Defective Human Retinoblastoma Protein Identified by Lack of Interaction with the E1A Oncoprotein

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ABSTRACT

Inactivating mutations of the retinoblastoma susceptibility gene (Rb) are involved in the pathogenesis of hereditary and sporadic retinoblas-toma. Alterations in the Rb gene have also been found in several other human tumors occurring with epidemiological incidence higher than that of retinoblastoma. Four human malignant glioma cell lines were examined for abnormalities in the retinoblastoma gene product (pRb), using a procedure based on the interaction of pRb with an in vitro-translated adenovirus E1A oncoprotein. In the CRS-A2 cell line, derived from a glioblas-toma multiforme, pRb did not bind with the in vitro-translated E1A protein. Restriction analysis of the CRS-A2 Rb gene and Rb mRNA expression provided patterns that could not be distinguished from the other glioma cell lines. Further investigation revealed the presence of a truncated pRb in the CRS-A2 cell line, due to a nucleotide insertion in the coding sequence at position 2550. In addition, this truncated Rb protein was indetectable in phosphorylated form. The binding assay with the in vitro-translated E1A was also used to study other cell lines with known mutations in the Rb gene. This method, which evaluates the interaction between in vitro-translated E1A and the pRb, is proposed as a rapid screening for detecting functional alterations in the retinoblastoma protein.

INTRODUCTION

The retinoblastoma susceptibility gene (Rb) is the prototype for a class of genes the inactivation of which appears to be causally related to cancer; these genes are therefore referred to as tumor suppressor genes (1–3). Although the precise function of pRb is still unknown, its activity is related to DNA synthesis (4, 5) or cell cycle regulation (6). Gross alterations in the Rb gene have been shown to occur regularly either in sporadic or in inherited forms of retinoblastoma, where deletion of chromosome 13 segments, containing the Rb locus, are commonly reported (7, 8). Alterations in the Rb gene structure, leading to either loss or functional inactivation of the gene product, have been observed in more common human tumors such as small cell lung cancer (9, 10), breast cancer (11, 12), osteogenic sarcomas (13, 14), leukemias (15), prostate (16) and bladder (17, 18) carcinomas, and malignant gliomas (19, 20). Southern blot analysis of tumor DNA probes with Rb cDNA3 is often not a sensitive enough method to provide evidence of such subtle alterations (17). Even the Rb-specific mRNA evaluation in Northern blot analysis does not seem to consistently assess the structural and functional status of Rb (17).

The retinoblastoma gene product, pRb, is a nuclear phosphoprotein with DNA-binding property (21, 22). Normal pRb shows a certain microheterogeneity in SDS-PAGE, due to different degrees of phosphorylation, so that the protein is usually found between 105 and 114 kDa of apparent molecular mass. The phosphorylation status of pRb oscillates between an unphosphorylated or an underphosphorylated form (fast-migrating), during the G1–G2 phases of the cell cycle, and a fully phosphorylated form (slow-migrating), when the cell reaches the G2–S boundary (4–6, 23). This microheterogeneous pattern is an index of asynchronism in the cell population and is the most common behavior, either in vivo or in vitro (4, 24).

The pRb is not detected in some tumor cell lines, even when Southern and Northern blot analyses provide a pattern that cannot be distinguished from the normal one (17, 22). Reinsertion of wild type Rb gene in human cancer cell lines with proven pRb impairment revert the neoplastic phenotype (16, 25, 26), thus ultimately confirming the oncosuppressor role of Rb.

The Rb gene product is capable of interacting with oncoproteins from DNA tumor viruses, such as the adenovirus E1A (24), the SV40 large T (27, 28), and the human papillomavirus E7 (29). This finding supports the hypothesis that these viruses can induce transformation by neutralizing the cellular mechanisms involved in growth suppression (24). The E1A-binding site of the pRb molecule has been also implicated in the interaction with the other DNA virus oncoproteins (30) as well as with endogenous factors, such as E2F, involved in important functions in cellular proliferation control mechanisms (30–32). Phosphorylated pRb interacts less strictly with viral oncoproteins (24, 28, 33) and releases bound E2F which, in its free form, can activate transcription (34).

A simple method was designed for the functional screening of the Rb gene product, based on its interaction with the E1A oncoprotein. Briefly, IVT-E1A was challenged with tumor cell lysates, to evaluate its binding with pRb by means of a communoprecipitation assay, using an anti-pRb monoclonal antibody. The method described in this paper permitted identification of a defective Rb protein in the CRS-A2 cell line, isolated in our laboratory starting from a surgical specimen of a glioblastoma multiforme. Further investigation revealed the presence of a nucleotide insertion in the CRS-A2 malignant glioma at position 2550 of the Rb coding sequence.

MATERIALS AND METHODS

Cell Culture

The LI (35–37), DF (37) (courtesy of Dr. G. Zupi, istituto Regina Elena, Rome, Italy), and ADF (courtesy of Dr. W. Malorni, istituto Superiore di Sanità, Rome, Italy) human malignant glioma cells; Y79 (38) and H209 SCLC cells (40) were grown in RPMI 1640 (Gibco) CRS-A2 cells, established in 1991 in our Institute from a surgical specimen of a glioblastoma multiforme, were grown in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (Gibco). SAOS-2 osteosarcoma cells (41) (courtesy of Dr. M. Zamanian, National Institute for Medical Research, London, United Kingdom) and 293 cells (42) were grown in Dulbecco’s modified

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The abbreviations used are: cDNA, complementary DNA; DOC, sodium deoxycholate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IVT-E1A, in vitro-translated adenovirus E1A protein; PCR, polymerase chain reaction; DGGE, denaturing gradient gel electrophoresis; nt, nucleotide.

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Eagle’s medium (Gibco). Media were supplemented with 10% fetal calf serum (15% for Y79 and WERI-Rb1 cells), 2% glutamine, and antibiotics. Cells were incubated at 37°C in a 5% CO₂ atmosphere.

**Cell Labeling and Lysis**

**Labeling with [³⁵S]Methionine.** Subconfluent cell cultures were washed twice with prewarmed 150 mM NaCl-10 mM sodium phosphate, pH 7.20, and incubated at 37°C in prewarmed methionine-free medium, plus 5% dialyzed fetal calf serum (Gibco) and 2% glutamine (6 ml of medium for each 75 cm² flask) for 180 min. The medium was then substituted with the same prewarmed medium (2.5 ml for each 75 cm² flask) plus 9.25 MBq (250 μCi) of [³⁵S]-methionine (Tran³⁵SLabel; ICN) and the incubation was carried out for 180 min at 37°C. Cells were then washed twice in ice-cold 150 mM NaCl-10 mM sodium phosphate, pH 7.20, and lysed in ice-cold lysis buffer (150 mM NaCl-50 mM Tris-HCl, pH 8.00-5.0 mM EDTA-1% Nonidet P-40-1 mM PMSF-1 μg/ml aprotinin-1 μg/ml leupeptin). One ml of the lysis buffer was added to each 75 cm² flask; when indicated, 0.1% SDS and 0.5% DOC were added to the lysis buffer. Flasks were placed on ice for 30 min with occasional shaking. Cell lysates were then collected in 1.5-ml centrifuge tubes and spun for 10 min at 14,000 rpm in an Eppendorf 5415 centrifuge at 4°C. Supernatants were collected for immunoprecipitation.

**Labeling with [³²P]Orthophosphoric Acid.** The procedure was similar to the [³⁵S]methionine labeling, except for the following modifications. The washings were carried out with 150 mM NaCl-10 mM Tris-HCl, pH 7.20, and the cells were incubated in phosphate-free medium plus 5% dialyzed fetal calf serum (Gibco) and 2% glutamine. To each flask 12.33 MBq (333 μCi) of [³²P]orthophosphoric acid (New England Nuclear) were added. Cells were then lysed in 20 mM NaCl-100 mM NaF-30 mM sodium pyrophosphate-3 mM sodium o-vanadate-50 mM Tris-HCl, pH 8.00-10.0 mM EDTA-1% Nonidet P-40-1 mM PMSF-1 μg/ml aprotinin-1 μg/ml leupeptin.

**Immunoprecipitation**

Cell lysates were preclotted for 60 min in a rotating device at 4°C by the addition of 100 μl/ml of protein A-Sepharose CL-4B (Phar-macia) 25% packed in lysis buffer, conjugated with affinity-purified rabbit anti-mouse IgGs (Cappel). Precleared lysates were then incubated for 180-240 min in a rotating device at 4°C with 25 μl/ml of protein A-Sepharose 25% packed in lysis buffer, conjugated first with rabbit anti-mouse purified IgGs (Cappel) and then with one of the following antibodies: 1 μg of purified C36 anti-pRb monoclonal antibody (24) (Oncogene Science); 1 μg of purified PAb416 anti-SV40 large T monoclonal antibody (43); 1 μg of purified PMG3-245 anti-pRb monoclonal antibody (27) (PharMingen); 1 μl of ascites of 18F anti-pRb monoclonal antibody (44) (courtesy of Dr. D. P. Lane, Medical Science Institute, The University of Dundee, Dundee, Scotland); 35 μl of supernatant of M73 anti-E1A monoclonal antibody (45) (courtesy of Dr. E. Harlow, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). After incubation, protein A-Sepharose beads were washed five times at 4°C in lysis buffer.

**SDS-PAGE, Staining, and Fluorography**

Thirty-five μl of electrophoresis sample buffer (62.5 mM Tris-HCl, pH 6.80-2% SDS-5% β-mercaptoethanol-10% glycerol) was added to drained protein A-Sepharose beads from the last step. Samples were heated at 95°C for 5 min and spun to separate protein A-Sepharose beads, and the supernatants were loaded onto a 7.5% or 10% acrylamide gel (46). After the bromophenol blue tracking dye reached the resolving portion, the gel was run overnight at 40 V (constant voltage), stained with Coomassie blue and, in the event of labeling with [³⁵S]methionine, treated for fluorography with Amplify (Amer sham) according to the manufacturer’s recommendations, then dried and exposed at ~70°C using Kodak XAR-5 film with Du Pont Cronex intensifying screens.

**Southern Blot Analysis**

Genomic DNA was prepared according to the method of Sambrook et al. (47), cleaved with HindIII, and resolved by electrophoresis through a 0.8% agarose gel; 10 μg of digested DNA were loaded onto each lane. After transfer to nitrocellulose filters (BA-85; Schleicher & Schuell), hybridization was carried out with a human Rb cDNA probe (24) labeled with the random priming technique (48). After a final high-stringency wash (0.1 × standard saline-citrate (1 × is 150 mM NaCl-15 mM citric acid, pH 7.0)-0.1% SDS at 60°C for 30 min), the nitrocellulose was exposed at ~70°C using Kodak XAR-5 film with Du Pont Cronex intensifying screens.

**Northern Blot Analysis**

Total RNA from subconfluent cell cultures of each cell line was prepared according to the method of Chomczynski and Sacchi (49) and resolved by electrophoresis through 1.2% agarose gel in the presence of formaldehyde (47). Ten μg of total RNA were loaded onto each lane. After transfer to nitrocellulose filters (BA-85), hybridization was carried out with a human Rb cDNA probe (24) labeled with a random priming technique (48). After a final high-stringency wash (0.2 × standard saline-citrate-0.1% SDS at 65°C for 30 min), the nitrocellulose was exposed at ~70°C using Kodak XAR-5 film with Du Pont Cronex intensifying screens. Normalization was obtained using a glyceraldehyde-3-phosphate dehydrogenase cDNA probe.

**In Vitro Transcription and Translation of Adenovirus 5 E1A**

The adenovirus E1A mRNA was synthesized in vitro using a plasmid containing the HindIII-BamHI fragment of pMTEB12S (50) cloned in the pSP64 vector cut with the same enzymes (courtesy of Dr. E. Morant, Cold Spring Harbor Laboratory, NY). The plasmid, carrying the complete adenovirus 5 125 E1A cDNA, was linearized with EcoRI and used in a preparative in vitro transcription system (Promega). Generally, 30-50 μg of RNA were synthesized in a 50-μl reaction using 100 units of SP6 polymerase. RNA was purified, ethanol precipitated, and stored at −20°C in distilled water. In vitro transcription was performed using 1 μg of RNA/50-μl reaction for 60 min at 30°C, using a pretreated rabbit reticulocyte lysate (Promega). Translations to generate radioactively labeled protein were done adding to the reaction 37 μCi (1.37 MBq) of [³⁵S]methionine (Amersham SJ 235) and a minus-methionine amino acid mixture. Translation reactions were stored at −80°C.

**Comunmunoprecipitations**

Comunmunoprecipitations were always done in a buffer containing no anionic detergents.

**Coprecipitation of pRB with IVT-E1A by the M73 Anti-E1A Antibody.** Precleared ³⁵S-labeled cell lysates and unlabelled in vitro translated E1A (IVT-E1A) were incubated for 60 min in a rotating device at 4°C. Immunoprecipitation was then carried out with the M73 anti-E1A monoclonal antibody, as described above. Twenty ml of IVT-E1A were added for each ml of cell lysate. Samples were analyzed by SDS-PAGE followed by autoradiography, as described above.

**Coprecipitation of IVT-E1A with pRB by the C36 Anti-pRB Antibody.** Precleared unlabeled lysates from 1 × 10⁶ cells for each cell line (1 ml) and 10 μl of ³⁵S-labeled IVT-E1A (4 μl × 10⁶ cpn of 5’-adenosine triphosphate radioactivity) were incubated for 60 min in a rotating device at 4°C. Immunoprecipitation was then carried out using the C36 anti-pRB monoclonal antibody, as described above. Samples were analyzed by SDS-PAGE followed by autoradiography. When immunoprecipitated samples were analyzed by liquid scintillation counting, 2 × 10⁶ cells were used.

**Analysis of the Rb mRNA**

The human Rb cDNA sequence used as a reference throughout the present paper is that published by Friend et al. (51) (GenBank No. M33647). One μg of total RNA was reverse transcribed using random hexadeoxiotides as primers in the GeneAmp RNA PCR kit (Perkin Elmer Cetus), according to the manufacturer’s instructions. One-tenth of the reaction mixture was then used for PCR amplification, using four pairs of oligonucleotide primers, synthesized following Horowitz et al. (22). After 40 cycles of amplification, the DNA fragments were analyzed by electrophoresis through 1% agarose gels. The DGGE analysis was done with a DGGE 2000 system (C.B.S. Scientific Company) (52). Samples were analyzed on perpendicular denaturing gradient gels, done in 1-mm-thick 6.5% polyacrylamide gels containing a 0-80% denaturant concentration gradient (5.6 M urea and 32% deionized formamide) perpendicular to the direction of the electrophoresis. The gels were run at 60°C C at 150 V for 6 h in 1 × 800 mM Tris base-400 mM sodium acetate-20 mM EDTA buffer, pH 8.0. For each fragment of Rb to be analyzed, both reference primers were used, and the PCR conditions were as follows:

- **A**: 3 μM primers (51) (GenBank No. M33647).
- **B**: 3 μM primers (51) (GenBank No. M33647).
- **C**: 3 μM primers (51) (GenBank No. M33647).
- **D**: 3 μM primers (51) (GenBank No. M33647).

After a final high-stringency wash (0.1 × standard saline-citrate-0.1% SDS at 60°C for 30 min), the nitrocellulose was exposed at ~70°C using Kodak XAR-5 film with Du Pont Cronex intensifying screens.
and sample cDNA were loaded onto the same gel and the separation pattern was visualized by silver staining (Bio-Rad).

Two different preparations of the cDNA fragment containing the 3' end of the coding sequence (nt 2275–nt 3046) were made blunt by treatment with T<sub>4</sub> polymerase and cloned in a BlueScript SK plasmid linearized by SmaI. The inserted cDNAs were then sequenced using an internal primer, spanning from nt 2847 to nt 2831.

RESULTS

**pRb Binding with E1A or IVT-E1A.** Control experiments for the binding of pRb with adenovirus E1A protein were carried out using the 293 cell line, derived from human embryonic cells transformed by a fragment of the adenovirus 5 genome (42) and constitutively expressing the E1A oncoprotein (24). The autoradiogram in Fig. 1, Lane B, shows the immunoprecipitation, after SDS-PAGE, of E1A from 35S-labeled 293 cells by the M73 anti-E1A monoclonal antibody (45). As demonstrated previously (24), E1A appears as various isoforms, ranging from 45 to 55 kDa of apparent molecular mass. Other proteins, such as pRb, p107, p130, and p300, are evident because of their communoprecipitation with E1A. The same 35S-labeled lysate from 293 cells was immunoprecipitated using the C36 anti-pRb monoclonal antibody (Fig. 1, Lane A).

The in vitro translated E1A protein was used to study the interaction between pRb and E1A. The translation product of the 12S E1A mRNA is a 243-amino acid polypeptide, able to immortalize (53) and transform cultured cells when coexpressed with the adenovirus E1B protein or with an activated Ha-ras cellular oncogene (54, 55). Radiolabeled IVT-E1A, compared with the E1A protein from 293 cells, displayed a pattern of bands of smaller apparent molecular mass; two of these, of approximately 38 and 43 kDa, appeared predominant (Fig. 1, Lane C) and both species could be fully recognized by the M73 monoclonal antibody (Fig. 1, Lane D). Moreover, IVT-E1A was able to interact with pRb. In fact, using the M73 anti-E1A monoclonal antibody, nonradiolabeled IVT-E1A, added to 35S-labeled lysate from the malignant glioma LI cell line, communoprecipitated with pRb and with another protein with a higher molecular mass, probably identifiable as p300 (Fig. 1, Lane E). It should be pointed out, however, that mainly unphosphorylated or underphosphorylated forms (bands with faster electrophoretic mobility) of pRb were communoprecipitated with IVT-E1A. This finding is in agreement with the results of Whyte et al. (24), describing the same pattern for pRb in 293 cells immunoprecipitated with M73, thus confirming the higher affinity of E1A (and IVT-E1A) for the less phosphorylated forms of the Rb protein. As a comparison, full pRb electrophoretic polymorphism is shown in Fig. 1, Lane F, where the same LI 35S-labeled lysate was immunoprecipitated using the C36 anti-pRb monoclonal antibody.

**IVT-E1A Binding with pRb from Malignant Glioma Cell Lines.** 35S-Labeled IVT-E1A was incubated with unlabeled cell lysates from four different human malignant gliomas, using as few as 1 × 10<sup>6</sup> cells for each cell line. The mixture was then immunoprecipitated choosing the C36 anti-pRb monoclonal antibody, as proposed by Whyte et al. (24), because of its ability to recognize the protein even when complexed with viral oncoproteins; the proteins were then analyzed by SDS-PAGE and subsequent autoradiography. Fig. 2 shows the communoprecipitation of 35S-labeled IVT-E1A with pRb in LI, DF, and ADF cell lines and the absence of communoprecipitation in the CRS-A2 cell line. In addition, Table 1 shows that the communoprecipitated samples from 2 × 10<sup>6</sup> cells contained specific radioactivity sufficient for liquid scintillation counting. SAOS-2 cells, expressing a truncated Rb gene product unable to bind with E1A (56), were used as a negative control. The fact that the only radioactive signal, according to Fig. 2, was the communoprecipitated 35S-labeled IVT-E1A, permitted abolishing SDS-PAGE analysis when a sufficient number of cells was available.

**Southern and Northern Blot Analysis.** Southern blot analysis of DNA from the LI, DF, CRS-A2, and ADF cell lines, after HindIII digestion and probing with Rb cDNA, displayed molecular sizes consistent with data reported by other investigators (17) (Fig. 3A). Moreover, no differences in band and intensity patterns were observed in the four cell lines.
ABERRANT Rb IN A HUMAN MALIGNANT GLIOMA

Table 1  Immunoprecipitation and coimmunoprecipitation of radiolabeled IVT-E1A evaluated by means of liquid scintillation counting

<table>
<thead>
<tr>
<th>Radiolabeled IVT-E1A added to</th>
<th>Immunoprecipitated with</th>
<th>cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer</td>
<td>MT3</td>
<td>79,650 ± 8,553</td>
</tr>
<tr>
<td>LI cells lysate</td>
<td>C36</td>
<td>5,948 ± 1,405</td>
</tr>
<tr>
<td>DF cell lysate</td>
<td>C36</td>
<td>3,771 ± 811</td>
</tr>
<tr>
<td>CRS-A2 cell lysate</td>
<td>C36</td>
<td>211 ± 36</td>
</tr>
<tr>
<td>ADF cell lysate</td>
<td>C36</td>
<td>4,415 ± 675</td>
</tr>
<tr>
<td>SAOS-2 cell lysate</td>
<td>C36</td>
<td>279 ± 12</td>
</tr>
</tbody>
</table>

In Northern blot analysis using Rb cDNA as a probe, all the cell lines displayed the presence of the message for the Rb gene product, with a molecular size of 4.7 kilobases (Fig. 3B, top), consistent with data from other laboratories (15, 57). Despite a lower amount of Rb message present in the CRS-A2 cell line, no important qualitative differences were evident among the four malignant glioma cell lines. Normalization using the glyceroldehyde-3-phosphate dehydrogenase cDNA probe permits comparing the amount of RNA loaded for each single lane (Fig. 3B, bottom).

Analysis of pRb by Immunoprecipitation. 35S-Labeled malignant glioma cell lysates were immunoprecipitated using the C36 anti-pRb monoclonal antibody; lysis and immunoprecipitation were carried out in a buffer containing no anionic detergents, such as SDS and DOC. Fig. 4A is an autoradiogram after SDS-PAGE, showing the standard pRb pattern in the LI, DF, and ADF cell lines. In fact, the protein was found in multiple forms between 105 and 114 kDa of apparent molecular mass; this microheterogeneity is essentially due to different degrees of phosphorylation and is considered normal for cycling, nonsynchronized cells (4–6, 24). CRS-A2 cells, instead, showed a band with a significatively lower apparent molecular mass, of about 99 kDa, with no discernible microheterogeneity pattern (Fig. 4A). To establish whether this abnormal band was an artifact, immunoprecipitation was done again using two more, highly specific, anti-pRb monoclonal antibodies, such as 1F8 (44) and PMG3-245 (27). The same immunoprecipitation pattern was observed with both antibodies, as with the C36 antibody, where immunoprecipitation was carried out either in the absence or in the presence of SDS and DOC (Fig. 4B). As a comparison, the standard pRb immunoprecipitation pattern by C36 is represented by the LI cell line. The 99-kDa CRS-A2 aberrant Rb protein was therefore recognized in immunoprecipitation by three different specific monoclonal antibodies against the human Rb protein and, in the case of C36, in the presence of anionic detergents.

The ability of the CRS-A2 aberrant protein to become phosphorylated was determined by immunoprecipitation after cell labeling with [32P]-orthophosphoric acid, using the same C36 antibody. As shown in Fig. 4C, phosphorylated pRb (ppRb) was clearly present in LI cells, but the same procedure failed to detect any phosphorylation signal corresponding to the migrating distance of the CRS-A2 Rb gene product.

IVT-E1A Binding with pRb from Other Malignant Cell Lines. To investigate the ability to recognize pRb abnormalities other than that reported for CRS-A2, the assay was extended to some other human tumor cell lines bearing described pRb deficiencies. Therefore IVT-E1A coimmunoprecipitation assay was done on two cell lines bearing truncated forms of pRb, such as SAOS-2 osteosarcoma cell line (58) and Y79 (38, 59); on WERI-Rbl retinoblastoma cell line, with a deletion of the Rb gene (39); and on the H209 small cell lung cancer cell line, bearing a point mutation leading to the substitution of a single amino acid in the pRb molecule (Cys 706 to Phe) (40). Results are shown in Fig. 5, where radiolabeled IVT-E1A, coimmunoprecipitated using the C36 antibody, is evident only in LI cells; the other cell lines failed to display any signal related to radiolabeled IVT-E1A. As a further control, a nonrelated monoclonal antibody, PA416, against SV40 large T-antigen (43), was used in LI cells.

Analysis of the Rb mRNA. Complementary DNA obtained from RNA extracted from the CRS-A2 and 293 cell lines was amplified by PCR using four oligonucleotide pairs, as described in “Materials and Methods.” The four amplified fragments from CRS-A2 cells, when compared by agarose gel electrophoresis, displayed the same size of those obtained from 293 cells (data not shown). This result excluded the possibility of large internal deletion of the coding sequence, suggesting the presence of a frame-shift mutation on the 3' end. Comparison by DGGE of the PCR fragments coding for the pRb COOH-terminal region (nt 2275–nt 3046) from CRS-A2 and 293 cells revealed the presence of a mutation (data not shown). This fragment from CRS-A2 cells was then cloned and sequenced, revealing a G insertion at position 2550. This base insertion can lead to the formation of a stop codon at the level of amino acid 853, producing a...
truncated Rb protein, lacking 75 amino acids. The experiment was done twice, starting from two different RNA preparations.

**DISCUSSION**

A functional analysis of the Rb gene in four human malignant glioma cell lines was done by an approach based on the coimmunoprecipitation of a complex formed by 35S-labeled IVT-E1A, pRb, and an anti-pRb monoclonal antibody. This allowed us to discover that an abnormal Rb gene product, unable to bind with IVT-E1A, was present in the CRS-A2 malignant glioma cells. Other methods, such as Southern and Northern blots, did not discriminate between CRS-A2 and the other malignant gliomas tested. Some investigators report that certain retinoblastomas express truncated Rb transcripts (60, 61); Venter et al. (19) find in a human malignant glioma a mRNA species with a smaller transcript which, however, seemed to be absent in all our cultured malignant glioma cell lines. Only more complex techniques, such as immunoprecipitation performed on total labeled cellular proteins or DNA sequencing, were able to detect an alteration in the CRS-A2 Rb gene product and to clarify its nature.

The method of radiolabeled IVT-E1A coimmunoprecipitation by the C36 monoclonal antibody, followed by SDS-PAGE and autoradiography, was quite sensitive, because as few as 1 x 10⁶ cultured cells were used for each single assay (see Fig. 2). Prolongation of the autoradiography exposure time beyond 24 h enhanced the sensitivity of the method, thus allowing a decrease of both the cell number and the amount of radiolabeled IVT-E1A (data not shown). Furthermore, it should be emphasized that the functional aspect of the assay permitted detecting different kinds of Rb gene product abnormalities, leading to defective binding with IVT-E1A (see Fig. 5).

In addition, a simple liquid scintillation counting after coimmunoprecipitation, instead of the more complex and time-consuming SDS-PAGE and autoradiography, might be sufficient for a rapid, preliminary screening (see Table 1), since any radioactive signal could be related only to the presence of coimmunoprecipitated IVT-E1A. We attempted to define more accurately the molecular defect impairing the E1A-binding properties in the CRS-A2 Rb gene product. The immunoprecipitation analysis of the CRS-A2 cell lysate using an anti-pRb antibody showed the presence of a pRb with a lower apparent molecular mass in SDS-PAGE, when compared with normal pRb. In addition, this protein displayed a low or a null degree of phosphorylation, perhaps due to its peculiar primary structure; the aberrant protein, in fact, might not become an efficient substrate for the specific kinases or might not be compartmentalized in the nucleus. These hypotheses are not mutually exclusive.

Binding with E1A takes place in a specific domain of the pRb molecule, functionally designed as “pocket,” spanning from amino acid 379 to 793, which is the minimal region necessary for E1A binding (62). Molecular damages at the level of the pocket region can lead to pRb malfunction in binding viral oncoproteins as well as endogenous transcriptional factors (22, 40, 41, 44, 63). The analysis of the CRS-A2 Rb cDNA sequence, revealing the presence of a G insertion at nt 2550, was consistent with our previous results. This insertion can lead, in fact, to the generation of a COOH-terminally truncated pRb, lacking the last 75 amino acids, with a computed molecular mass of 97,663 Da. The molecular damage found in CRS-A2 pRb could be the cause of the lack of interaction with IVT-E1A and of the lack of phosphorylation as well. Unless there are sense point mutations in the unsequenced upstream region, leading to the substitution of critical amino acids, these data suggest that the 75-amino acid COOH-terminal region may be necessary for the E1A binding.

In addition, the CRS-A2 Rb gene product was detected in immunoprecipitation by the 1F8 monoclonal antibody, which does not recognize SAOS-2 pRb. The defective Rb protein present in the SAOS-2

![Graphical representation](image-url)
osteogenic sarcoma cell line is a 95-kDa protein that lacks exons 21–27. It is unable to bind with SV40 large T and E1A oncoproteins, is found only in an underphosphorylated form and is recognized in the cytoplasm by immunofluorescence using the PMG3-245 monoclonal antibody, but not the 1F8 (41, 44, 56). The epitope of the 1F8 antibody is supposed to lie within the pRb segment encoded by exons 21-27, and the IVT-E1A binding assay could be considered as a functional test for the Rb protein, based on its binding properties with the E1A oncoprotein. Since endogenous transcription factors, such as E2F, share the DNA sequence encoding for its epitope is between nt 2110 (the beginning of the IVT-E1A binding assay could be considered as a functional test for the Rb protein, based on its binding properties with the E1A oncoprotein. Since endogenous transcription factors, such as E2F, share the DNA sequence encoding for its epitope is between nt 2110 (the beginning

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