*Proc. Natl. Acad. Sci. USA*
Vol. 87, pp. 6–10, January 1990
Biochemistry

# C-terminal truncation of the retinoblastoma gene product leads to functional inactivation

(tumor suppression/osteosarcoma)

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*Communicated by J. Edwin Seegmiller, September 15, 1989*

**ABSTRACT** **Mutational inactivation of the retinoblastoma (RB) gene has been implicated in the genesis of retinoblastoma, osteosarcoma, and other human tumors. Our strategy has been to characterize naturally occurring mutants from tumor cells to pinpoint potential domains of RB protein crucial for tumor suppression. We show here that osteosarcoma cell line Saos-2 contains an abnormal endogenous RB protein of 95 kDa (p95) that is located mainly in the cytoplasm. This protein was identified by antibodies recognizing several different RB epitopes, but not by one directed solely against the C terminus, suggesting C-terminal truncation. This conclusion was supported by analysis of mRNA and genomic DNA, which revealed that a transcriptionally active RB allele had a deletion of exons 21–27. In contrast to normal RB protein, this truncated protein was not phosphorylated and did not bind to the large tumor (T) antigen encoded by simian virus 40. We previously reported that introduction of normal RB protein into Saos-2 cells suppressed their neoplastic phenotype, indicating functional inactivation of their endogenous RB genes. These results provide an initial step to elucidate domains crucial to the cancer-suppression function of RB protein; its C-terminal portion is evidently important for this activity.**

The retinoblastoma (RB) gene is the first cloned human tumor-suppressor gene (1–3). This gene contains 27 exons dispersed within 200 kilobases (kb) of genomic DNA (4) and ubiquitously expresses a 4.7-kb mRNA transcript in all normal tissues (2). The RB gene product, pp110$^{RB}$, has been identified by using specific anti-RB antibodies (5). Preliminary characterization of the protein indicated that it is a nuclear phosphoprotein associated with DNA-binding activity (5). It was further shown to form a specific complex with simian virus 40 (SV40) large tumor (T) antigen and other transforming proteins of DNA tumor viruses (6–8). Although the relationship between the biochemical properties of the RB protein and its biological activity in tumor suppression is unknown, these results suggest that the RB gene product may play an important role in regulating genes involved in oncogenesis.

Mutations of the RB gene have been observed not only in retinoblastomas, but also in several other tumor types such as osteosarcoma, synovial sarcoma, small-cell lung carcinoma, and adenocarcinoma of the breast (9–11). It has been postulated that complete inactivation of the RB gene may lead to tumorigenesis. Loss of RB function is unequivocal in tumors that lack the RB protein; however, abnormal RB proteins have been found occasionally in some tumor samples. If this hypothesis of gene inactivation is to be rigorous, it is necessary to demonstrate that the abnormal RB protein is indeed functionally inactivated. Recently, we developed a biological assay for RB function by introducing an exogenous RB gene into tumor cells through retrovirus-mediated gene transfer (12). In tumor cells with mutated endogenous RB genes, reintroduction of normal exogenous RB gene induced profound effects on the neoplastic phenotype, whereas it had no significant effect on tumor cells with apparently normal RB gene products. This assay makes it possible to study the relationship between the structure and the tumor-suppression function of the RB gene.

We previously demonstrated that the neoplastic properties of osteosarcoma cell line Saos-2 were suppressed by the introduction of exogenous normal RB protein (12). In this communication, we show that Saos-2 contains an endogenous RB protein that is unphosphorylated, is truncated to 95 kDa, is localized mainly in the cytoplasm, and does not bind to SV40 large T antigen. This protein is translated from a shortened 3.8-kb mRNA that lacks exons 21–27. These results imply that a 20-kDa C-terminal region of the RB protein has an important role in its tumor-suppression function.‖

## MATERIALS AND METHODS

**Cell Cultures.** Osteosarcoma cell lines U-2OS and Saos-2 were obtained from the American Type Culture Collection. COS-7, a derivative of monkey kidney cell line CV-1, has been transformed by SV40 and expresses T antigen. Saos-2/Rb-SC1 is a derivative of Saos-2 infected with a retrovirus carrying the RB gene and expresses exogenous normal RB protein (12). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 5–10% fetal bovine serum.

**Origin of DNA Probes and Antibodies.** Probe RB3.8 was used in DNA and RNA blotting analysis as described (13). Probe RB1.8, containing exons 18–27 (4), was generated by digesting RB3.8 with *Bgl* II. This probe was used to isolate λ clone OS5 with deletion junctions from a Saos-2 genomic library constructed as described (14). DNA fragments containing deletion junctions were sequenced by the dideoxynucleotide chain-termination method (15).

Polyclonal antibodies anti-fRB, anti-B, and anti-C were prepared as described (5, 16). Anti-fRB recognizes epitopes within the region encoded by RB exons 9–16. The B peptide is encoded by part of RB exon 20, while the C peptide represents the extreme C terminus of the RB protein. Monoclonal antibody PMG3-245 recognizes one epitope encoded within exons 9–16, and monoclonal antibody PAb419 recog-

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Abbreviations: RB, retinoblastoma; SV40, simian virus 40; T antigen, tumor antigen; RT-PCR, reverse transcription–polymerase chain reaction.

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession nos. M29005 and M29006).

nizes SV40 large T antigen (Oncogene Sciences, New York). These antibodies were used either to immunoprecipitate RB proteins or to perform Western blotting analysis according to published procedures (5, 17).

**Reverse Transcription–Polymerase Chain Reaction (RT–PCR).** This method has been described (18). Briefly, single-stranded cDNA was prepared by incubating 5 μg of total RNA or 2 μg of polyadenylylated RNA and 1.5 μg of oligo(dT) as primer with 60 units of reverse transcriptase from avian myeloblastosis virus (Seikagaku America, Saint Petersburg, FL) for 90 min at 42°C. One-tenth of the resulting cDNA was amplified in the presence of specific primers (0.5 μM each) with 0.5 unit of *Thermus aquaticus (Taq)* polymerase (Perkin–Elmer/Cetus) for 30 cycles on a programmable cyclic reactor (Ericomp, San Diego, CA). Each cycle included denaturation at 94°C, reannealing of primer at an appropriate temperature, and primer extension at 72°C. Aliquots of the PCR reaction mixture were analyzed by electrophoresis in a 5% polyacrylamide gel.

**Cell Fractionation.** The procedures to separate membrane, nuclear, and cytoplasmic fractions were adapted from Radke *et al.* (19) as described (5). All three fractions were then assayed for RB protein content and for an enzyme marker (5′-nucleotidase and or NADH diaphorase) in order to verify the composition of each fraction.

**SV40 Binding Assay.** This assay has been described by Ludlow *et al.* (20). Basically, about $10^7$ Saos-2 cells were transfected by the lipofection method (21) with 10 μg of plasmid pRSV40T DNA (provided by S. Subramani, University of California at San Diego), which contains a long terminal repeat (LTR) promoter from Rous sarcoma virus and the SV40 early region. After 48 hr of DNA transfection, Saos-2 cells were labeled with [$^{35}$S]methionine for 90 min and then lysed in 50 mM Tris/HCl buffer (pH 7.4) containing 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 50 mM NaF, 1 mM phenylmethanesulfonyl fluoride, and 0.05 mg of aprotinin per ml. After clarification by centrifugation, equal amounts of lysate were immunoprecipitated with specific antibodies against RB protein and T antigen.

## RESULTS

**Detection of a Truncated RB Protein Lacking the Normal C Terminus.** As a general approach to determine RB gene inactivation, antibodies have been developed against the RB gene product to examine tumor cells for the presence or absence of normal RB protein (16). A 95-kDa protein (p95) and some other proteins were detected in osteosarcoma cell line Saos-2 after immunoprecipitation with anti-fRB antibody (Fig. 1*A*). Another osteosarcoma cell line, U-2OS, contained the normal-sized, 110-kDa RB protein (pp110$^{RB}$). Most of the background bands in both U-2OS and Saos-2 could be eliminated by boiling the lysates in 1% SDS before immunoprecipitation. This result suggested that the anti-fRB antibody indeed recognized a smaller protein in Saos-2. To further demonstrate that p95 was a mutated version of the RB protein, several antibodies recognizing different epitopes of the RB protein were used to immunoprecipitate proteins from these cell lysates (Fig. 1*B*). Anti-fRB again immunoprecipitated both the normal and the truncated RB protein. The anti-B antibody, which was prepared against a peptide encoded by part of RB exon 20, also recognized the truncated Saos-2 RB protein. Thus, the same 95-kDa protein in Saos-2 was recognized by two different anti-RB antibodies, suggesting that this protein was a mutated version of the RB protein. In contrast, the anti-C antibody, which was prepared against the C-terminal peptide encoded by part of RB exon 27, reacted with normal 110-kDa RB protein (Fig. 1*B*) but failed to detect the truncated RB protein in Saos-2. The data indicated that the C-terminal region of the RB protein of Saos-2 might be modified.

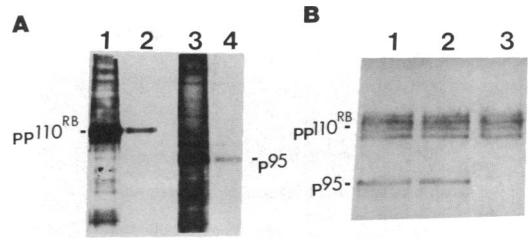


FIG. 1. Identification of truncated RB protein p95 in Saos-2 cells. (*A*) U-2OS (lanes 1 and 2) and Saos-2 (lanes 3 and 4) cells were labeled with [$^{35}$S]methionine and lysates were immunoprecipitated with anti-fRB in nondenaturing conditions (lanes 1 and 3) or in denaturing condition (boiled with 1% SDS) (lanes 2 and 4). Immunoprecipitates were separated by SDS/10% PAGE and fluorographed for 3 days. A 95-kDa truncated RB protein was detected in Saos-2 cells. (*B*) U-2OS ($10^7$) and Saos-2 ($5 \times 10^7$) cells were mixed and then lysed in lysis buffer. Aliquots of lysate were immunoprecipitated with anti-fRB (lane 1), anti-B (lane 2), or anti-C (lane 3) antibody. Following separation of the immunoprecipitable proteins by SDS/7.5% PAGE and transfer to nitrocellulose filters, the blots were probed with monoclonal antibody PMG3-245. p95 from Saos-2 was precipitated by anti-fRB and anti-B antibodies but failed to react with anti-C antibody.

**A Shortened, 3.8-kb RB Transcript Was Generated by a 3′ Deletion.** The simplest mutation of Saos-2 that can explain the protein data above is a 3′ deletion or mutation of the RB gene. To confirm this supposition, genomic DNA from U-2OS and Saos-2 was digested with restriction endonuclease *Hin*dIII and probed with RB3.8, which contains exons 9–27 (Fig. 2*A*). In comparison to the normal genomic DNA pattern, Saos-2 DNA completely lacked the 10-kb, 6.2-kb, and 2.1-kb *Hin*dIII fragments that contain exons 20–27, but showed an aberrant 12-kb fragment. The 10-kb *Hin*dIII fragment was known to contain exons 20–23; therefore the 12-kb band was plausibly explained as a deletion-junction fragment. Northern blotting analysis with the same probe RB3.8 revealed a single shortened RB transcript of 3.8 kb, compared to normal RB mRNA of 4.7 kb (Fig. 2*B*).

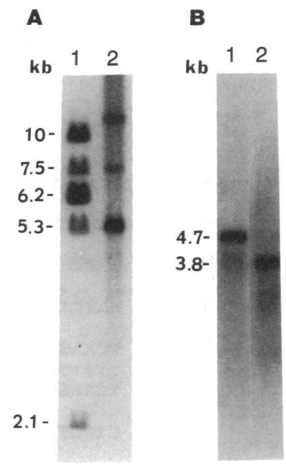


FIG. 2. DNA and RNA blotting analysis of the RB gene in Saos-2. (*A*) DNA (3 μg per lane) from U-2OS (lane 1) or Saos-2 (lane 2) was digested with restriction enzyme *Hin*dIII and analyzed by DNA blotting with cDNA probe RB3.8. RB exons 24–27 as represented by *Hin*dIII fragments of 6.2 kb and 2.1 kb were missing in Saos-2 cells. The 10-kb fragment covering exons 20–23 was also missing and a new 12-kb fragment was generated. (*B*) Polyadenylylated RNA (5 μg per lane) from U-2OS (lane 1) and Saos-2 (lane 2) were analyzed with cDNA probes for the RB gene. A shortened, 3.8-kb transcript was detected in Saos-2 cells.

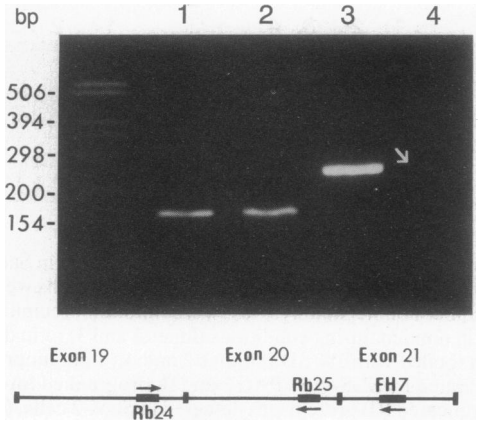


FIG. 3. Analysis of Saos-2 mRNA by RT–PCR. DNA fragments generated from single-stranded cDNA (synthesized from RNA by reverse transcriptase) were amplified by 25–30 cycles of PCR with each primer pair. Fragments of 161 base pairs (bp) and 242 bp were generated from normal cell cDNA by using Rb24/Rb25 and Rb24/FH7 primer pairs, respectively (lane 1 and 3). In contrast, the reactions with Saos-2 cDNA generated the 161-bp but not the 242-bp fragment (lane 2 and 4; expected size of fragments indicated by arrow), indicating that exon 21 is not present in mutated RB mRNA. The locations of primers are shown in the diagram at the bottom.

We used the RT–PCR technique to further characterize the 3.8-kb RB mRNA. Since it seemed likely that the deletion junction was in the region of exons 20–23, we synthesized primer pairs for a stepwise progression from the 5′ end of exon 20 toward the 3′ deletion junction. Two pairs of primers (Rb24/Rb25 and Rb24/FH7) were initially synthesized according to the RB cDNA sequence (5). Rb24, Rb25, and FH7 were localized at exons 19, 20, and 21, respectively (4). We predicted that if any exon was deleted out of the 3.8-kb RB mRNA, the corresponding primer in that exon would fail to serve as a primer in the PCR reaction, resulting in no amplified DNA. An amplified DNA fragment with the expected size was obtained from both the normal and the shortened RB mRNA by using primer pair Rb24/Rb25 (Fig. 3, lanes 1 and 3). However, no DNA fragment was amplified from Saos-2 by using primer pair Rb24/FH7, compared with the expected result in normal RB mRNA. FH7, located in exon 21, was unable to serve as primer in RT–PCR, suggesting that the 3.8-kb RB mRNA had a deletion containing exon 21. From the concept of colinearity of a gene and its product, we deduced that the truncated RB protein lacking the C-terminal region was translated from the shortened, 3.8-kb mRNA, which was in turn transcribed from at least one RB allele with a deletion from exon 21 to exon 27.

**Isolation and Analysis of the Deletion-Junction Clones.** To precisely characterize the Saos-2 deletion junction and recombination mechanism, we isolated two identical clones, OS5 and OS4, by screening the Saos-2 genomic library with probe RB1.8. Restriction mapping of the clones revealed that the normal 6.5-kb *Eco*RI fragment was changed to 3.4 kb. This result indicated that the recombination junction had occurred in intron 20 and had led to the deletion of exons 21–27 (Fig. 4*A*). To exclude the possibility of a cloning

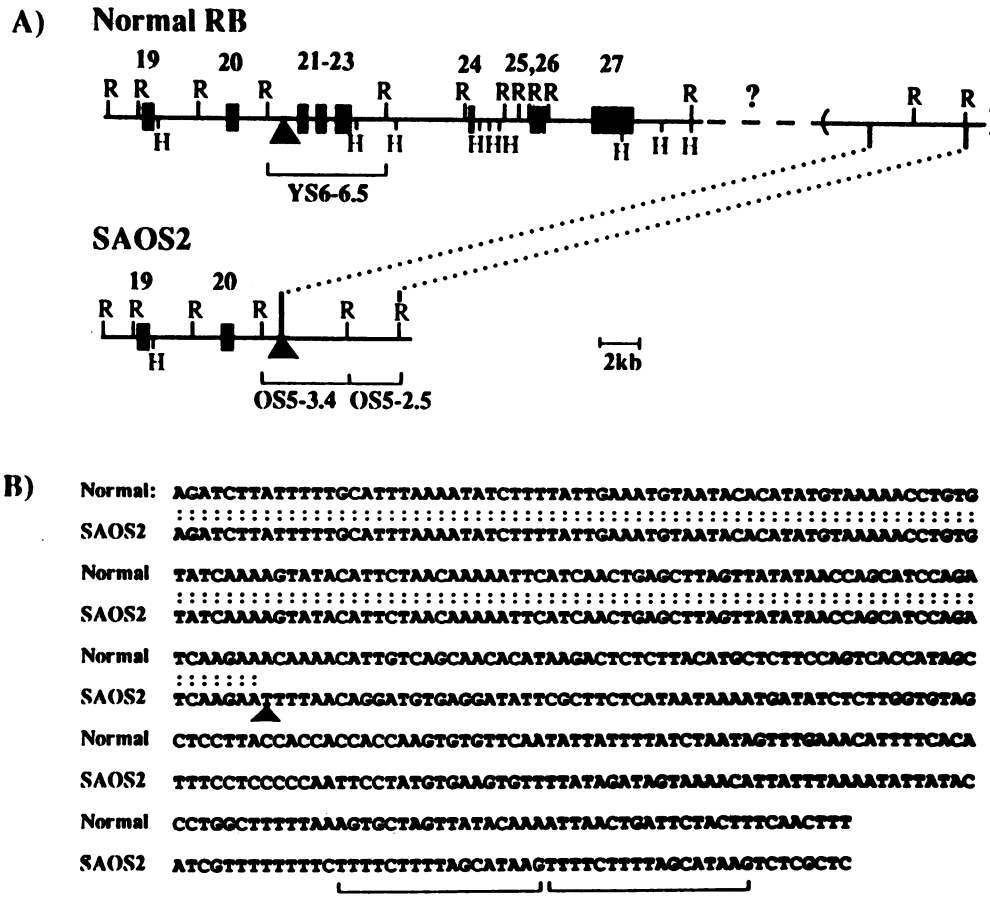


FIG. 4. Deletion-junction map of the RB gene in Saos-2 cells. The restriction map (R, *Eco*RI; H, *Hin*dIII) of exons 19–27 of the normal RB gene was compared with that of the deletion clone (OS5) isolated from a Saos-2 genomic library as shown in *A*. The 6.5-kb *Eco*RI fragment (YS6-6.5) from normal genomic clone YS6 and the 3.4-kb fragment (OS5-3.4) from OS5 covering the deletion site (solid triangle) were further characterized and sequenced to reveal the deletion junction *B*. Two 17-bp tandem repeats (underlined) were found 130 bp downstream of the deletion site (triangle). The highly repetitive *Alu* and *Kpn* I units were not detected. The normal clone corresponding to OS5-3.4 was not isolated.

artifact in generating this abnormal fragment, a unique 1.4-kb *Eco*RI–*Sph* I fragment from OS5-2.5 was used as probe to detect a 12-kb band in Saos-2 DNA digested with *Hin*dIII, confirming that the isolated clones corresponded to the original genomic DNA (data not shown). The DNA fragments containing the potential recombination junction from both normal and Saos-2 genomes were sequenced (Fig. 4*B*). We could not identify involvement of any repetitive unit such as an *Alu* or a *Kpn* I sequence in the recombination junction region. However, 130 bp downstream from the deletion junction were two 17-bp tandem repeats. Whether these repeats have any relation to this recombination remains unclear.

**The Truncated RB Protein Is Unphosphorylated and Cytoplasmic.** Normal RB protein has been shown to be a phosphoprotein localized in the nucleus (5), presumably in relation to its function. To characterize the mutated p95 protein further, we used Saos-2/Rb-SC1 cells, which were derived from Saos-2 by infection with a retrovirus carrying the RB gene and which express the normal RB protein from that gene, to examine whether p95 could be phosphorylated. Cells were separately labeled with [$^{35}$S]methionine and [$^{32}$P]orthophosphate and immunoprecipitated with anti-fRB antibody. Both exogenous normal RB protein and truncated endogenous RB protein (p95) were detected when the cells were labeled with [$^{35}$S]methionine (Fig. 5*A*, lane 1). However, only the normal exogenous RB protein but no truncated endogenous RB protein (p95) could be detected in $^{32}$P-labeled cell lysates (Fig. 5*A*). These results indicated that p95 was unphosphorylated. When Saos-2 cells were fractionated into nuclear, membrane, and cytoplasmic components, the truncated RB protein was found mainly in the cytoplasmic fraction (Fig. 5*B*). Immunostaining of Saos-2 cells with anti-RB antibodies also showed strong cytoplasmic fluorescence (data not shown). These results suggested that the majority of the truncated protein was located in the cytoplasm instead of the nucleus. Therefore, the C-terminal region is necessary for nuclear localization. Whether lack of phosphorylation results from loss of phosphoryl acceptor sites, abnormal protein conformation, or the abnormal location of the protein remains to be resolved.

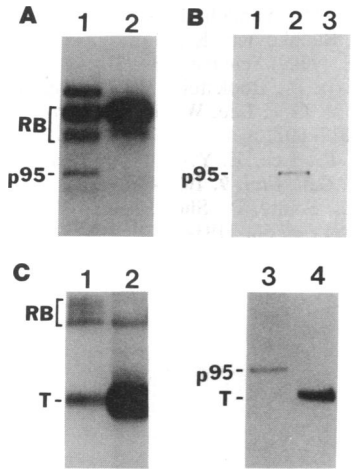


FIG. 5. Characterization of p95 in Saos-2 cells. (*A*) p95 is unphosphorylated. Saos-2/Rb-SC1 cells ($5 \times 10^6$) were labeled with [$^{35}$S]methionine (lane 1) or [$^{32}$P]orthophosphate (lane 2). The cell lysates were immunoprecipitated with anti-fRB antibody and analyzed by SDS/7.5% PAGE. p95 was labeled by $^{35}$S but not by $^{32}$P. (*B*) p95 is located in the cytoplasmic fraction. Saos-2 cells ($2 \times 10^7$) were separated into membrane (lane 1), cytoplasmic (lane 2), and nuclear (lane 3) fractions. Each fraction was then immunoprecipitated with anti-fRB antibody and analyzed by the Western blotting procedure. The blot was probed with monoclonal antibody against RB protein, PMG3-245. (*C*) p95 fails to bind SV40 large T antigen. COS-7 (lanes 1 and 2) and pRSV40T-transfected Saos-2 cells (lanes 3 and 4) were labeled with [$^{35}$S]methionine and lysates were immunoprecipitated with anti-fRB (lanes 1 and 3) or with monoclonal antibody against SV40 T antigen, PAb419 (lanes 2 and 4). The immunoprecipitates were subjected to SDS/7.5% PAGE and fluorographed for 30 hr. Normal RB protein was coprecipitated with T antigen by either of the specific antibodies (lanes 1 and 2), whereas p95 was not (lanes 3 and 4).

**p95 Fails to Form a Complex with T Antigen.** RB protein forms a tight complex with transforming proteins of DNA tumor viruses such as E1A of adenovirus and large T antigen of SV40 (6, 7). A specific complex can be formed by coexpression of both proteins in cultured cells (*in vivo* assay) or by addition of purified T antigen to cell lysates containing RB protein (*in vitro* assay) (20). These assays provided a simple way to determine the binding activity of the truncated RB protein with SV40 large T antigen. A tight complex between unphosphorylated RB protein and SV40 large T antigen was formed when cellular lysates were prepared from COS-7 cells, which express T antigen (Fig. 5*C*). Such a complex was not detected in Saos-2 cells transfected with pRSV40T DNA. Similar results were obtained in the *in vitro* assay when Saos-2 lysate was mixed with purified T antigen (data not shown). For comparison, a 103-kDa truncated RB protein resulting from deletion of exon 21 in the bladder carcinoma cell line J82 also failed to form a complex with E1A protein (22) or T antigen (data not shown). These results clearly indicated that truncation of the C-terminal region of RB protein abolished its complex formation with SV40 large T antigen.

## DISCUSSION

A shortened RB protein of 95 kDa (p95) was found in osteosarcoma cell line Saos-2. The protein was truncated at the C terminus. It was restricted to the cytoplasmic compartment of the cell, was unphosphorylated, and failed to form a complex with SV40 large T antigen. p95 was expressed from a mutated RB allele with deletion of exons 21–27. The expected molecular mass of an RB polypeptide lacking these exons was 85 kDa rather than 95 kDa, suggesting that a portion of the aberrant protein was derived from an unknown reading frame 3′ to the RB gene.

Mechanisms of RB gene deletion may be inferred from examination of sequences around the ends of the deleted region. In Saos-2, the deletion junction and endpoints of the transcriptionally active RB allele were characterized in detail. The sequences directly involved in recombination appeared not to be homologous to each other or to *Alu* or *Kpn* I sequences, suggesting that the deletion occurred by illegitimate recombination. Two other RB gene deletions have been described at the molecular level, and homology between deletion endpoints was not observed in these cases either (23, 24). Despite the complete deletion of exons 21–27 apparent in Fig. 2*A*, Saos-2 cells appear to have two different deletions affecting its two RB alleles. Preliminary Southern blotting results have shown that the other allele may have a deletion from exon 14 to exon 27 (unpublished data). p95 cannot originate from this allele because the protein was recognized by antibodies made specifically against peptide B, which is encoded by exon 20. In addition, analysis by RT–PCR demonstrated that Saos-2 mRNA contained exon 20 but lacked exon 21. Thus the two alleles of the RB gene in Saos-2 cells were functionally inactivated by two different deletions. Similar heterozygous mutations have been observed in other RB$^-$ tumors (13, 23) and may not be uncommon.

Several biochemical properties of p95 were concomitantly altered, indicating that one or more of these features might be critical for RB protein function. For example, binding of RB to SV40 large T antigen or adenovirus E1A protein has been

proposed to inactivate the RB protein, thereby mimicking its inactivation by mutation (7, 20). Loss of this binding property was observed in another aberrant RB protein, from bladder carcinoma cell line J82, which lacks amino acid residues 703–737, encoded by exon 21 (22). Since p95 also lacks this region, its inability to form a complex with large T antigen was consistent with the observations in J82. The RB protein is normally located in the nucleus and is associated with DNA-binding activity, suggesting that it may function to regulate the expression of other genes (5). The cytoplasmic location of p95 may result from loss of nuclear translocation signals, which are as yet undefined. The relatively low level of p95 in Saos-2 cells may result from instability due to its truncation at the C-terminal region. Since the function of RB protein may be modulated by phosphorylation (20, 25), loss of phosphorylation of p95 might indicate that many phosphorylation sites are in the C terminus or that phosphorylation elsewhere in the protein may depend on the integrity of the C-terminal region. Further characterization of other naturally mutated RB proteins will be very useful in understanding the inactivation mechanisms of the RB gene. This study provides an initial step to elucidate structural domains crucial to the tumor-suppression function of RB protein.

We thank R. Bookstein, Jean Y. J. Wang, and P. Scully for critical reading of the manuscript and Su Huang for providing Saos-2/Rb-SC1 cells. This study was supported by grants from the National Eye Institute (EY05758) and the American Cancer Society (CD373).

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