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Mutations in the *RB1* Gene and Their Effects on Transcription

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Inactivation of both alleles of the *RB1* gene during normal retinal development initiates the formation of a retinoblastoma (RB) tumor. To identify the mutations which inactivate *RB1*, 21 RB tumors isolated from 19 patients were analyzed with the polymerase chain reaction or an RNase protection assay or both. Mutations were identified in 13 of 21 RB tumors; in 8 tumors, the precise errors in nucleotide sequence were characterized. Each of four germ line mutations involved a small deletion or duplication, while three somatic mutations were point mutations leading to splice alterations and loss of an exon from the mature *RB1* mRNA. We were unable to detect expression of the mutant allele in lymphoblasts of three bilaterally affected patients, although the mutation was present in the genomic DNA and transcripts containing the mutations were obvious in the RB tumors in the absence of a normal *RB1* allele. The variations in the level of expression of mutant transcripts suggest deregulation of *RB1* transcription in the absence of a functional *RB1* gene product.

The Knudson two-hit hypothesis (21) outlined the genetic changes associated with the development of heritable and nonheritable retinoblastoma (RB). In heritable cases, one allele at the *RB1* locus on chromosome 13 is inactivated in the germ line; the second event involves somatic inactivation of the second allele. Since heritable cases require only a single somatic mutation to initiate tumor formation, they most commonly develop bilateral and multifocal tumors. In the nonheritable cases, both alleles of the *RB1* gene must acquire somatic inactivating mutations in a single retinal precursor cell. Because of the low frequency of somatic mutation, nonheritable RB is always unilateral and unifocal. As initially predicted by Knudson (21) and later shown by Cavenee et al. (4), all RB tumors have mutations in both alleles of the *RB1* gene. In 70% of tumors, the second mutation involves the somatic loss of the normal allele, often by mitotic recombination and loss of heterozygosity (LOH) in the *RB1* locus (4, 9).

A cDNA corresponding to the *RB1* locus has been cloned and sequenced (2, 11, 12). The mRNA is ubiquitously expressed, producing a 928-amino-acid, 105-kilodalton nuclear phosphoprotein, detectable in a variety of tissues (12, 24). Homozygous deletion of *RB1* has been noted in several RB tumors and in other tumor types (12, 13, 17, 18, 23, 24). However, most RB tumors express a full-length *RB1* transcript and do not contain *RB1* gene rearrangements when analyzed on Northern (RNA) and Southern blots (17). The two-hit model of Knudson predicts that both *RB1* alleles in these tumors must contain subtle mutations leading to functional inactivation of the *RB1* gene product. Previously, we demonstrated that subtle mutations, detectable only by RNase protection, were the predominant types of mutations in RB tumors (10). To extend our characterization of the mutations in *RB1*, we have used RNase protection of *RB1* transcripts to locate probable mutations, followed by polymerase chain reaction (PCR) to amplify and sequence the mutant allele.

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MATERIALS AND METHODS

Cell culture and nucleic acid isolation. RB tumor cell lines (14, 15) and Epstein-Barr virus (EBV)-transformed lymphocytes (34) were grown as described previously. Fresh surgical RB tumor specimens and cultured cells were lysed in guanidine thiocyanate and layered on a CsCl gradient. The RNA and DNA were isolated as described before (6, 17).

PCR oligonucleotides. The oligonucleotides were synthesized with an Applied Biosystems model 380A DNA synthesizer.

First-strand cDNA synthesis from total RNA, using specific primers. Oligonucleotides JD1, JD3, and JD5 (0.5 pmol each) were added to 10 to 20 μg of total RNA in 10 μl of 200 mM NaCl–40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], pH 6.5–1 mM EDTA and heated to 70°C for 2 min, followed by incubation at 42°C for 4 h. cDNA synthesis was initiated by the addition of 40 μl of RTase buffer containing 60 mM Tris hydrochloride, pH 8.3, 12.5 mM $MgCl_2$, 50 mM KCl, 2.5 mM $NaPP_i$, 2.5 mM dithiothreitol, a 0.5 mM concentration of each deoxynucleoside triphosphate, 25 U of RNAguard (Pharmacia), 20 U of avian myeloblastosis virus (Boehringer Mannheim Diagnostics), and 22 U of murine (Pharmacia) reverse transcriptases. Reaction mixtures were incubated at 40°C for 2 h and then frozen at −20°C until needed.

Amplification of cDNA and genomic DNA, using the PCR. Genomic DNA (1 μg) or cDNA (1/10 of first-strand synthesis) was denatured in 0.2 M NaOH for 5 min at room temperature and then neutralized with HCl prior to amplification. PCR was performed as described before (28), using three constant-temperature water baths and a robot arm to move the samples between water baths. For amplifications of ≤500 base pairs (bp), we used 80-s denaturation at 93°C, 100-s annealing at 56°C, and 60-s extension at 72°C. For longer products, the extension times were increased. The extension times were increased to 5 to 10 min on the last cycle to ensure complete synthesis. Typically, 30 cycles were sufficient for cloning and RNase protection experiments. The PCR reaction mixture was brought to 5 mM EDTA and 300 mM NaOAc and extracted sequentially with equal volumes of phenol and chloroform-isoamyl alcohol

TABLE 1. Summary of mutations in RB tumors and constitutional cells from patients

RB	Cells	LOH	DNA		RNA	
			Allele 1	Allele 2	Allele 1	Allele 2
Bilateral[a]	RB429	Yes[b]	(5-bp deletion)	(5-bp deletion)	5-bp deletion	5-bp deletion
Bilateral	EBV538	No	55-bp duplication	Normal	No transcript	Normal
	RB538	Yes	55-bp duplication	55-bp duplication	55-bp duplication	55-bp duplication
Bilateral	EBV543	No	10-bp deletion	Normal	No transcript	Normal
	RB543	Yes	10-bp deletion	10-bp deletion	10-bp deletion	10-bp deletion
Bilateral	EBV570	No	9-bp deletion	Normal	No transcript	Normal
	RB570B	Yes	9-bp deletion	9-bp deletion	9-bp deletion	9-bp deletion
	RB570C	No	9-bp deletion	Point mutation	No transcript	Exon 22 deletion
	RB570D	No	9-bp deletion	Unknown	9-bp deletion	Unknown
Unilateral	EBV571	No	Normal	ND[c]	Normal	ND
	RB571	No	Point mutation	Unknown	Exon 12 deletion	No transcript
Unilateral[a]	RB600	Yes	Point mutation	Point mutation	Exon 12 deletion	Exon 12 deletion

[a] Normal cells not available from these patients.
[b] Inferred LOH.
[c] ND, Not determined.

(24:1). The mixture was precipitated with 2.5 volumes of ethanol, pelleted by centrifugation, and suspended in TE (10 mM Tris hydrochloride, 1 mM EDTA, pH 7.4) prior to further manipulation.

Agarose gel electrophoresis of PCR products. Typically, 1/10 of the PCR reaction was separated on 1.2% agarose gels for products ≥800 bp in length or 1% agarose–2% NuSieve composite gels for smaller products. Gels were electrophoresed, and the DNA was visualized as described previously (25).

RNase protection assay. Antisense RNA (cRNA) probes were generated in vitro as described previously (10). In a volume of 30 μl, 1/50 of the PCR reaction was hybridized to 10^5 cpm of labeled RNA probe. RNase digestion and electrophoresis were performed as described before (10).

Cloning of PCR products. The PCR products were ligated into the *Eco*RV site of plasmid Bluescript SK (Stratagene) and used to transform XL-1 Blue (Stratagene) *Escherichia coli*. Colonies containing the insert were identified by their inability to cleave the X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) substrate.

DNA sequencing. Double-stranded plasmid templates containing the cloned PCR products were isolated as described previously (26). Dideoxynucleotide chain termination sequencing reactions were performed on the plasmid DNA, using a Sequenase kit (version 2.0) according to the specifications of the supplier (U.S. Biochemical Corp.).

RESULTS

Detection of mutations. The *RB1* gene has 27 exons spanning approximately 180 kilobases of genomic DNA and transcribes a 4.7-kilobase mRNA. To simplify the initial screening of tumors, we focused our analysis on the coding sequence in the *RB1* transcripts; most tumors (85%) express detectable transcripts and are suitable for analysis with this strategy. Mutations were identified in 13 of the 21 tumors that were screened by RNase protection of *RB1* RNA transcripts. Eight of these 13 RB tumors were studied in greater detail, using PCR to isolate and sequence the mutant portion of the genome (Table 1). Of the eight RB tumors analyzed by PCR, two were from patients with unilateral disease and six were from four patients with bilateral RB tumors. EBV-transformed lymphoblast cell lines from four RB patients and the parents of three of the bilaterally affected patients were also studied.

The Taq DNA polymerase requires a DNA template (28); therefore, the *RB1* mRNA was first converted to cDNA by reverse transcriptase. Three antisense oligonucleotides (JD1, JD3, and JD5) (Fig. 1) were hybridized to total cellular RNA to prime first-strand cDNA synthesis. Three separate PCR reactions, each covering approximately one-third of the coding region, were performed on the first-strand cDNA (Fig. 1), and the PCR-cDNA products were analyzed by agarose gel electrophoresis and the RNase protection assay. Once the approximate location of the mutation was identified, the appropriate PCR-cDNA fragment was cloned and sequenced. To determine whether or not the mutations

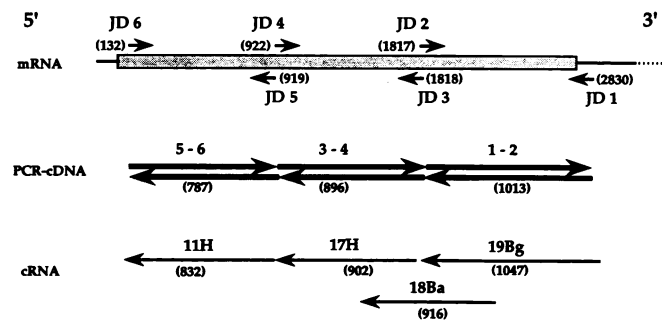

FIG. 1. Diagram of *RB1* mRNA, PCR oligonucleotides, PCR-cDNA products, and cRNA antisense probes. The coding region of the *RB1* mRNA is shown as a filled box. The positions of the oligonucleotide primers used in the initial PCR reactions are shown as small arrows above (sense) and below (antisense) the mRNA. Numbers in parentheses indicate the number of nucleotides from the start methionine. The three PCR-cDNA products are shown as opposing arrows, with the numbers of the primers used above and the length of the normal product below. The antisense cRNA probes used to screen the total RNA and PCR products are shown as solid arrows, with designation above and the length of protected product below.

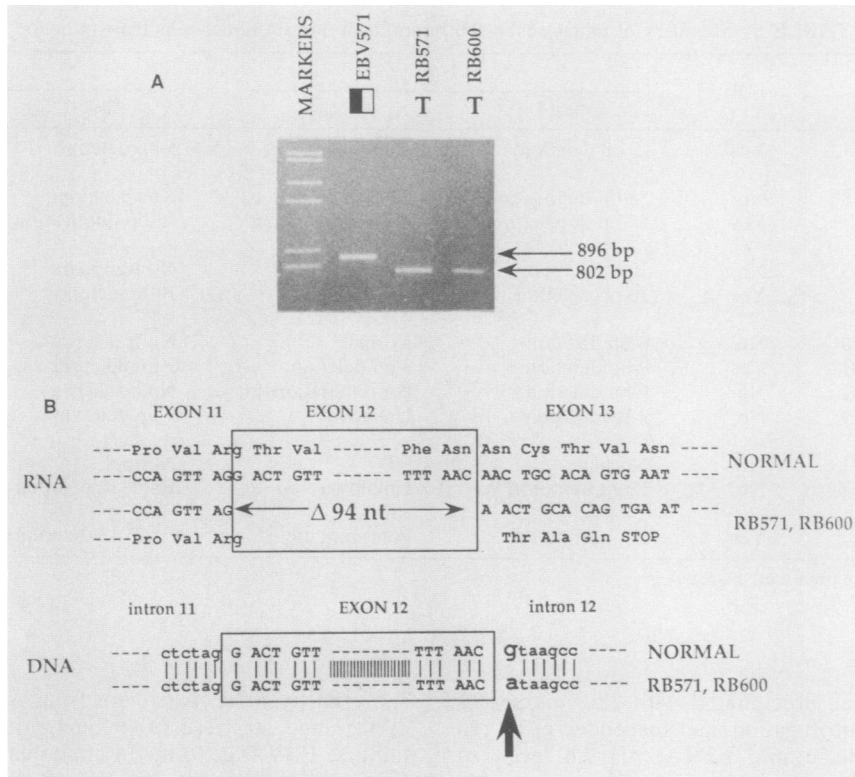

FIG. 2. Evidence that point mutation causes loss of exon 12 from *RB1* mRNA in RB571 and RB600. First-strand cDNAs were amplified and separated by agarose gel electrophoresis as described in Materials and Methods. (A) Ethidium-stained gel of PCR-cDNA amplified with oligonucleotides JD3 and JD4 from constitutional cells of patient 571 (EBV571), RB571, and RB600. *Eco*RI- and *Hin*dIII-digested λ DNA were run as size markers. The length of the fragments is indicated on the left. A product of decreased length was detected in the RB571 and RB600 cDNAs. (B) Nucleotide sequences of wild-type and mutant *RB1* mRNA and genomic DNA at the intron boundaries of exon 12. The deletion of exon 12 in RB571 and RB600 RNAs causes a translational frame shift and a termination codon in exon 13. The DNA mutation, a G-to-A transition at the splice donor site of exon 12, is indicated with a wide arrow.

detected in the RNA were present in the DNA, the relevant regions in genomic DNA from tumor cells and lymphoblasts were amplified by PCR, using oligonucleotides flanking the mutant region. The genomic sequence of *RB1* was provided by T. Dryja (25a). For yet unknown reasons, we have been unable to amplify the sequences of exon 1 from either cDNA or genomic DNA, and we were unable to characterize three tumors with mutations in this region (10).

Identical somatic point mutations result in loss of exon 12 from two unrelated RB tumors. The unilateral and unifocal tumors RB571 and RB600 were isolated from two unrelated patients with no family history of RB. Analysis by agarose gel electrophoresis of the PCR-cDNA products (primed with JD3-4) of RB600, RB571, and EBV571 (an EBV-transformed lymphoblast line from patient 571) showed an anomalous band migrating at a lower molecular weight in both tumors compared with the control (Fig. 2A). The PCR-cDNA products of RB571 and RB600 were cloned into plasmid vectors and sequenced. All 10 cDNA clones isolated from both tumors were missing exon 12, consistent with splice site mutations. Loss of exon 12 introduced a frameshift into the reading frame which could produce a truncated protein of 379 amino acids.

To identify the exact mutation responsible for the aberrant splicing, we amplified and sequenced the genomic region surrounding and including exon 12. Oligonucleotides corresponding to genomic sequences flanking exon 12 were used in PCR amplification of the genomic DNA isolated from RB571, RB600, and the constitutional cells (EBV571) of patient 571. Constitutional cells from patient 600 were not available. All 10 clones isolated from EBV571 contained the normal sequence. Clones representative of each allele were isolated from the RB571 tumor: two contained the normal sequence and three contained a G-to-A transition at the splice donor site of exon 12 (Fig. 2B). These results confirm the restriction fragment-length polymorphism analysis showing that RB571 retains both maternal and paternal alleles. Using the sensitive PCR technique, we were unable to detect any cDNAs which contained exon 12; apparently, the mutation on the other allele prevents synthesis of a detectable transcript. The genomic PCR products from RB600 contained the same G-to-A transition in all 10 clones isolated, suggesting that LOH was the second genetic event in this tumor.

Identification of germ line mutations. RB tumors isolated from patients with bilateral tumors must have one mutant *RB1* allele in the germ line. The four tumors from bilaterally affected individuals without a family history of RB (RB429, RB538, RB543, and RB570B) all demonstrate LOH (35), so that the allele remaining in each tumor should carry the germ line mutation. The constitutional cells from three of these patients contained the mutation detected in the RB tumor; constitutional cells of patient 429 or her parents were not available. None of the parents of the other three patients carried the mutated allele, confirming that these children have new germ line mutations.

(i) RB429. RNase protection of the *RB1* mRNA and sequencing the PCR-cDNA demonstrated a 5-bp deletion in

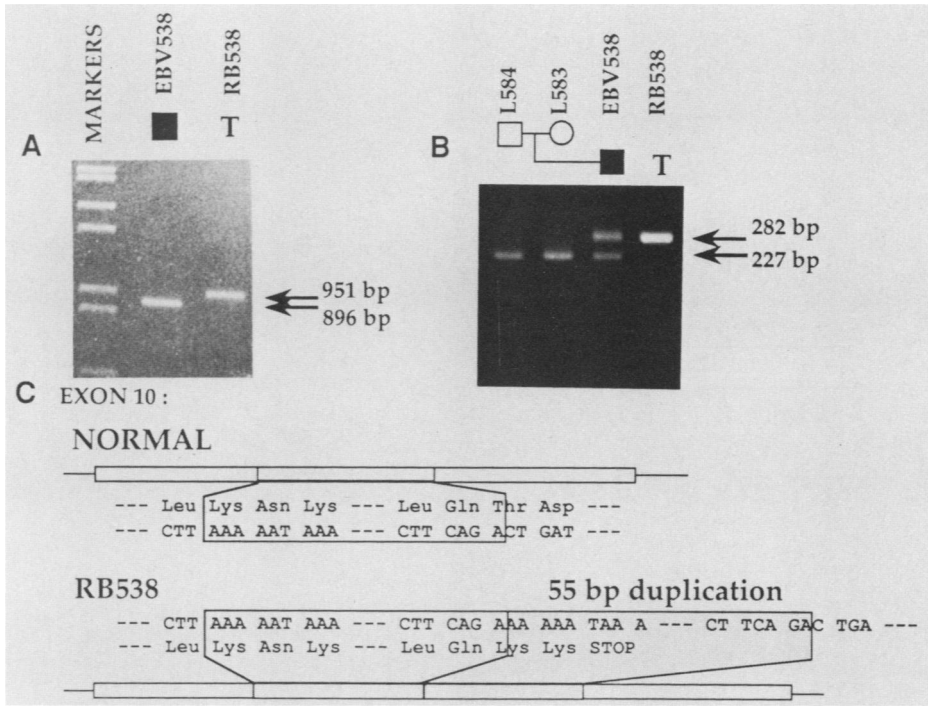

FIG. 3. Duplication of 55 bp of DNA in the constitutional cells (EBV538) and RB538 tumor from bilaterally affected patient 538. (A) Ethidium-stained gel of PCR-cDNA amplified with oligonucleotides JD3 and JD4 from constitutional cells of patient 538 (EBV538) and RB538. *Eco*RI- and *Hin*dIII-digested λ DNA were run as size markers. The length of the fragments is indicated on the right. An anomalous fragment of increased length in the RB538 lane is not detectable in EBV cells from the patient. (B) Ethidium-stained 3% NuSieve agarose composite gel of PCR-amplified genomic DNA from the patient's constitutional cells, RB538, and his parents' lymphocytes. (C) Nucleotide sequence of the region of exon 10 containing the 55-bp duplication in patient 538. The box indicates the 55 bp of DNA which is duplicated. The duplication introduces a frameshift and termination TAA shown below.

exon 8. The absence of a normal sequence in this region confirmed the LOH for this tumor (29). The deletion shifted the reading frame and resulted in a new TGA termination codon within exon 8. The predicted truncated protein would contain 268 amino acids. The constitutional cells were not available from this patient to confirm the 5-bp deletion as the germ line mutation.

(ii) RB538. Figure 3A shows agarose gel electrophoresis of the PCR-cDNA products of EBV538 and RB538, generated by using the JD3 and JD4 primers. A band of increased molecular weight is seen in the RB tumor. Southern blot analysis and RNase protection of these PCR products indicated no mutant product in the lymphoblasts (data not shown). Cloning and sequencing of the PCR-cDNA from the tumor revealed a 55-bp duplication within exon 10. This duplication altered the reading frame and introduced a novel TAA termination codon at position 346 (Fig. 3C). According to the two-hit model, the 55-bp duplication should be the germ line mutation; however, no transcripts containing this mutation were detected in the constitutional cells of the patient. To resolve this discrepancy, oligonucleotides homologous to the intron sequences flanking exon 10 were used for PCR amplification of genomic DNA from RB538, the constitutional cells of patient 538 and his parents. Agarose gel electrophoresis of the PCR-amplified products from genomic DNA are shown in Fig. 3B. The two bands in the constitutional cells of the patient indicate one allele with the 55-bp duplication and one normal allele; as expected, the RB tumor contained only the 55-bp duplication. Since neither parent carried this mutation, the 55-bp duplication must be a new germ line mutation. It is noteworthy that no mutant mRNA was detected in the lymphoblasts, while a mutant mRNA was easily detectable in the RB tumor (see Discussion).

(iii) RB543. We previously localized a mutation in RB543 to exon 18 of the *RB1* gene (10). The tumor had undergone LOH and contained only mutant transcripts. We were unable to detect the predicted germ line mutation in the RNA of a patient's constitutional cells. Cloning and sequencing the PCR-cDNA defined the mutation in RB543 as a 10-bp deletion within exon 18. The deletion shifted the reading frame and resulted in a TAA termination codon. The predicted truncated protein would contain 586 amino acids. PCR amplification of genomic DNAs from constitutional cells of patient 543 and her parents and from tumor RB543 were performed with oligonucleotides flanking the mutation. RNase protection of these products detected the 10-bp deletion on one allele of the patient's constitutional cells (EBV543), confirming that it was the germ line mutation (Fig. 4A). RB543 contained only the 10-bp deletion. Since neither parent had the mutation, the 10-bp deletion must be a new germ line mutation in their child.

(iv) RB570B. Patient 570 presented with advanced tumors requiring enucleation of both eyes. Four separate tumors, RB570A, -B, -C, and -D, were isolated from the two eyes. Analysis of these four tumors with RFLP probes on chromosome 13 indicated that RB570A and -B (left eye) showed LOH for markers within *RB1*. RB570C and -D (right eye) remained heterozygous for all RFLPs tested (35). RNase protection was performed on total RNA from tumors RB570B, -C, and -D and EBV570 (data not shown). All transcripts in RB570B contained a mutation in exon 19;

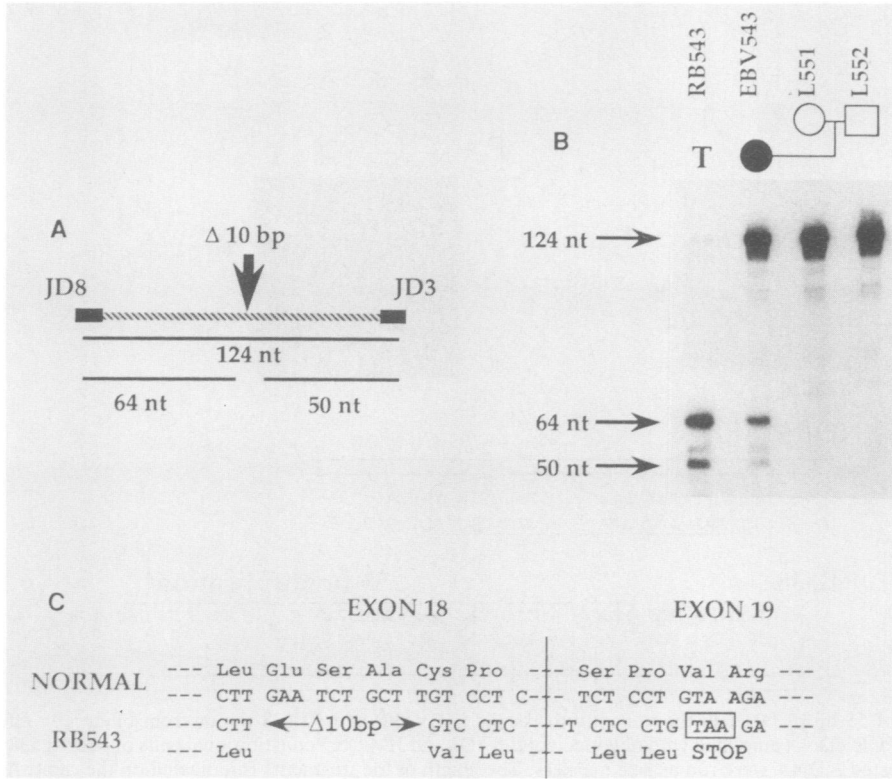

FIG. 4. A 10-bp deletion in the germ line and RB tumor of bilaterally affected patient 543. (A) Diagram of PCR products, using oligonucleotides JD3 and JD8. The position of the 10-bp deletion is indicated above, and the length of protected cRNA probes is shown below. (B) RNase protection of PCR-amplified genomic DNAs performed as described in Materials and Methods. Antisense cRNA probe 18Ba was used to protect the amplified products of the constitutional cells of patient RB543 and the lymphocytes of the parents. The sizes of protected fragments are given on the left. (C) Nucleotide sequence of the region of exon 18 which contains the 10-bp deletion in RB543 and EBV543 cells. The deletion results in a translation frameshift and a TAA termination codon in exon 19.

transcripts from RB570D contained this mutation and an apparently normal transcript; all RB570C transcripts contained a mutation in exon 22; and EBV570 contained only normal transcripts. Since the mutation in exon 19 was present in tumors isolated from different eyes, it was assumed to be in the germ line, even though transcripts with this mutation were not observed in the patient's lymphoblasts or in RB570C (see below).

Oligonucleotide primers homologous to intron sequences flanking exon 19 were used to amplify genomic DNA from constitutional cells of patient 570 and her parents and from tumors RB570B, -C, and -D. RNase protection of the PCR products confirmed that a 9-bp deletion (see below) was the germ line mutation carried by the patient and present in the three tumors studied (Fig. 5A). Both parents carried only normal alleles, indicating that this patient had a new germ line mutation. Since the 9-bp deletion in exon 19 led to the formation of a novel, in-frame TAA termination codon, translation of this message would result in a truncated protein of 649 amino acids.

Somatic mutations in different tumors from a patient with bilateral RB. All tumors arising in a patient with bilateral RB should contain the same germ line mutation but should have different somatic mutations, since each tumor arises independently. As shown above, the normal cells and all tumors from patient 570 contain a 9-bp deletion in exon 19; clearly, this is the predisposing germ line mutation. The second somatic mutations in all four tumors are different, confirming their independent origin. In tumors RB570A and -B, the second somatic mutation was LOH on chromosome 13, in RB570A by mitotic recombination between the *RB1* gene and the most proximal informative RFLP (p7F12) and in RB570B by LOH for all informative RFLPs (35). By using primers JD1 and JD2, two different clones were isolated from the PCR-amplified cDNA of RB570D: one carried the normal sequence, while the other contained the 9-bp deletion within exon 19. The somatic mutation which initiated formation of RB570D has not yet been identified. Agarose gel electrophoresis of the PCR-cDNAs generated with primers JD1 and JD2 from the constitutional cells of patient 570 and the three tumors RB570B, -C, and -D is shown in Fig. 6A. The anomalous band of lower molecular weight was seen only in sample RB570C, a tumor in which transcripts with the 9-bp deletion were not detected. The location (defined by RNase protection) and size of the mutant cDNA were consistent with a deletion of exon 22 from the mature mRNA. This observation was confirmed by cloning and sequencing the PCR-cDNA product from RB570C. All clones were missing exon 22. Since, using oligonucleotides JD1 and JD2, the PCR-cDNA product contains both exon 19 and 22, it was clear that the allele transcribing the exon-22-deleted message was not the allele which contained the germ line 9-bp deletion. Oligonucleotides flanking exons 22 and 23 were used to amplify genomic DNA from EBV570 and RB570C. One allele of the tumor showed a single-base-pair change: the AG of the splice acceptor site of exon 22 was changed to AA. The other allele from the tumor and both alleles from the EBV cell line showed the normal AG.

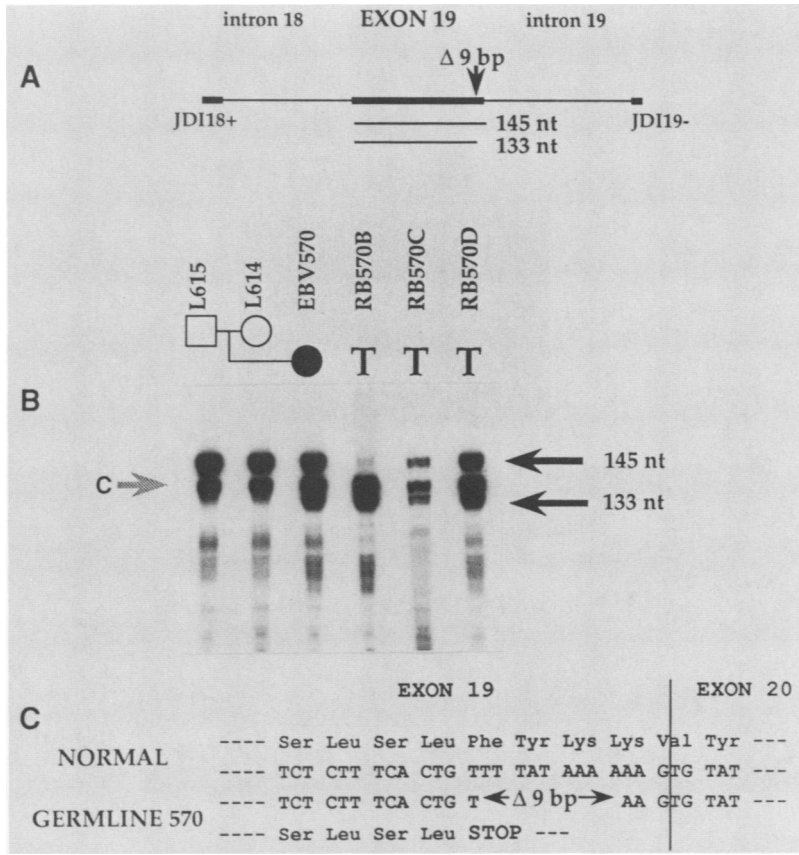

FIG. 5. A 9-bp deletion in the germ line and three individual RB tumors isolated from a bilaterally affected patient. (A) Diagram of PCR products, using oligonucleotides JDI18+ and -I19−. The position of the 9-bp deletion is indicated above, and the length of protected cRNA probes is shown below. (B) RNase protection of PCR-amplified genomic DNAs performed as described in Materials and Methods. Antisense cRNA probe 18Ba was used to protect the amplified products of the constitutional cells of the patient (EBV570), RB570B, -C, and -D and the parents' lymphocytes. The sizes of protected fragments are given on the right. Since only three nucleotides remain beyond the deletion in exon 19, the mutant transcript protects a smaller fragment 133 nucleotides in length. The reduced signal in RB570C was due to the limiting amount of DNA available. (C) Nucleotide sequence of the region of exon 19 which contained the 9-bp deletion in RB570B, -C, -D, and EBV570. The deletion resulted in the formation of an in-frame TAA termination codon at the site of the deletion.

Ligation of exon 21 to exon 23 leaves the mRNA in frame and should result in a truncated *RB1* protein missing only the 38 amino acids encoded by exon 22.

DISCUSSION

Characterization of mutations in *RB1*. The Knudson two-hit model (21) makes several clear predictions on the state of the *RB1* alleles in RB patients and their tumors. The first prediction is that all bilateral RB patients carry one mutated and one normal allele in their constitutional cells. We have detected mutations of one allele of *RB1* in the constitutional cells from four bilaterally affected RB patients: EBV247 (shown previously [10]), EBV538, EBV543, and EBV570.

The second prediction is that a somatic mutation inactivates the remaining normal allele of the recessive *RB1* gene to initiate tumorigenesis. Earlier studies have shown that, in 70% of RB tumors, this second hit was loss of the normal allele and duplication of the mutant allele (5), often by mitotic recombination. Molecular demonstration of this event is presented here for RB538, RB543, and RB570B; both alleles in these tumors carry the germ line mutation detected on one allele of the EBV line. In the remaining 30% of tumors, the somatic mutation has been assumed to be different from the first mutation. The mutation in RB570C validates this conclusion: in this tumor, one allele has the germ line 9-bp deletion and a somatic point mutation present in the other allele results in aberrant splicing and the loss of exon 22.

The third prediction of the Knudson model is that the multiple tumors arising in patients with bilateral RB will all contain the same germ line mutation, but different somatic mutations will inactivate the remaining normal allele. Patient 570 had four independent tumors. All tumors and the lymphocytes have a 9-bp deletion in exon 19. As described above, the second somatic mutation was different in each tumor (Table 1).

The fourth prediction is that most patients with unilateral RB develop tumors from the occurrence of two somatic mutations in a single retinoblast cell and will have two normal alleles in their constitutional cells. We show that RB571 and RB600, unilateral tumors, had the same point mutation in the splice donor site of exon 12, resulting in a mature mRNA lacking the entire exon 12. Since patient 600 was unavailable and one mutation in RB571 was undefined, we were unable to demonstrate unequivocally the presence of two independent somatic mutations in unilateral RB.

A splice donor mutation similar to that seen in RB571 and -600, resulting in skipping an exon, has been documented in

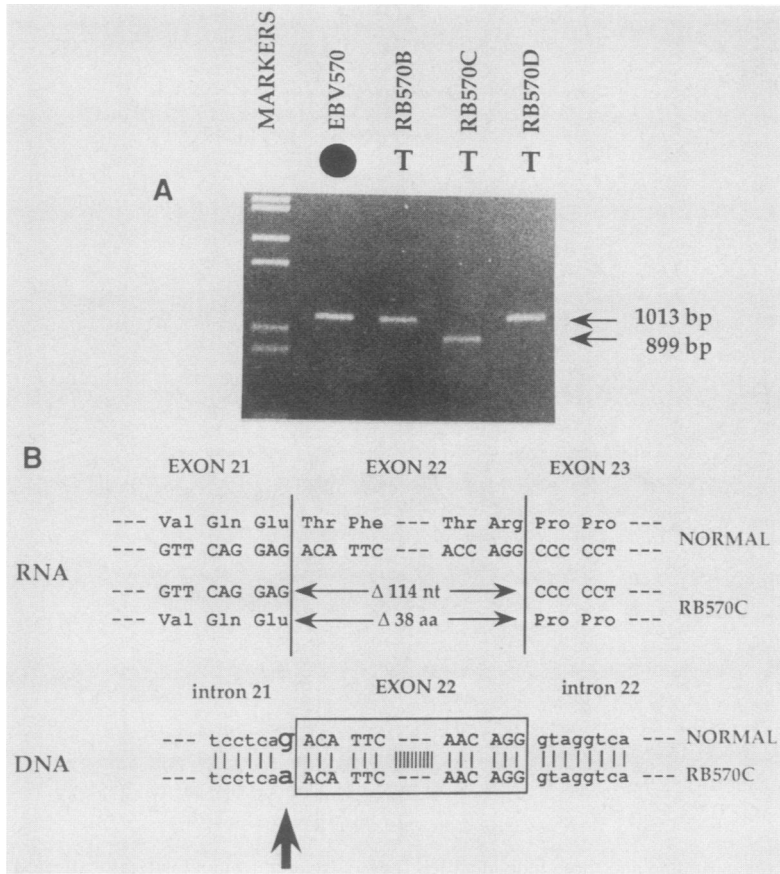

FIG. 6. Evidence that a point mutation resulting in an in-frame deletion of exon 22 is the second somatic mutation in RB570C. (A) Ethidium-stained gel of PCR-cDNA amplified with oligonucleotides JD1 and JD2 of constitutional cells of the patient (EBV570) and RB570B, -C, and -D. *Eco*RI- and *Hin*dIII-digested λ DNA were run as size markers. The length of the fragments is indicated on the right. (B) Nucleotide sequence of normal and RB570C PCR-cDNA and genomic DNA at the intron boundaries of exon 22. The deletion of exon 22 results in the in-frame deletion of 38 amino acid residues from the *RB1* coding sequence. The DNA mutation, a G-to-A transition at the splice accepter site of exon 22, is indicated with an arrow.

thalassemia (31). However, in that instance, the majority of the transcripts of the cloned mutant used a cryptic splice donor 3′ of the exon, resulting in a transcript of increased length. Only a fraction of the transcripts were truncated by skipping of the exon. In contrast, in RB571 and RB600, only the truncated transcript missing exon 12 was detected. This may be due to the absence of an adequate cryptic splice donor in intron 12 or to instability of transcripts created from a cryptic splice donor site.

Naturally occurring mutations which lead to disease states have revealed much about the predisposing genes. Analysis of the mutations in a low-density lipoprotein receptor gene in familial hypercholesterolemia (3), α- and β-globin genes in thalassemia and sickle cell anemia (27), the gene for phenylalanine hydroxylase in phenylketonuria (8), and the hypoxanthine phosphoribosyltransferase gene in Lesch-Nyhan syndrome (32) have delineated functional domains and suggested mechanisms of mutation. Analysis of mutations in *RB1* has not yet provided such information (19, 22), although Horowitz et al. did correlate loss of exon 21 in a bladder carcinoma cell line with decreased stability of the *RB1* protein and with loss of the ability to bind adenovirus E1A protein.

RB tumors present a unique opportunity to study spontaneous germ line and somatic mutations in the same gene. It is interesting that all four germ line mutations involve small deletions or duplications. The three point mutations leading to exon loss by aberrant splicing are all somatic mutations. Deletions or duplications may be the result of double-strand breaks followed by the DNA repair characteristically associated with meiotic recombination (30). Some diseases with a high frequency of new germ line mutations, such as Duchenne muscular dystrophy, also often involve deletions and duplications (1, 20). Patients with Lesch-Nyhan syndrome have germ line mutations in the hypoxanthine phosphoribosyltransferase gene; mutations can be small deletions, splice mutations (16), or point mutations (33). Diseases with a low frequency of new germ line mutations, such as the β thalassemias (27), are more often point mutations than deletions.

Our failure to detect single-base-pair changes within the coding region of the gene may be due to our selection by RNase protection of tumors for analysis. Deletions and insertions are the mutations most easily detectable by RNase protection, while single-base changes may be undetectable by the conditions we used for RNase protection. We are now sequencing the coding region of the tumors which do not show mutations by RNase protection; we expect to observe single-base-pair mutations in these tumors.

Effects of mutations on *RB1* transcription. Results from this study and our previous work indicate the presence of only normal transcripts in the constitutional cells of bilaterally

affected RB patients, e.g., F462, EBV538, EBV543, and EBV570; except for EBV247 (10), we did not detect transcripts from the mutant allele in lymphocytes. In the RB tumors of these patients, the mutant mRNA was easily detectable. It is difficult to explain these results without postulating regulation of transcription of the *RB1* gene. Two assumptions are required to construct a model which will fit the available data. First, a functional *RB1* gene product must directly or indirectly regulate its own transcription rate. In support of this assumption, adenovirus-transformed human retina cells express high levels of *RB1* transcripts (17); we suggest that the binding of the *RB1* gene product by the E1a protein of adenovirus prevents feedback regulation and leads to increased transcription of the *RB1* gene in transformed cells. Second, the *RB1* transcripts produced from the mutant allele must be less stable than the normal transcript. The instability of transcripts with premature termination codons has been documented for other genes (7, 27).

We suggest that the following changes in transcription occur in the cells of patients with a germ line mutation in one *RB1* allele. In normal cells, i.e., lymphocytes, the transcription rate may increase twofold to compensate for the functional loss of *RB1* product from the mutant allele. If the mutant transcript is unstable, its steady-state level will be much less than that of the wild-type transcript, making the mutant transcript undetectable in normal tissues. In RB tumors, however, both alleles are mutant, and the complete absence of functional *RB1* protein will result in maximal transcription rates from both mutant alleles, allowing the accumulation of detectable levels of unstable mutant mRNAs in the tumor cells. This model can explain the unexpected observations made on patients 538 and 543. The germ line mutation in patients 538 (55-bp duplication) and 543 (10-bp deletion) produce premature termination of translation, probably leading to unstable transcripts which are undetectable in lymphoblasts. However, the tumors in both patients show LOH and contain only the mutant allele. The absence of functional *RB1* protein results in very high rates of transcription from the mutant alleles and accumulation of levels of *RB1* transcripts which are easily detectable despite their instability.

Patient 570 presents a unique opportunity to test the assumptions of increased transcription of *RB1* in tumors and instability of mutant *RB1* messages. As with the other patients, the mutant allele (9-bp deletion) does not produce detectable transcripts in lymphocytes. Three different tumors from this patient all show different patterns of *RB1* transcripts. Tumor RB570B shows LOH and contains only the allele with the germ line mutation; mutant transcripts are easily detectable in this tumor. RB570D retains the mutant germ line allele and has an uncharacterized mutation in the remaining allele. Transcripts from both alleles are detectable in this tumor at approximately the same level, indicating that the somatic mutation in RB570D results in a transcript whose stability is comparable to that containing the germ line mutation. Tumor RB570C contains the germ line mutation on one allele and a somatic splice accepter mutation on the other allele, leading to loss of exon 22 from the mature mRNA. This tumor contains only transcripts with exon 22 deleted; we detected no transcripts with the 9-bp deletion. We predict that the transcripts with the exon 22 deletion are more stable than the ones with the 9-bp deletion because the exon 22 deletion does not lead to premature termination. Furthermore, we expect that the transcription rate of *RB1* in RB570C will be less than in RB570B and -D.

It is perhaps significant that, of the four tumors isolated from patient 570, only RB570C has not grown in culture or in the anterior chamber of a nude mouse eye (14). The cells, which remain alive in culture, have formed Flexner-Wintersteiner rosettes, indicative of differentiating retinoblast cells. Perhaps the removal of the 38 amino acid residues from exon 22 only partially inactivates the protein, allowing it to carry out some of its functions, and permits the tumor to undergo some differentiation.

The importance of *RB1* in the developing retina is apparent from the observation that mutations in *RB1* prevent normal differentiation and lead to uncontrolled growth of retinal stem cells. A number of tumors from a variety of tissues have been shown to contain mutated *RB1* alleles, underlining the importance of *RB1* in regulating growth or differentiation in many cell types. Given the general importance of *RB1*, it is likely that its expression requires precise regulation. Our paper provides indirect, but convincing, evidence in support of a model of transcriptional regulation of *RB1*.

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