes, consistent with the heterozygous deletion shown in Fig. 3*A*. Finally, probe p6NR0.5 was used in DNA blotting of genomic DNA from retinoblastoma cell lines Y79, RB355 (21), WERI-27, and WERI-24 and normal DNA (Fig. 3*C*). Normal DNA was heterozygous for a polymorphic *Bam*HI site with alleles of 2.2 and 4.5 kb. Y79, RB355, and WERI-27 contained two allelic fragments, a small 2.2-kb allele (normal) and a larger allele of ≈23 kb. WERI-24 demonstrated a single copy of the small allele. *Sst* I (Fig. 3*C*), *Pst* I, *Hin*dIII, and *Eco*RI digestion of RB355 and WERI-27 DNA yielded aberrant fragments identical to those of Y79 DNA, whereas three other retinoblastoma lines had only normal-sized fragments (data not shown). In the context of deletion junction clones isolated from Y79, these data indicate the presence of similar, heterozygous DNA deletions in the three separate cell lines, Y79, WERI-27, and RB355. Deletion junctions differ by less than 1 kb (the maximal error in comparing DNA fragments in this size range by agarose gel electrophoresis). WERI-24 was previously known to have only one copy of the *RB* gene (figure 3, lane 4 in ref. 10).

Rearrangements of the *RB* Gene in Fibroblasts from Retinoblastoma Patients. Given the high incidence and specificity of deletions described above, we used probe p6NR0.5 to examine DNA of fibroblasts from seven patients with bilateral retinoblastoma as well as DNA from six nonretinoblastoma tumor lines. Two fibroblast samples demonstrated apparently heterozygous genomic rearrangements near p6NR0.5, as indicated by novel bands after digestion with several restriction endonucleases (Fig. 4). F1 fibroblasts showed faint, aberrant bands of 12 and 13 kb by *Eco*RI and *Hin*dIII digestion, re- spectively, which were similar in size to those seen in Y79 (Fig. 3*B*). The weak band intensity (compared to the normal allele) might be explained by a deletion that removes most of the probe-hybridizing sequences from the deleted allele. The rearrangement in F2 fibroblasts was completely different from the other four cases but still occurred near intron 1. The remaining fibroblasts and tumor lines had normal hybridization patterns (data not shown).

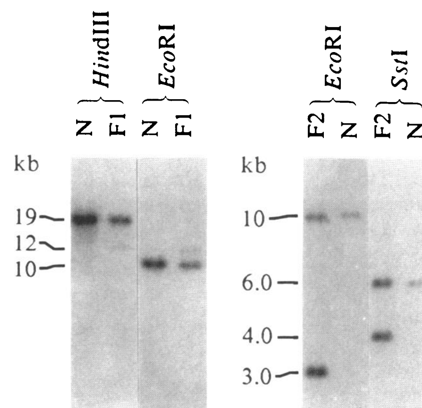


FIG. 4. Genomic rearrangement near p6NR0.5 in retinoblastoma patients' fibroblasts. DNA (3 μg per lane) of fibroblast lines from two hereditary retinoblastoma patients (F1 and F2) and from representative nonretinoblastoma tumor lines (N) was digested with restriction endonuclease *Hin*dIII, *Eco*RI, or *Sst* I and was processed for DNA blotting with probe p6NR0.5 exactly as described in Fig. 3*B*.

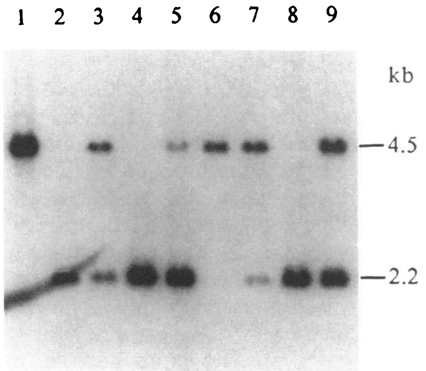


FIG. 5. *Bam*HI restriction fragment length polymorphism detected by p6NR0.5. DNA (5 μg per lane) from nine arbitrarily selected nonretinoblastoma tumor lines was digested with restriction endonuclease *Bam*HI and processed for DNA blotting with probe p6NR0.5 as described in Fig. 3*B*. Unequal band intensities in lanes 5 and 7 indicate the presence of more than two copies of the *RB* gene.

DNA Polymorphism for a *Bam*HI Site in Intron 1. Genomic DNA from nine nonretinoblastoma tumor lines was digested with *Bam*HI and hybridized with probe p6NR0.5 in DNA blotting analysis (Fig. 5). Four heterozygous and five homozygous genotypes were observed, which in diploid individuals would result from 10 small (2.2 kb) and 8 large (4.5 kb) alleles. Examination of two pedigrees confirmed codominant inheritance (data not shown). These results suggest that nearly 50% of individuals are heterozygous, which is optimal for an informative two-allele polymorphism.

DISCUSSION

Several lines of evidence indicate that mutational inactivation of both alleles of the *RB* gene is required for retinoblastoma genesis. The structural analysis presented herein was undertaken to provide a framework for characterizing these mutations. We screened both normal and mutant genomic libraries with the intent of possibly detecting a subtle mutation in the latter; in this regard, the endeavor was quite successful. Since both normal and mutant clones were used to assemble the normal gene map, it may be argued that another unsuspected genomic rearrangement of Y79 might be incorporated inadvertently. However, the map correctly predicts the linear order and sizes of all exon-containing *Hin*dIII fragments seen in genomic DNA blotting (10–12). A previously described 14- or 14.5-kb band at the 5′ end (10, 12) is now measured at about 19 kb with more careful regard to sizing (unpublished observations), in agreement with clones from the normal library.

Two gaps of unknown size remain in the gene map, indicating that the *RB* gene is larger than 150 kb. We have confirmed that these gaps contain only intron sequences since exons adjacent to the gaps are also directly adjacent in the cDNA sequence. Using pulsed-field gel electrophoresis (22), we have preliminarily es-

tablished that the middle and 3′ contigs share a common 250-kb *Sfi* I fragment while the 5′ end is on an adjacent 50-kb *Sfi* I fragment (unpublished results). Accordingly, we estimate the overall size of the *RB* gene at 200 to 225 kb.

The Y79 genomic library yielded two clones that defined a partial deletion of one *RB* allele. While such deletions are expected to result in a 50% reduction of hybridization intensity on DNA blotting with selected cDNA probes, no such reduction can be appreciated in two previous independent investigations (10, 12). One possible explanation is that Y79 might contain more than two copies of the *RB* gene, with only one copy carrying a deletion. However, the simple two-allele appearance of Y79 DNA in Fig. 3 argues against this. Alternatively, the missing exons may be translocated elsewhere in the genome rather than completely lost. Our cDNA probe RB0.9 (exons 2–8) could not detect alteration of the 19-kb *Hin*dIII band (exons 1 and 2) because exon 2 was removed by the deletion. We conclude that deletions beginning and ending in introns of large genes are not ideally detected by cDNA probes. Of eight retinoblastoma tumors examined in this and our previous report (10), five have now been observed to have complete or partial DNA deletions.

The aberrant restriction pattern (indicating heterozygous deletion) seen in genomic DNA from Y79 was exactly duplicated in DNA from RB355 and WERI-27. Previous studies have shown that these three cell lines all contain shortened RB mRNA transcripts of ≈4.0 kb and lack the normal 4.7-kb transcript (figure 3, lanes 1, 2, and 5 in ref. 10). A cDNA library made from Y79 has been screened, and a 3.8-kb cDNA clone has been isolated that corresponds to the genomic deletion of exons 2–6 observed in Y79 (unpublished results). Therefore, the deleted alleles in RB355 and WERI-27 most likely give rise to shortened RB mRNAs as well. Since retinoblastoma genesis is postulated to require inactivation of both *RB* alleles, we must assume that undetected mutations have occurred in the other alleles, resulting in a lack of detectable 4.7-kb transcripts (10) and a total absence of RB protein (13).

While containing similar deletions, these three cell lines were established in different cities from tumors of unrelated patients. Y79 and WERI-27 were derived from patients with bilateral tumors (ref. 14; unpublished data), whereas RB355 was from a unilateral tumor (21). Most hereditary cases result from new germline mutations (2); common mutant alleles are not widely dispersed as in some recessive genetic disorders. Given these circumstances, the possibility of an inherited, common deletion allele is exceptionally remote. The cell lines themselves are karyotypically distinct with different, unique marker chromosomes (refs. 21 and 23; unpublished data). We conclude that three independent deletion events occurred at approximately the same genetic site near p6NR0.5. Similar rearrangements were detected in somatic cells from two bilateral retinoblastoma patients, demonstrating that mutations of this type are involved in the heritable predisposition to retinoblastoma (5).

Common sites for genomic rearrangement are of interest in understanding molecular mechanisms of natural mutagenesis. Deletions of the low density lipoprotein receptor gene and the γ,δ,β-globin gene complex occur frequently at *Alu* repetitive sequences (24), presumably by unequal crossing-over. The same mechanism may explain partial reduplication of the low density lipoprotein gene (25). Identifying such common sites also has clinical application: at present, there is no genetic method to distinguish unilateral hereditary retinoblastoma patients from unilateral nonhereditary cases. The former can transmit retinoblastoma susceptibility to offspring and are at risk for second primary malignancies, whereas the latter are not (1). If the retinoblastoma gene is commonly inactivated by genomic rearrangements, then probes specific for these rearrangements may be used to detect predisposing mutations in DNA from patients' somatic cells. This technique might be extended to other types of common mutations if these exist. Panels of mutation probes could then supplement restriction fragment length polymorphism analysis in retinoblastoma pedigrees, since *RB* gene mutations would be detected directly rather than by linkage. Probe p6NR0.5 also detects a useful two-allele restriction fragment length polymorphism, so patients without DNA rearrangements in this region have a 50% chance of being heterozygous and informative for pedigree analysis.

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