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Genetics

Molecular mechanism of retinoblastoma gene inactivation in retinoblastoma cell line Y79

(nonhomologous recombination/mutation/cancer genetics)

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ABSTRACT **Formation of retinoblastoma, a cancer arising in the retinas of young children, is determined by mutational inactivation of an autosomal gene (*RB*), which has been molecularly cloned. Whereas all normal tissues and many tumor cells express an RB mRNA of 4.7 kilobases, six of six retinoblastomas were previously found either to lack *RB* gene expression or to have RB transcripts of abnormal (reduced) length. To further characterize the latter type of mutation, we chose to examine retinoblastoma cell line Y79, which expressed a shortened RB mRNA of about 4.0 kilobases. RB cDNA clones isolated from a library constructed with Y79 mRNA demonstrated an internal loss of 470 nucleotides near the 5′ end, which corresponded to a deletion of exons 2–6. Genomic clones containing the deletion junction were isolated from a library made with Y79 DNA, which allowed precise localization and sequencing of deletion endpoints in introns 1 and 6. These regions had no apparent homology to each other or to the *Alu* family of repetitive sequences, implying that the deletion must have occurred by a mechanism other than recombination of homologous sequences. Deletion of exons 2–6 would interrupt the open reading frame in RB mRNA and would result in premature termination of translation. Since no normal RB protein was detected by immunoprecipitation with specific antibody, the other, apparently normal RB allele in Y79 cells was necessarily inactivated by a different mutation.**

It has become increasingly evident that some aspects of the cancer phenotype necessarily involve genetic alterations within tumor cells (1–3). Some of these alterations may occur in somatic cells during the life of an individual, whereas other mutations might be inherited from a parental germline. The latter type of inheritance would explain cases of familial cancer and inherited cancer predisposition (4). The clearest example of a heritable cancer predisposition is that afforded by retinoblastoma, a highly malignant but readily treatable cancer of the retina that occurs in young children. In its hereditary form, retinoblastoma can be transmitted as an autosomal-dominant cancer susceptibility trait: each offspring of a carrier parent has a 50% chance of inheriting the trait, and 90% of carriers will develop retinoblastoma (5, 6). Because of its clear-cut heritability, retinoblastoma has been a prototypic model for the study of genetic determination in cancer (4).

The retinoblastoma susceptibility (*RB*) gene has been localized to chromosome band 13q14 by linkage analysis of retinoblastoma pedigrees (7) and by examination of cases having cytogenetic deletions in somatic cells (8–10). A gene on chromosome 13 was recently identified that had properties expected of the *RB* gene (11–13). It encoded a 4.7-kilobase (kb) mRNA transcript that is expressed in most normal cells including fetal retina. Partial or complete deletion of this gene was detected in a substantial fraction of retinoblastomas. At the level of gene expression, we demonstrated that RB mRNA was altered in six of six retinoblastomas: of four tumors with apparently normal genes, three contained a shortened mRNA message (4.0 kb instead of 4.7 kb) and one had no mRNA expression (12). These findings reflect heterogeneity in *RB* gene mutation, as expected for genes suffering many independent mutational events (6). Complete loss of *RB* gene function in retinoblastomas was further confirmed at the protein level. Specific antibody was generated that immunoprecipitated a phosphoprotein of 110 kDa in many nonretinoblastoma tumor cells but not from five of five retinoblastomas, thereby identifying the *RB* gene product (14). This protein was located in the cell nucleus and was associated with DNA-binding activity, which supports its proposed role in regulating other genes (14, 15).

Examination of natural mutations of the low density lipoprotein receptor in familial hypercholesterolemia (16–18), the globin genes in thalassemias (19), and phenylalanine hydroxylase in phenylketonuria (20) has revealed important features of these genes, such as mechanisms of mutation and mRNA processing and functional domains of gene products. We have studied three retinoblastoma cell lines, Y79 (21), RB355 (22), and WERI-27 (23), that expressed RB mRNA transcripts of ≈4.0 kb (12). Preliminary analysis with Southern genomic blotting indicated that all three contained heterozygous rearrangements of the *RB* gene (23). To further characterize these mutations, RB cDNA and genomic clones were isolated from libraries constructed with Y79 mRNA and DNA, and the sequence of selected portions was determined.‡ We found that the shortened transcript in Y79 reflected the deletion of exons 2–6 in one *RB* allele and that this deletion must have occurred by a mechanism other than recombination of homologous sequences. Since normal RB protein was completely absent from Y79 cells, the second *RB* allele must have been inactivated by a different mutation, perhaps at the transcriptional regulatory region.

MATERIALS AND METHODS

Probes and Cell Lines. A normal RB cDNA fragment, RB4.5, was constructed from two shorter, overlapping clones, RB-1 and RB-5 (12), and was subcloned into plasmid pGEM-1. The RB4.5 insert was cleaved once by *Eco*RI into 5′ (RB0.8, 0.8 kb) and 3′ (RB3.8, 3.8 kb) fragments. RB1.8 (1.8 kb) was derived from RB3.8 by *Bgl* II digestion (12). All cDNA probes were labeled with [α-^{32}P]dATP by the random primer method (24). Retinoblastoma cell line Y79 was obtained from American Type Culture Collection. This cell line

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‡The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03809).

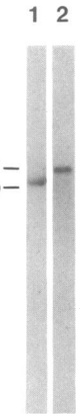

FIG. 1. RNA blotting analysis of retinoblastoma cell line Y79 and normal fetal retina. Five micrograms of polyadenylylated RNA prepared from Y79 cells (lane 1) and fetal retina (lane 2) were separated by electrophoresis in 1% formaldehyde/agarose gels and transferred to nitrocellulose filters with 20× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7). Filters were hybridized with ^{32}P-labeled RB4.5. The transcript sizes (in kb) are given at left.

originated from a patient with a maternal family history of retinoblastoma (21).

cDNA Cloning. Total cytoplasmic RNA was isolated by using the guanidine isothiocyanate method (25). cDNA complementary to Y79 polyadenylylated RNA was generated by using reverse transcriptase from avian myeloblastosis virus or Moloney murine leukemia virus and was cloned in λgt11 arms as previously described (26).

DNA Sequencing. A combination of three methods was used to sequence Y79 cDNA clones. First, cDNA inserts were subcloned into M13 and were sequenced with a universal primer and dideoxynucleotides (27). Second, double-stranded DNA sequencing was performed with selected clones as templates and synthetic oligonucleotides from the normal sequence as primers (28). Third, a G+C-rich sequence was confirmed by chemical sequencing (29). Genomic clones were sequenced by subcloning into M13 or pGEM-1.

Immunoprecipitation Analysis of ^{32}P-Labeled RB Protein. About 1 × 10^6 Y79 or LAN-1 cells were incubated in phosphate-free medium with 10% dialyzed serum for 30 min and then were metabolically labeled for 3 hr in the same medium containing 250 μCi (1 Ci = 37 GBq) of [^{32}P]phosphoric acid per ml. Cellular lysates were immunoprecipitated by IgG directed against RB protein (14) and were analyzed by 7.5% $NaDodSO_4$/polyacrylamide gel electrophoresis as previously described (30).

RESULTS

RNA Blotting Analysis and Cloning of RB cDNAs from Y79. RNA blotting analysis of poly(A)-selected RNA from retinoblastoma cell line Y79 and fetal retina (Fig. 1) demonstrated a shortened RB transcript of ≈4.0 kb in Y79 as compared to the normal 4.7-kb transcript in fetal retina. Y79 genomic DNA initially appeared to have no gross abnormalities by DNA blotting analysis with RB cDNA probes (12). To clarify the genesis of the shortened transcript, a cDNA library of Y79 mRNA was constructed with λgt11 vector and was screened with cDNA probes RB0.8 and RB3.8 from the normal *RB* gene. Five cDNA clones were isolated and were characterized by restriction endonuclease mapping (Fig. 2). Alignment of these clones to each other and to the normal cDNA was confirmed by hybridization with oligonucleotides synthesized according to the normal RB cDNA sequence.

Sequence Analysis of Y79 RB cDNAs. The longest cDNA clone, Y451, contained an insert of 3.8 kb. A significant portion of Y451 was sequenced by dideoxy chain termination, with confirmation of a C+G-rich region (14) by chemical sequencing methods. Sequence identity was found to two separate regions of the normal RB cDNA sequence (Fig. 3). The larger region of identity included the unique internal *Eco*RI site in both Y451 and normal cDNA, thus establishing that this site was homologous in both clones. In addition, nucleotides 243–275 of Y451 were identical to the first 33 base pairs (bp) of the normal RB sequence. Nucleotides 1–242 of Y451 were not found in the normal RB cDNA sequence, and Y451 lacked 470 nucleotides present in the normal sequence. These data were preliminarily interpreted as follows. Primer extension studies had suggested that our normal cDNA clones lacked about 200 bp from the 5′ end (data not shown). The initial 275 nucleotides of Y451, which terminated in 33 nucleotides identical to the normal RB sequence, were considered to represent the 5′-most portion of normal RB mRNA. This was confirmed by sequencing genomic clones containing exon 1 (unpublished data) as well as an independently cloned normal RB cDNA (31). The following 470 nucleotides missing from Y451 thus suggested a deletion in the Y79 mRNA transcript. If the normal RB cDNA sequence is renumbered to reflect the additional 5′ nucleotides (Fig. 3), then this deletion removes nucleotides 276–745 relative to the normal gene. Additional sequence analysis demonstrated the same deletion in two other RB cDNA clones, Y382 and Y383 (Fig. 2). Preliminary mapping of exon/intron junctions (H.-J. S. Huang and W.-H.L., unpublished data) showed that the missing nucleotides in these clones corresponded precisely to loss of exons 2–6. When a cDNA fragment containing the deleted exons was used as a probe in RNA blotting analysis, no mRNA transcript was detected in Y79 cells, whereas LAN-1 neuroblastoma cells expressed the expected 4.7-kb RB mRNA (data not shown).

Sequence Analysis of Genomic DNA. Internal deletion of the RB mRNA in Y79 cells could arise either by mutation(s) at splicing sites or by true DNA deletion. To resolve these two alternatives, Y79 genomic DNA clones containing the 5′ region of the *RB* gene were isolated. Restriction map analysis of two overlapping clones, Y2.3 and Y7.3, suggested the presence of a potential deletion junction (23). DNA blotting analysis of Y79 DNA confirmed an internal deletion of one

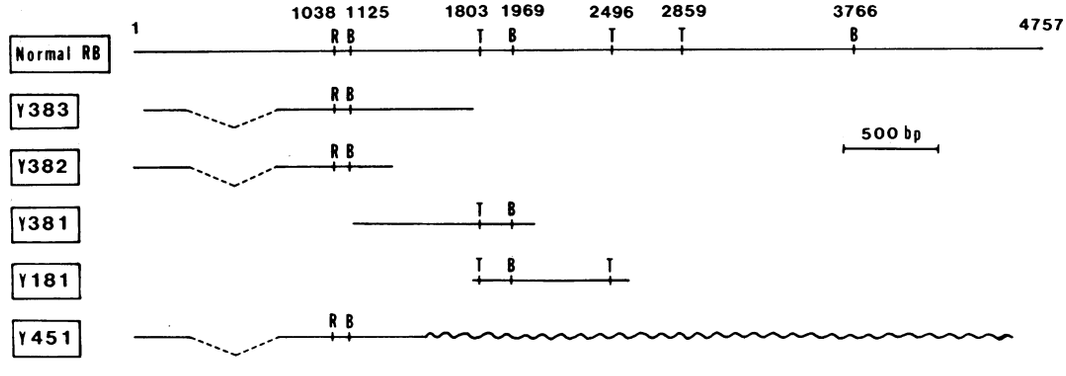

FIG. 2. Comparison of restriction maps of normal (RB4.7) and Y79 RB cDNA clones. Dashed lines indicate regions missing from Y79 RB cDNAs. Wavy lines represent regions not hybridizing with normal RB cDNA. B, *Bgl* II; R, *Eco*RI; T, *Taq* I.

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Y79  TTCCGGTTTTTCTCAGGGGACGTTGAAATTATTTTTGTAACGGGAGTCGGGAGAGGACGGGGCGTGCCCCGCGTGCGCGCGCGTCGTCCTCCCCGGCGCTCCTCCACAGCTCGCTGGCTC  120

                  M  P  P  K  T  P  R  K  T  A  A  T  A  A  A  A  A  A  E  P  P  A  P  P  P  P  P  P  P  E  E  D  P  E
Y79  CCGCCGCGGAAAGGCGTCATGCCGCCCAAAACCCCCCGAAAAACGGCCGCCACCGCCGCCGCTGCCGCCGCGGAACCCCCGGCACCGCCGCCGCCGCCCCCTCCGTAGGAGGACCCAGAG  240
                  M  P  P  K  T  P  R  K  T  A  A  T  A  A  A  A  A  A  E  P  P  A  P  P  P  P  P  P  P  E  E  D  P  E  (34)

     Q  D  S  G  P  E  D  L  P  L  V  R
Y79  CAGGACAGCGGCCCGGAGGACCTGCCTCTCGTCAG
Nor    GGACAGCGGCCCGGAGGACCTGCCTCTCGTCAG    GCTTGAGTTTGAAGAAACAGAAGAACCTGATTTTACTGCATTATGTCAGAAATTAAAGATACCAGATCATGTCAGAGAGAGA  357
     Q  D  S  G  P  E  D  L  P  L  V  R    L  E  F  E  E  T  E  E  P  D  F  T  A  L  C  Q  K  L  K  I  P  D  H  V  R  E  R  (73)

Nor  GCTTGGTTAACTTGGGAGAAAGTTTCATCTGTGGATGGAGTATTGGGAGGTTATATTCAAAAGAAAAAGGAACTGTGGGGAATCTGTATCTTTATTGCACGAGTTGACCTAGATGAGATG  477
     A  W  L  T  W  E  K  V  S  S  V  D  G  V  L  G  G  Y  I  Q  K  K  K  E  L  W  G  I  C  I  F  I  A  R  V  D  L  D  E  M  (113)

Nor  TCGTTCACTTTTACTGAGCTACAGAAAAACATAGAAATCAGTGTCCATAAATTCTTTAACTTACTAAAAGAAATTGATACCAGTACCAAAGTTGATAATGCTATGTCAAGACTGTTGAAG  597
     S  F  T  F  T  E  L  Q  K  N  I  E  I  S  V  H  K  F  F  N  L  L  K  E  I  D  T  S  T  K  V  D  N  A  M  S  R  L  L  K  (153)

Nor  AAGTATGATGTATTGTTTGCACTCTTCAGCAAATTGGAAAGGACATGTGAACTTATATATTTGACACAACCCAGCAGTTCGATATCTACTGAAATAAATTCTGCATTGGTGCTAAAAGTT  717
     K  Y  D  V  L  F  A  L  F  S  K  L  E  R  T  C  E  L  I  Y  L  T  Q  P  S  S  S  I  S  T  E  I  N  S  A  L  V  L  K  V  (193)

                        G  S  I  T  N  G  R  ---
Y79                     GGGAAGTATTACAAATGGAAGATGATCTGGTGATTTCATTTCAGTTAATGCTATGTGTCCTTGACTATTTTATTAAACTCTCACCTCCC
Nor  TCTTGGATCACATTTTTATTAGCTAAAG    GGGAAGTATTACAAATGGAAGATGATCTGGTGATTTCATTTCAGTTAATGCTATGTGTCCTTGACTATTTTATTAAACTCTCACCTCCC  834
     S  W  I  T  F  L  L  A  K     G  E  V  L  Q  M  E  D  D  L  V  I  S  F  Q  L  M  L  C  V  L  D  Y  F  I  K  L  S  P  P  (232)

Y79  ATGTTGCTCAAAGAACCATATAAAACAGCTGTTATACCCATTAATGGTTCACCTCGAACACCCAGGCGAGGTCAGAACAGGAGTGCACGGATAGCAAAACAACTAGAAAAT...etc
Nor  ATGTTGCTCAAAGAACCATATAAAACAGCTGTTATACCCATTAATGGTTCACCTCGAACACCCAGGCGAGGTCAGAACAGGAGTGCACGGATAGCAAAACAACTAGAAAAT...etc to 4757
     M  L  L  K  E  P  Y  K  T  A  V  I  P  I  N  G  S  P  R  T  P  R  R  G  Q  N  R  S  A  R  I  A  K  Q  L  E  N ...etc to (928)
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FIG. 3. Comparison of sequences of Y79 RB cDNA clones with normal (Nor) RB cDNA. Clones Y451, Y383, and Y382 were sequenced and were aligned to the normal RB cDNA sequence. Line labels (at left) identify the cell type from which the sequence was derived. Nucleotides and amino acids are numbered (at right). The first 242 nucleotides of Y451 are presumed to represent the normal sequence as well, due to the 33-bp overlap at nucleotides 243–275. Above and below each nucleotide sequence are the deduced amino acid sequences (one-letter code) for Y79 and normal RB proteins, respectively, based on use of the first methionine as the start codon. The large gap in the Y79 sequence represents exons 2–6, which are missing from Y79 RB cDNAs. The first and second methionine codons are boxed. ---, stop codon.

RB allele (23). DNA fragments containing the deletion junction and deletion endpoints in introns 1 and 6 were subcloned and sequenced. These sequences were aligned with the normal intron sequences to reveal the precise deletion junction (Fig. 4). There was perfect sequence identity to the corresponding normal introns on both sides of the deletion joint. Sequences of the deletion endpoints demonstrated no apparent homology to each other or to the *Alu* family; therefore, we cannot explain the deletion by recombination between homologous sequences, as has been proposed for some deletions of the low density lipoprotein receptor (17). A computer-assisted search for homologous sequences in the GenBank data base[§] was also uninformative about the nature of the deletion endpoints. The endpoint in intron 1 has a unique sequence since a probe containing it hybridized to a single band in Southern blotting with human genomic DNA (23).

Sequence Divergence of Y451. One Y79 cDNA clone, Y451, differed from the others in that a large portion (≈2.5 kb) did

[§]EMBL/GenBank Genetic Sequence Database (1987) GenBank (IntelliGenetics, Mountain View, CA), Tape Release 48.0.

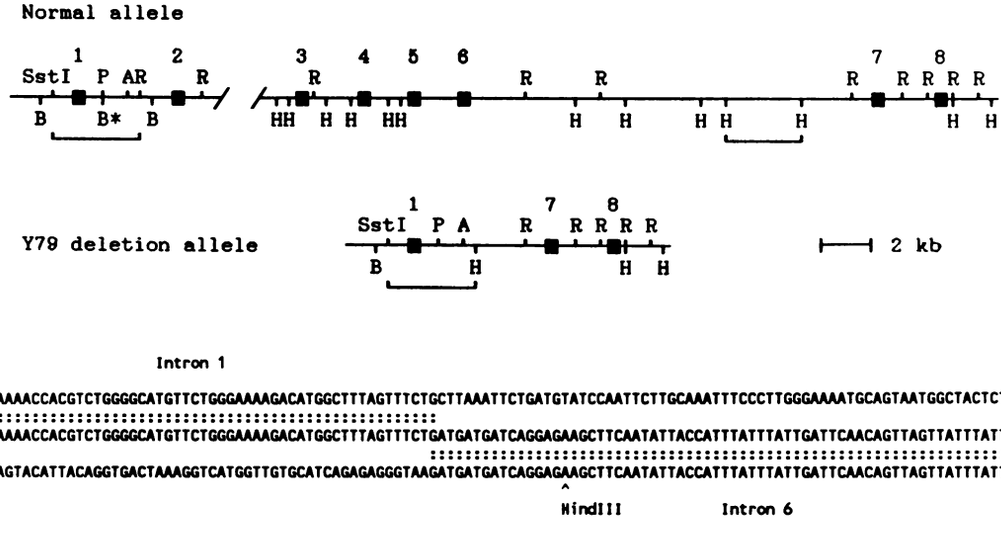

FIG. 4. Characterization of the Y79 deletion junction. (*Upper*) Regional restriction maps of normal and Y79 deletion alleles. Exons (solid boxes) are not drawn to scale. Preliminary exon numbering was established by sequence characterization of exon/intron junctions (H.-J. S. Huang and W.-H.L., unpublished results). Fragments encompassing deletion endpoints and junctions (brackets) were subcloned and sequenced; sequencing was also continued beyond the 3′ *Hin*dIII site in genomic clones. A, *Acc* I; B, *Bam*HI; H, *Hin*dIII; P, *Pst* I; R, *Eco*RI. Only selected *Acc* I and *Pst* I sites are shown. (*Lower*) The sequences of normal introns 1 and 6 and the deletion junction in Y79 are shown. Sequence identity is indicated by double dots.

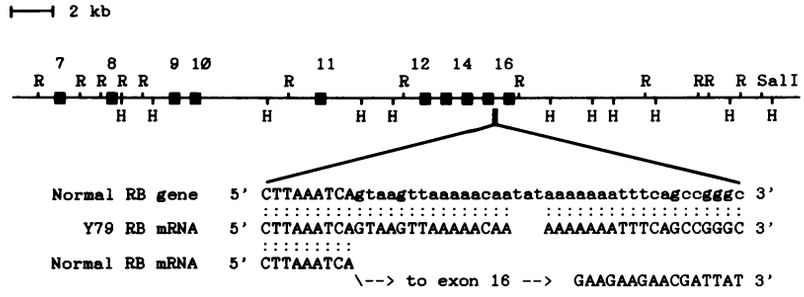

FIG. 5. Origin of divergent sequences in Y451. (*Upper*) A regional restriction map of either normal or Y79 alleles. Exons are indicated as in Fig. 4. Y451 and normal cDNA sequences diverged after exon 15 within a 7.5-kb *Hin*dIII fragment. H, *Hin*dIII; R, *Eco*RI. (*Lower*) Sequence comparison of normal or Y79 *RB* genes and their transcripts at the 15th exon/intron junction. Intron 15 from a normal *RB* gene is shown (lowercase nucleotides); a corresponding region in Y79 had the same sequence. The splice donor site conforms to the consensus sequence. Sequence identity (double dots) was found between intron 15 and Y451 cDNA except that three nucleotides were missing from Y451, representing a partially unprocessed RNA transcript presumably encoded by one Y79 allele.

not hybridize to normal RB cDNA; in particular, the region homologous to RB1.8 (nucleotides 1969–3766) was completely absent (Fig. 2). However, other cDNA clones such as Y383, Y381, and Y181 did contain this region. The RNA blotting analysis of Fig. 1 was repeated with RB1.8 as probe, and identical results were obtained. Conversely, probes from the divergent region of Y451 did not detect a significant amount of the 4.0-kb mRNA transcript (data not shown). This demonstrated that the majority of mRNA transcripts did not contain the divergent region of Y451. Additional sequencing showed that the point of divergence in Y451 corresponded to nucleotide 1529 of the normal cDNA sequence (Fig. 5).

To address the origin of divergent Y451 sequences, we examined phage clones from both normal and Y79 genomic libraries (23) that encoded this region of cDNA (Fig. 5). The relevant fragments from both normal and Y79 genomic clones were again subcloned and sequenced by using as primer a 20-mer oligonucleotide synthesized according to the normal cDNA sequence (nucleotides 1466–1485). A typical splice donor site (CAgtaa) (32) was identified in both genomic clones that coincided precisely with the start of divergence between Y451 and normal cDNA sequences (Fig. 5). The subsequent 200 or more nucleotides of Y451 were derived from intron 15, whereas the normal cDNA sequence proceeded to exon 16 (H.-J. S. Huang and W.-H.L., unpublished data). Clearly, Y451 was generated from RNA that had not been spliced at this exon/intron junction, which resulted in retention of intron sequences. Although intron 15 sequences were identical in both Y79 and normal genomic clones, Y451 lacked three nucleotides (TAT) 15 bp downstream from the splice junction (Fig. 5). Loss of the three nucleotides as an artifact of reverse transcriptase or by posttranscriptional modification of Y79 mRNA was considered highly unlikely. Therefore we inferred that one *RB* allele in Y79 lacked 3 bp in intron 15 relative to other known *RB* alleles (as represented in our clones). Because none of the other cDNAs from Y79 contained intron 15 sequences (Fig. 2), it was tentatively concluded that Y451 represented a partially or improperly processed minor transcript and that the 3-bp deletion in intron 15 did not significantly impair mRNA splicing (32).

Hypothetical Translation of Y79 RB cDNA. The addition of 242 nucleotides from Y79 RB cDNA to the beginning of the normal RB sequence resulted in a revised normal sequence length of 4757 nucleotides, with candidate initiation codons at nucleotides 139 and 475 (Fig. 3). The second methionine codon is missing from Y79; if translation is initiated at the first methionine codon, the loss of exons 2–6 results in a frame shift and premature termination in exon 7 (Fig. 3). We labeled Y79 cells and LAN-1 (neuroblastoma) cells with [^{32}P]phosphoric acid and immunoprecipitated lysates with antibody specific for the RB protein (14). The *RB* gene product, pp110^{RB}, was detected in the control cell line LAN-1 but was absent from Y79 cells (Fig. 6). Identical results were obtained with three additional polyclonal antisera against other epitopes of the RB protein (unpublished data). The total lack of RB protein indicated that both alleles of the *RB* gene were inactivated. The nondeleted allele was thus inactivated by a different, unknown mechanism.

DISCUSSION

The above data on retinoblastoma cell line Y79 demonstrate that its shortened RB mRNA transcript was derived from one allele of the *RB* gene containing an intragenic deletion, while the nondeleted allele was apparently inactive. Since the size of the deletion is ≈50 kb (R.B. and W.-H.L., unpublished data), it is far too small to be cytogenetically visible, in concordance with previous studies (21, 33). We have found two other retinoblastoma cell lines with heterozygous deletions very similar to that of Y79 (23); in addition, DNA rearrangements involving intron 1 were also observed in fibroblasts from two hereditary retinoblastoma patients. Since rearrangements in this region appear to be a common event in inactivating the *RB* gene, it was of interest to understand the molecular mechanism of these mutations in detail.

Unequal crossing-over by meiotic or mitotic recombination between repetitive elements has been proposed as one mechanism for deletions of eukaryotic genes. Eight large deletions in the globin and low density lipoprotein receptor genes have *Alu* repeats at one or both ends (17, 19). Furthermore, the breakpoints in *Alu* sequences are not located at random; certain sequence-specific configurations may facilitate aberrant recombination. Other deletions in the globin genes reportedly do not involve *Alu* sequences at either endpoint (19, 34). In these cases there is no homology, either direct or inverted, between 200-nucleotide sequences at deletion endpoints. The large *RB* gene deletion described above is another case that does not involve recombination by

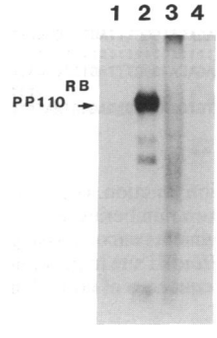

FIG. 6. Immunoprecipitation of RB protein. LAN-1 (lanes 1 and 2) and Y79 (lanes 3 and 4) cells were labeled with [^{32}P]phosphoric acid for 4 hr. Cell lysates were immunoprecipitated with either preimmune serum (lanes 1 and 3) or an affinity-purified polyclonal antibody (lanes 2 and 4) that recognizes the RB protein (14). Immunoprecipitates were separated in 7.5% NaDodSO$_4$/polyacrylamide gels that were autoradiographed overnight.

means of homologous sequences. Darras and Francke (35) and Vanin *et al.* (34) have suggested that some deletions may depend on organization of chromatin loops by chromosome "scaffolding" proteins (36). Scaffolding-associated DNA sequences have been defined in *Drosophila* and may be sites of topoisomerase II activity. Like scaffolding-associated DNA sequences, our deletion endpoints are A+T-rich; however, we do not find topoisomerase II consensus sequences (37). Characterization of commonly deleted regions will further the understanding of mutagenesis in the human genome.

The presence of intron sequences in one cDNA clone, Y451, suggested that perhaps a minor proportion of RB transcripts in Y79 were abnormally processed. Abnormal or alternative mRNA processing (38) in Y79 cells may also account for a number of faint bands that appear on longer exposure of the RNA gel blot of Fig. 1 (data not shown). Although a 3-nucleotide deletion was found in the retained intron relative to two other introns 15, this change was not of major significance for mRNA processing (32) since the majority of transcripts and cDNA clones spliced this intron correctly.

In 1971, Knudson proposed the "two-hit" hypothesis to explain the difference in average age of onset in sporadic and familial retinoblastoma patients (39). In familial cases, the first hit was conceived as a mutation inherited from an affected parent. Comings (15) proposed that these two hits might inactivate both alleles of a single gene, leading to deregulation of downstream oncogenes. Current data clearly support the concept that both alleles of the *RB* gene are inactivated in retinoblastomas (12, 14, 40). Cavenee *et al.* (41) observed loss of chromosome 13 heterozygosity in some retinoblastomas and inferred that chromosomal events such as mitotic nondisjunction could produce two mutated *RB* alleles in tumors; these two alleles would have identical mutations. In Y79, both *RB* alleles are necessarily inactivated because RB protein is absent; however, the two mutations are clearly different. Given the presence of an apparently normal allele, a mutation of its promoter is considered most plausible.

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