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Definition of the Minimal Simian Virus 40 Large T Antigen- and Adenovirus E1A-Binding Domain in the Retinoblastoma Gene Product

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It has previously been demonstrated that the simian virus 40 large T antigen and adenovirus E1A proteins can form complexes with the retinoblastoma susceptibility gene product (RB). We studied the ability of these proteins to bind to mutant RB proteins in vitro. A region of RB spanning residues 379 to 792 was found to be both necessary and sufficient for binding to T or E1A. Furthermore, this region of RB contains sufficient structural information to mimic wild-type RB in its ability to distinguish between wild-type T and the transformation-defective T mutant K1. The results of competition experiments with peptide analogs of the RB-binding sequence in T suggest that this region of RB makes direct contact with a short colinear region of T, i.e., residues 102 to 115, previously implicated in both transformation and RB binding.

The existence of tumor suppressor genes was first suggested by experiments in which the fusion of normal cells with their malignant counterparts was shown to suppress the ability of the latter to form tumors in vivo (22, 27). Furthermore, these fused cells could revert to a tumorigenic phenotype after losing specific chromosomes derived from the normal parent (22, 27). A number of human tumors have been characterized by the frequent, nonrandom loss of specific chromosomal segments detectable either by karyotypic analysis and/or by Southern blotting with DNA probes of known chromosomal location (13, 47). This, again, implies that specific gene loss is linked to the evolution of key aspects of a neoplastic phenotype.

Retinoblastoma is a pediatric tumor which occurs in both sporadic and familial forms (28). Previous studies indicated that the risk of developing retinoblastoma was linked to abnormalities, including deletions, of the long arm of chromosome 13 (2, 7, 16, 48). Further studies suggested that inactivation of both alleles of a sequence mapping to chromosome 13q14 was a necessary event in the pathogenesis of this tumor, consistent with the notion that this region harbored a tumor suppressor gene (4, 40). The retinoblastoma susceptibility gene (RB) was subsequently cloned and sequenced (12, 15, 31). It encodes a nuclear protein of 928 amino acids which exists in both phosphorylated and un(der)-phosphorylated forms (32, 34). Huang et al. (25) have demonstrated growth inhibition in RB−/− human retinoblastoma and osteogenic sarcoma cell lines infected with an RB-encoding retrovirus, in keeping with the suggested function of the gene. RB expression has been noted in a variety of normal cell types (31). Indeed, RB inactivation may play a role in the pathogenesis of a wide range of human tumors, including breast, bladder, and small-cell lung carcinomas, osteosarcomas, and soft tissue sarcomas (14, 19, 24, 33, 50, 51, 53, 57). These observations underscore the potential importance of RB as a cell growth regulatory element.

How RB carries out its growth suppressor function is unknown. The protein has DNA-binding activity in vitro (32), although at the present time it is unknown whether it is capable of recognizing specific DNA sequences. Recently the products of three different DNA tumor viral transforming genes, i.e., the adenovirus E1A protein, the simian virus 40 large T antigen (T), and the human papillomavirus type 16 E7 protein, have been found to have specific RB-binding activity (5, 8, 54). Previous studies have shown that the binding of T to RB requires an intact T sequence extending from residue 105 to 114, a region which has previously been shown to be necessary for transformation (5). In T, this sequence is both necessary and apparently sufficient for RB binding, although the quaternary structure of T also contributes to complex formation (6, 35). Both E1A and E7 contain short colinear sequences which are highly homologous to $T_{105\ to\ 114}$, and these sequences specifically contribute to the RB binding and transforming activities of both proteins (6, 8, 10, 11, 18, 55).

In the present study, we asked whether a limited colinear segment of the RB sequence constituted a minimal T-binding domain and, if so, whether, as expected, this domain operates similarly in E1A binding. This information should prove useful for subsequent investigations into the possibility that the RB-binding segments of E1A, T, and E7 are related to an analogous region of a cellular protein with which RB normally interacts.

MATERIALS AND METHODS

In vitro transcription. A 2-μg portion of linearized plasmid was transcribed in vitro in the presence of 40 mM Tris (pH 7.5)–6 mM $MgCl_2$–2 mM spermidine–10 mM NaCl–10 mM dithiothreitol–0.5 mM ribonucleoside triphosphate (Pharmacia Inc.)–100 U of RNAse inhibitor (Promega Biotec)–10 to 20 U of the appropriate RNA polymerase (SP6 or T7; Pharmacia), in a 100-μl reaction volume (38). The reaction was incubated for 60 to 120 min at 37°C.

In vitro translation. RNA (2 to 8 μl; concentration, 0.5 to 2.0 μg/μl) was added to 1 μl of 1 mM amino acids (minus methionine; Promega)–5 μl of [^{35}S]methionine (1,200 Ci/mmol, 10 mCi/ml; Dupont, NEN Research Products)–35 μl of micronuclease-treated rabbit reticulocyte lysate (Promega) and sufficient diethylpyrocarbonate (Sigma Chemical

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Co.)-treated H_2O to bring the reaction volume to 50 μl (42). The reaction was incubated at 30°C for 60 min.

Cell culture. 293 cells, a human embryonic cell line transformed by a fragment of the adenovirus 5 genome (17), and D_2C_2 cells, a T-transformed cell line derived from CV-1P monkey kidney cells (34), were grown in Dulbecco modified Eagle medium with 10% fetal calf serum (GIBCO Laboratories). BALB/c 3T3 CIA31 (A31) cells were grown in Dulbecco modified Eagle medium supplemented with 10% bovine serum (Colorado Serum Co.). C57B1/6 primary mouse embryo fibroblast (MEF) lines expressing wild-type (MEFΨTex) and mutant (MEFΨK1) forms of T were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (GIBCO) and G418 (150 μg/ml) (9). All cells were grown at 37°C in a humidified 10% CO_2-containing atmosphere. For preparation of cell lysates, cultures were grown to confluence in 100-mm culture dishes and washed twice with ice-cold Tris-buffered saline (25 mM Tris [pH 8.0], 150 mM NaCl). The cells were then lysed for 30 min at 4°C in 1 ml of ice-cold EBC buffer (50 mM Tris [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 μM sodium orthovanadate) containing 10 μg of the following protease inhibitors per ml: aprotonin (Sigma), phenylmethylsulfonyl fluoride (Sigma), and leupeptin (Boehringer Mannheim Biochemicals). The lysates were then centrifuged at 14,000 × *g* in an Eppendorf microcentrifuge for 15 min at 4°C to clear the lysate of nuclei and debris.

Antibodies. Tissue culture supernatants were the source of PAb 419, M73, and M58 (20, 21).

Polymerase chain reaction. Polymerase chain reaction (PCR) was carried out in a 100-μl reaction volume containing approximately 1 ng of DNA template, 50 mM KCl, 10 mM Tris (pH 8.3), 0.5 to 3.5 mM $MgCl_2$, 0.01% gelatin, 200 μM deoxynucleoside triphosphate (Pharmacia), 1 μM of each oligonucleotide primer, and 2.5 to 5 U of *Taq* polymerase (Perkin-Elmer Cetus) (43). This reaction mixture was overlaid with mineral oil and incubated at 94°C for 90 s to denature the template, followed by 30 cycles consisting of 37°C for 2 min (annealing), 72°C for 3 min (extension), and 94°C for 1 min (denaturation) by using a programmable heat block (MJ Research). Following a final incubation at 72°C for 10 min, a sample of the reaction mixture was analyzed by agarose gel electrophoresis and ethidium bromide (Sigma) staining. PCR products which were to undergo in vitro transcription were subcloned into the vector pGEM-4Z (Promega).

Protein expression using baculovirus. Use of the baculovirus system was as previously described by Summers and Smith (49). Briefly, *Spodoptera frugiperda* cells (SF9) were grown at 27°C in Grace insect media (GIBCO) supplemented with 10% heat-inactivated fetal calf serum, lactoalbumin (Difco), and Yeastolate (Difco). A recombinant *Autographa californica* nuclear polyhedrosis virus (AcMNPV) containing the coding region for large T antigen (41) was used to infect fresh log-phase SF9 cells growing in suspension. At 3 to 5 days after infection, the cells were lysed in EBC and analyzed for protein expression by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver staining.

Immunoaffinity chromatography. Immunoaffinity chromatography was essentially as described by Simianis and Lane (46). Briefly, 1 ml of protein-A Sepharose beads (Pharmacia) was equilibrated in NETN (20 mM Tris [pH 8.0], 500 mM NaCl, 1 mM EDTA, 5% Nonidet P-40) and then rocked overnight at 4°C in 50 to 100 ml of PAb 419 tissue culture supernatant. Protein A was covalently linked to PAb 419 with 40 mM dimethylpimelimidate dihydrochloride (Sigma) in 0.1 M borate buffer (pH 9.0). The cross-linked beads were rocked for 1 h at room temperature and then washed in 40 mM ethanolamine (Sigma) and once with 0.1 M borate (pH 8.0). Whole cell lysates (cleared of nuclei and debris by centrifugation) were then loaded onto the column, and after washing the column, T was eluted with 0.2% triethylamine (Sigma)–10% glycerol–50 μg of phenylmethylsulfonyl fluoride per ml. Column fractions were neutralized with 1/10 volume of 300 mM Tris (pH 7.5)–1 M NaCl–1 mM dithiothreitol–50 μg of phenylmethylsulfonyl fluoride per ml and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

In vitro RB-binding assay. Samples (5 to 10 μl) of in vitro translate containing ^{35}S-labeled RB products were mixed with either immunoaffinity-purified T antigen (1 to 2 μg) or 25 to 500 μl of the appropriate cell lysate in 500 μl of NETN at 4°C (8). After 2 to 4 h, 25 to 50 μl of PAb 419 (anti-T) or 25 μl of M73 plus 25 μl of M58 (anti-E1A) were added. The mixture was rocked for an additional 1 h, after which 25 μl of a 1:1 mixture of protein-A Sepharose beads (Pharmacia) previously washed and suspended in NETN plus 4% bovine serum albumin (Sigma) were added. After a 30-min incubation at 4°C, the beads were washed five times with NETN. Immunoprecipitates were subjected to sodium dodecyl sulfate-gel electrophoresis. The gels were then fixed, treated with 1 M sodium salicylate (Sigma), dried, and exposed to X-ray film at −70°C.

DNA manipulations. Purification and modification of cloned DNA fragments were essentially as previously described (1, 36).

DNA sequencing. Double-stranded DNA sequencing was carried out by using a Sequenase 2.0 kit (United States Biochemical Corp.) with the protocol provided by the manufacturer.

RESULTS

An in vitro RB-binding assay, originally described by Dyson et al. (8), was used to determine which region of RB is necessary for binding to T and to E1A. RB was transcribed in vitro by using the plasmid pBSK-RB1 (a generous gift of Steven Friend), which contains a full-length RB cDNA inserted downstream of the phage T7 promoter. Following transcription, translation was carried out in vitro in the presence of [^{35}S]methionine. An autoradiogram of the RB translation products generated in this manner is shown in Fig. 1, lane 1. Full-length RB was not detected among the translation products. Instead, a nested set of N-terminal deletions was generated by virtue of translation initiation occurring at internal methionines, as previously determined by Whyte et al. (54).

The in vitro translate was mixed with 1 or 2 μg of purified T and, after incubation, was immunoprecipitated with PAb 419, a monoclonal anti-T antibody (Fig. 1, lanes 3 and 4, respectively). Immunoprecipitation of the translate in the absence of T was performed in parallel (lane 2). In the presence of T, those RB proteins ~56 kilodaltons or greater in size were specifically coimmunoprecipitated. Similar results were obtained when a lysate prepared from the T-transformed monkey cell line D_2C_2 (34) was studied as a source of T in place of the purified protein (data not shown). A similar assay was performed in an effort to learn whether E1A could interact with these truncated RB products. In this case, a crude extract of 293 cells (which express E1A) was mixed with the RB translation products, and immunoprecip-

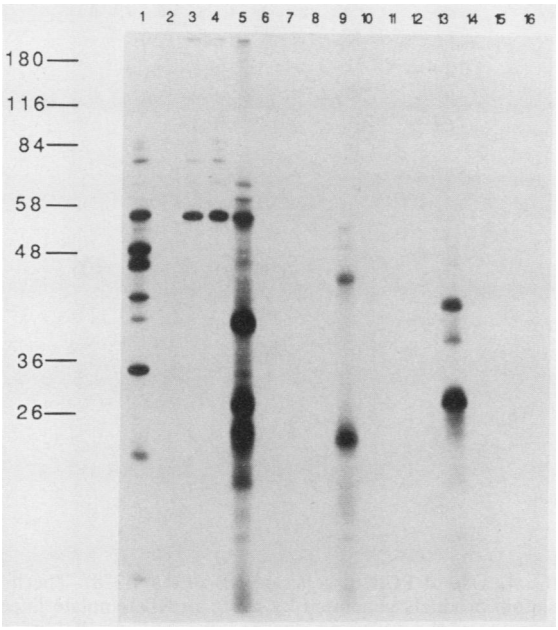

FIG. 1. In vitro binding of N-terminal and C-terminal RB dl mutants to T. The in vitro translation products generated by using standard RB cRNA terminating at nucleotide 4162, which is past the normal RB stop codon, are shown in lane 1. For binding studies, the translation products were mixed with 0, 1, or 2 μg of immunoaffinity-purified T antigen (lane 2, 3, and 4, respectively) and immunoprecipitated with an anti-T antibody (PAb 419). Lanes 5, 9, and 13 depict the in vitro translation products generated by using an RB cRNA terminating immediately 3′ of the codons for amino acids 767, 656, and 514, respectively. Each set of translation products was mixed with 0 μg (lanes 6, 10, and 14), 1 μg (lanes 7, 11, and 15), or 2 μg (lanes 8, 12, and 16), respectively, of T and immunoprecipitated with PAb 419 as above. In vitro translation was carried out in the presence of ^{35}S, and proteins were resolved in a 10% sodium dodecyl sulfate-polyacrylamide gel. Molecular masses of marker proteins (in kilodaltons) are indicated at the left.

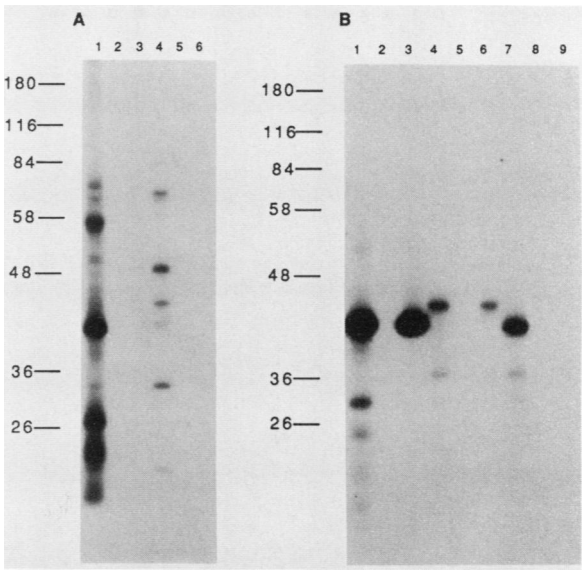

FIG. 2. In vitro binding of RB mutants to E1A. (A) The RB in vitro translation products of an RB cRNA template truncated immediately 3′ of the codon for amino acid 767 (lane 1) or bearing an in-frame deletion corresponding to amino acids 622 to 668 (lane 4) are shown. For binding studies, the translation products were mixed with 100 μg of 293 cell lysate (lanes 3 and 6, respectively) and immunoprecipitated with a mixture of anti-E1A antibodies (M73 and M58). Parallel immunoprecipitations of these translates were performed without the prior addition of cell lysate (lanes 2 and 5, respectively). (B) The RB cDNA encoding amino acids 379 to 792 (lane 1), 379 to 816 (lane 4), and 409 to 816 (lane 7) was amplified by using PCR and subcloned downstream of an SP6 promoter, following which in vitro transcription and translation were performed. These translation products were mixed with 0 μg (lanes 2, 5, and 8) or 100 μg (lanes 3, 6, and 9) of a crude 293 cell lysate and immunoprecipitated with M73 and M58 as described above. Immunoprecipitates were resolved in a 10% gel. Molecular masses of marker proteins (in kilodaltons) are indicated at the left.

itation was performed with a mixture of two anti-E1A monoclonal antibodies (20). The same bands which coimmunoprecipitated with T also coimmunoprecipitated with E1A (data not shown). Identical results were reported earlier by Harlow's laboratory for E1A, T, and E7 (8). Thus, given the known size of the RB protein (928 residues), one can conclude that approximately 40% of the N terminus can be deleted without altering binding to T and E1A.

The next question was how much of the C terminus of RB is needed for T binding. By using convenient restriction sites, the RB cDNA template used for transcription was truncated at three sites (encoding residues 768, 657, and 515) upstream of the natural stop codon to generate RB deletion (dl) 768-928, RB dl 657-928, and RB dl 515-928. The translation products of these templates are shown in Fig. 1, lanes 5, 9, and 13, respectively. By using the same conditions as described above, RB dl 768-928 was inactive in T binding (Fig. 1, lane 8). Similar results were obtained with respect to E1A binding (Fig. 2A, lane 3). The N-terminal boundary of this deletion is 30 amino acids downstream of the exon 21 deletion recently described by Horowitz et al. (24) in a bladder carcinoma cell line. The latter was shown to eliminate binding of RB to E1A (24). As can be seen in Fig. 1, the deletions present in RB dl 657-928 and RB dl 515-928 eliminated all detectable T-RB binding (Fig. 1, lanes 12 and 16, respectively). Thus, from these data, one could conclude that if there are unnecessary C-terminal region sequences, they must be downstream of residue 768.

In addition to the N-terminal and C-terminal deletions described above, four internal deletion mutants, namely, RB dl 622-668, RB dl 417-620, RB dl 516-668, and RB dl 417-515 were generated by excising convenient restriction fragments from pBSK-RB1 followed by religation of the vector with, when necessary, synthetic oligonucleotides to restore the proper reading frame. The restoration of the reading frame was confirmed by sequencing the relevant portion of each RB mutant. The in vitro translation products obtained from these four mutant RB templates are shown in Fig. 3, lanes 4, 7, 10, and 13, respectively. These translation products were then immunoprecipitated with PAb 419 after having been mixed with purified T (Fig. 3, lanes 6, 9, 12, and 15). Parallel reaction mixtures to which T was not added were also immunoprecipitated (Fig. 3, lanes 5, 8, 11, and 14). Each of these mutants failed to bind to T in this assay. These four mutants also failed to bind to E1A when the assay noted above was used. An example is shown in Fig. 2A, lane 6.

On the basis of its molecular weight, we predicted that the smallest T-binding N-terminal RB mutant arose from translation initiation occurring at a cluster of three methionines located at amino acids 379 to 387. To test this hypothesis, we generated an N-terminal deletion mutant (RB dl 1-378) by amplifying the coding region extending from residue 379 to

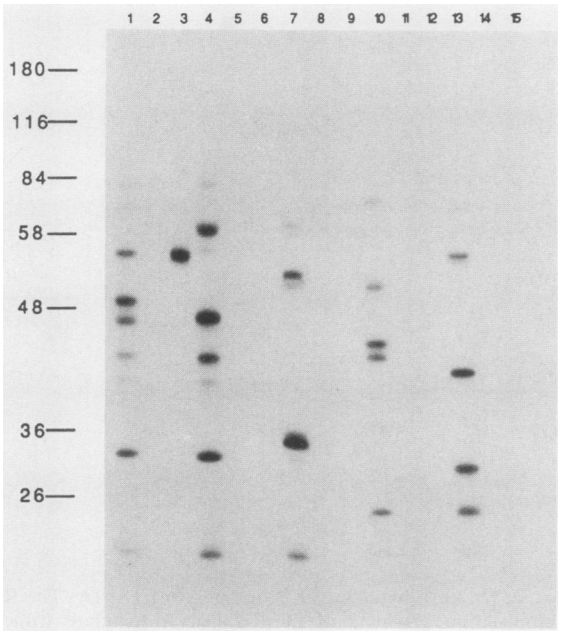

FIG. 3. In vitro binding of RB internal deletion mutants to T. The in vitro translation products generated by using a full-length RB template linearized downstream of the authentic stop codon are shown in lane 1. For binding studies, the translation products were mixed with 0 μg (lane 2) or 1 μg (lane 3) of immunoaffinity-purified T and immunoprecipitated with PAb 419. Lanes 4, 7, 10, and 13 depict the in vitro translation products generated by using RB templates bearing deletions corresponding to amino acids 622 to 668 (lane 4), 417 to 620 (lane 7), 516 to 668 (lane 10), or 417 to 515 (lane 13). These sets of translation products were mixed with 0 μg (lanes 5, 8, 11, and 14) or 1 μg (lanes 6, 9, 12, and 15) of T and immunoprecipitated with PAb 419 as above. In vitro translation products were resolved in a 10% gel. Molecular masses of marker proteins (in kilodaltons) are indicated at the left.

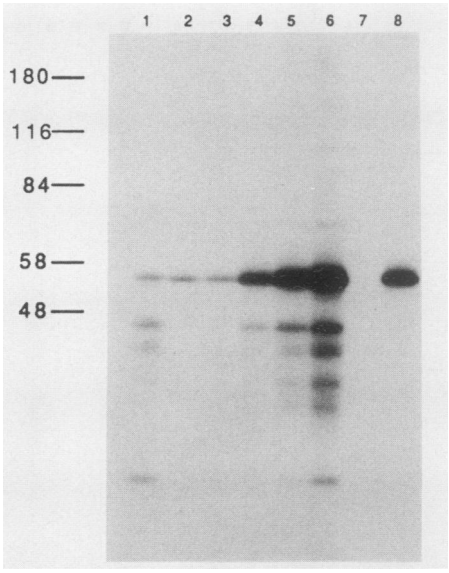

FIG. 4. Use of PCR to generate RB dl (aa 1-378). The in vitro translation products generated by using an RB template linearized downstream of the authentic stop codon are shown in lane 1. The higher-molecular-weight species expected in this set of products and seen in Fig. 1 and 2 were not detected due to underexposure. The RB cDNA encoding a segment extending from residue 379 to 928 was amplified by using PCR and subcloned downstream of an SP6 promoter. In vitro transcription was then performed, followed by in vitro translation in the presence of 0.5 μg (lane 2), 1 μg (lane 3), 2 μg (lane 4), 4 μg (lane 5), or 8 μg (lane 6) of RNA. A portion of protein from the in vitro translation corresponding to lane 4 was mixed with 0 μg (lane 7) or 2 μg (lane 8) of T and immunoprecipitated with PAb 419. Products were electrophoresed in a 10% gel. Molecular masses of marker proteins (in kilodaltons) are indicated at the left.

the C terminus by using the PCR (43). The upstream primer was designed so that the reaction product incorporated a new translation initiation sequence conforming to the rules proposed by Kozak (30). The results of transcribing this PCR product and then of translating the relevant RNA in vitro are shown in Fig. 4, lanes 2 through 6. In lane 1 are shown the products of translation of the native template. The dominant translation product of the PCR-generated template comigrated with the ~56-kilodalton T-binding RB mutant derived from the native template and, as was noted for the latter, bound specifically to T (compare lanes 4, 7, and 8). None of the other translation products of the PCR-generated mutant template bound to T, including the next largest member of the set, an ~45-kilodalton polypeptide. It was assumed that the band which did bind was likely initiated at one or more of the aforementioned set of three methionine codons and, therefore, that the N-terminal boundary of the T and E1A (T/E1A)-binding sequence is at or very close to Met-379.

PCR was also used to generate RB mutants RB dl 1-378;793-928, RB dl 1-378;817-928, and RB dl 1-408;817-928. The in vitro translation products for these three PCR-generated RB mutants are shown in Fig. 5, lanes 4, 7, and 10, respectively. Also shown are the results obtained after immunoprecipitating each in vitro translate with PAb 419 after preincubating it in the presence (lanes 6, 9, and 12) or absence (lanes 5, 8, and 11) of purified T. As can be seen from this figure, each of these mutants, with the exception of RB dl 1-408;817-928 (lanes 10 to 12), retained the ability to bind to T. Identical results were obtained for E1A binding to these mutants in a separate experiment in which the same batch of in vitro translate was used (Fig. 2B). Therefore, one can place the C-terminal boundary of the minimal T/E1A-binding sequence between residues 769 and 793 and the N-terminal boundary between Met-378 and residue 408. A summary of the T/E1A in vitro binding data is shown in Fig. 6.

A short colinear T sequence, residues 105 to 114, is essential for both transformation and RB binding (5). Mutations in this region which interfere with transformation also abrogate RB binding and vice versa (5). Analogous sequences which control RB binding are present in the E1A and human papillomavirus E7 proteins (6, 8, 10, 11, 18, 55). Moreover, a synthetic peptide corresponding to this sequence bound specifically to RB isolated from crude monkey cell extracts (6). To test whether this sequence interacts directly with the T/E1A-binding region of RB defined in vitro, we synthesized a short peptide spanning amino acids 102 to 115 of T and determined whether this peptide would compete with intact T for binding to RB in the in vitro translation assay. As a control, an identical peptide bearing a Glu-107-Lys substitution was tested in parallel. This element is a copy of this sequence in the T mutant K1 (6). K1 neither transforms nor binds RB in various assays (5, 26; S. Chen and E. Paucha, unpublished data). As noted in Fig. 7A, preincubation of the in vitro RB translate with 10 μg of the T peptide (lane 3) inhibited the binding of the relevant truncated RB species to T. By contrast, an identical quantity of the K1 peptide was inactive (lane 2).

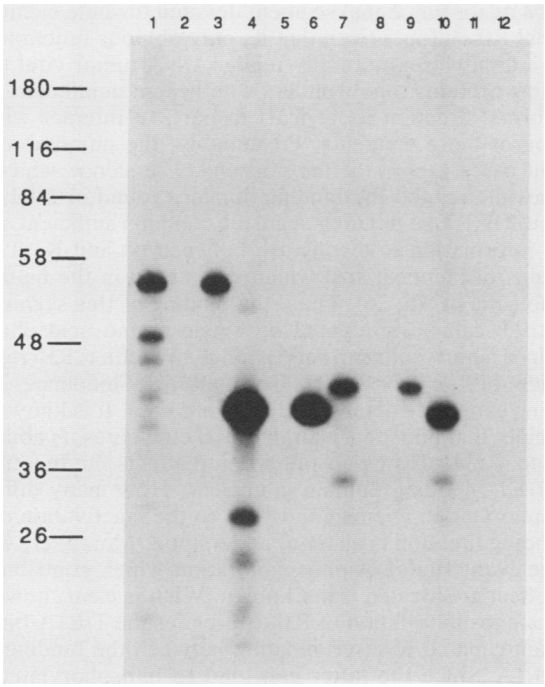

FIG. 5. In vitro binding of PCR-generated RB mutants to T. The RB cDNA encoding amino acids 379 to 928 (lane 1), 379 to 792 (lane 4), 379 to 816 (lane 7), and 409 to 816 (lane 10) was amplified by using PCR and subcloned downstream of an SP6 promoter. Each set of translation products was mixed with 0 μg (lanes 2, 5, 8, and 11) or 1 μg (lanes 3, 6, 9, and 12) of T and immunoprecipitated with PAb 419 as above. Products were electrophoresed in a 10% polyacrylamide gel. Molecular masses of marker proteins (in kilodaltons) are indicated at the left.

We also asked whether the wild-type synthetic peptide could promote the dissolution of a preformed RB-T antigen complex. The results of such an experiment are shown in Fig. 7B. A sample of the same batch of in vitro RB translate studied in the experiment depicted in Fig. 7A and purified T (final concentration 1 nM) were preincubated for 2 h, following which 15 μg of either the T peptide (lane 3) or K1 peptide (lane 2) was added. After a further incubation of 4 h, immunoprecipitations were performed with PAb 419, as described above. Pilot control experiments indicated that the binding of T to RB was complete after 2 h over the range of T concentrations used in these studies (data not shown). As noted in Fig. 7B, lane 3, a dramatic reduction in RB bound was detected after the addition of the T peptide. The K1 peptide, by contrast, had no effect.

Finally, we asked whether the smallest identified T-binding RB mutant, RB dl 1-378;793-928, contains sufficient structural information to bind selectively to the wild-type (wt) 105 to 114 sequence. Thus, the ability of the wt and K1 peptides to compete with T for binding to limiting quantities of this RB mutant was tested. Only the wt peptide was active in this regard, in keeping with its effect on binding to the 378 to 928 RB polypeptide (data not shown). Since it is possible that the T-RB interaction is influenced by the milieu of the putative RB-binding motif in T, we also studied the ability of some of the aforementioned RB mutants to bind to wt T and to K1 T. Portions of in vitro translate were mixed with ~150 μg of a lysate of naive A31 mouse fibroblasts or A31 derivatives that stably produce either wt T (MEFΨTex) (9; S. Chen and E. Paucha, unpublished data) or K1 T (MEFΨK1) (9; S. Chen and E. Paucha, unpublished data). Equivalent amounts of wt T and K1 T were found to be present in these lines, as defined in parallel, antibody excess immunoprecipitation experiments, in which the amount of immunoprecipitable K1 or T was determined by silver staining (data not shown). As can be seen from Fig. 8, each

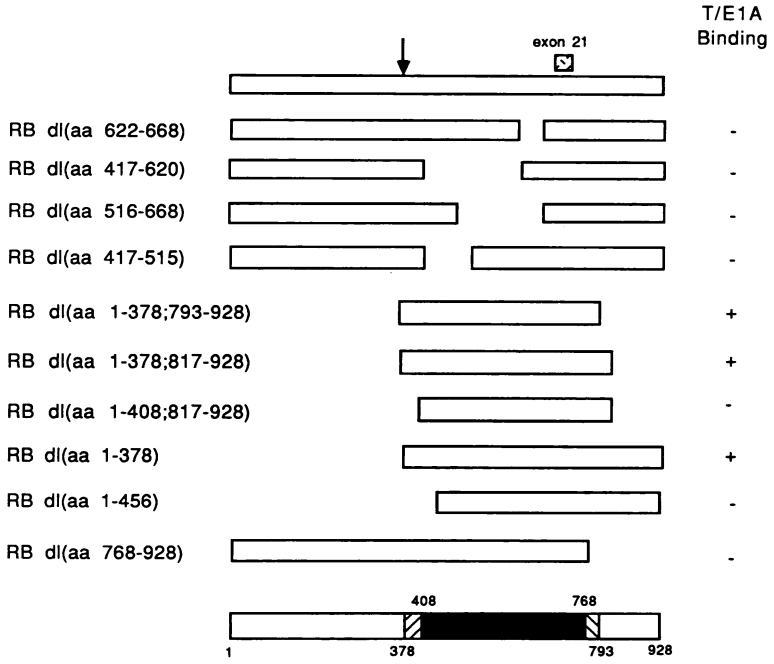

FIG. 6. Summary of RB mutant binding data. □, Unnecessary for T/E1A binding; ▨, contains N-terminal AA necessary for T/E1A binding; ▧, contains C-terminal AA necessary for T/E1A binding; ■, proposed T/E1A-binding domain.

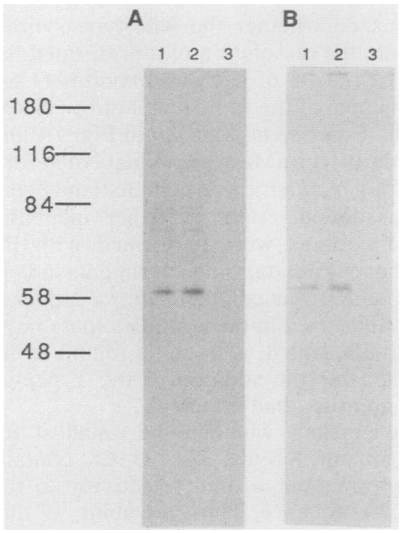

FIG. 7. Competition between T and various T-derived peptides for RB binding. (A) RB cRNA translation products were premixed for 2 h with no peptide (lane 1), 10 μg of K1 peptide (lane 2), or 10 μg of T peptide (lane 3), following which 50 ng of T antigen was added. After 2 h of incubation at 4°C, immunoprecipitation was carried out with PAb 419. Bound proteins were electrophoresed through a 10% gel. (B) RB translate was premixed with 50 ng of T antigen. After 2 h, no peptide (lane 1), 10 μg of K1 peptide (lane 2), or 10 μg of T peptide (lane 3) was added. After an additional 4 h at 4°C, immunoprecipitation was carried out with PAb 419 and bound proteins were electrophoresed through a 10% acrylamide gel. Molecular masses of marker proteins (in kilodaltons) are indicated at the left.

RB mutant which bound specifically to wt T failed to bind to the K1 derivative. The decrease in band intensity observed in lane 12 was not a reproducible finding (data not shown).

DISCUSSION

Our data suggest that a large region located in the C-terminal half of RB, encompassing residues 379 to 792, must be relatively intact for T binding to occur. Since, at a minimum, only ~10 T residues make contact with this segment, it seems likely that much of this ~400-amino-acid region of RB is devoted to maintaining its functional conformation and not to directly contacting T sequences. Further definition of the RB sequences in this segment which are needed for T binding will likely require analysis of smaller internal deletions as well as of point mutations. In this regard, it is noteworthy that data from Hu et al. (24a) and W.-H. Lee et al. (personal communication), who have performed experiments similar to those described here, suggest that a short subsegment of this region, residues 573 to 645, can be deleted from the RB protein without qualitatively altering binding to T or E1A, provided a spacer sequence is reinserted. Conceivably, this short segment may serve as an interval or hingelike sequence needed to maintain the proper geometry of the flanking subsegments of the T/E1A-binding domain. The data from these two groups are in agreement with the C-terminal and N-terminal boundaries of the T/E1A-binding region identified in this report (24a; W.-H. Lee, personal communication).

It is notable that T or E1A binding by RB is, in large measure, a product of a domain comprising nearly 40% of the primary structure of the protein. Given this observation, one might speculate that the T/E1A-binding domain constitutes a major functional segment devoted to some element of normal RB action. Given that its only obvious function is to bind a family of structurally related DNA tumor viral transforming proteins, one wonders whether, in uninfected cells, its normal function is, at least in part, to interact with an analog of these elements. Presumably, the putative analog would carry a copy of the consensus sequence which describes the related RB-binding domains found in T/E1A.

The RB T/E1A-binding segment contains sufficient structural information to distinguish between wt and K1 T, two proteins of identical size which differ only in the nature of amino acid 107 (5, 26). Thus, the binding of this segment of RB to T can be abrogated by single amino acid changes within a short colinear region of T. Whether RB regions outside of the 379 to 792 segment also influence T-RB binding cannot be determined from our data. It is known, for example, that phosphorylation of RB eliminates its ability to bind to T (34). How phosphorylation affects the function of the T/E1A-binding domain is unclear. How many different phosphorylation events contribute to the inactivation of the T-binding function is also not understood. Moreover, where the relevant site(s) of phosphorylation which contribute to this effect are located is not known. What is clear, however, is that phosphorylation of RB can render the T/E1A-binding domain capable of discriminating between the binding of T and E1A, since the latter can bind to phosphorylated RB (54).

The 105 to 114 sequence of T can operate as a specific RB ligand without a contribution from other T sequences (6). That observation was confirmed here in experiments that showed that the wt 102 to 115 peptide could effectively compete with intact T for binding to both the 379 to 928 and the 379 to 792 truncated RB species. These observations corroborate earlier genetic data (5) which suggested that the

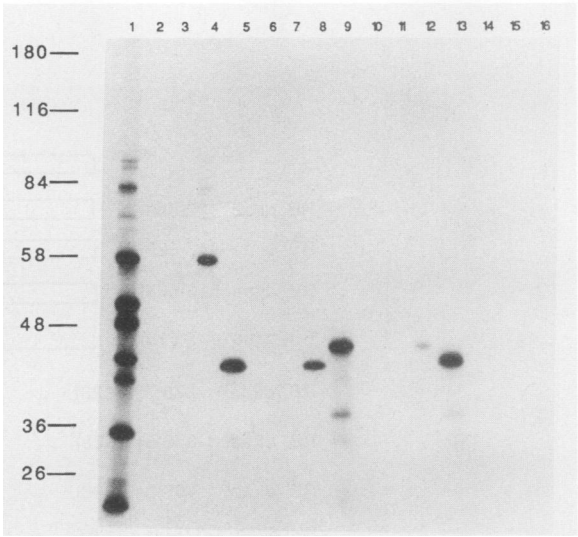

FIG. 8. Binding of RB mutants to wt or K1 T synthesized in murine cells. The RB in vitro translation sets using either the RB1 cDNA (lane 1) or PCR-generated cDNA fragments corresponding to amino acids 379 to 792 (lane 5), 379 to 816 (lane 9), and 409 to 816 (lane 13) are shown. These in vitro translation products were mixed with ~150 μg of A31 whole cell lysate (lanes 2, 6, 10, and 14), MEFΨK1 cell lysate (lanes 3, 7, 11, and 15), or MEFΨT cell lysate (lanes 4, 8, 12, and 16) and immunoprecipitated with PAb 419. Immunoprecipitated products were electrophoresed in a 10% gel. Molecular masses of marker proteins (in kilodaltons) are indicated at the left.

binding of RB to T requires that this region of T be structurally intact. That this sequence provides a considerable portion of the free energy underlying the T-RB interaction is further supported by the finding that the presence of the peptide in sufficiently high concentration could lead to the apparent dissolution of a preexisting T-RB complex. At the same time, it should be noted that a large molar excess of peptide relative to T was required to demonstrate competition for RB binding. While this may merely reflect the inability of the synthetic peptide to assume the higher order structural characteristics of the in situ sequence, it raises the possibility that other, as yet undefined, T sequences influence its binding to RB. Indeed, the study of the E1A protein has led to the identification of two distinct RB-binding domains, only one of which is homologous to T 105 to 114 (N. Dyson and E. Harlow, personal communication). Furthermore, the data of Ludlow et al. (35) have shown that aspects of T-antigen quaternary structure, as reflected by the preferential binding of RB to oligomeric, as opposed to monomeric, T also contribute to RB binding. It seems likely, then, that more than one structural feature of T contributes to its RB-binding function. Little more can be said of how quaternary structure participates at present, since the primary sequences needed for proper T oligomerization are not known. Among the obvious questions in this regard is whether the 105 to 114 T sequence contributes both to normal T quaternary structure and to T-RB complex formation.

The human RB gene is complex, spanning greater than 200 kilobases and containing 27 exons (23, 37). The T/E1A-binding region identified here is encoded by exons 12 to 23. It is of interest that exon 20 can be predicted to encode a leucine zipper structure (3, 23, 37), a motif which has been implicated in certain protein-protein interactions which underlie the function of some transcriptional regulation factors (29, 39). Furthermore, a number of DNA-binding proteins bind to DNA only after leucine-zipper-mediated homo- or heterodimer formation (29, 39). Presently, one does not know whether RB dimerizes in uninfected cells nor is it established that RB can recognize specific DNA sequences. Yet, these are obvious possibilities in need of definitive testing. It should also be noted that most leucine-zipper-containing DNA-binding proteins contain a basic region, N terminal to the zipper motif, and the former is thought to form the DNA contact surface (39, 52). RB lacks such a sequence (37). On the other hand, leucine-zipper-containing DNA-binding proteins which lack this conserved basic region do exist, including *myc* and OCT-2 (39).

The products of exons 17, 18, 20, and 21 contain histidine-cysteine pairs which have, in other settings, been identified as elements of certain zinc-finger motifs which may play a role in the DNA-binding function of other proteins (23, 32, 39). It will be interesting to determine whether DNA binding by RB is indeed the product of a discrete domain. If that were true, its structural relationship to the T/E1A-binding domain and any evidence that T or E1A binding perturbs DNA binding would be of considerable interest. One of the immediate questions is whether T and E1A perturb RB growth-controlling function by modulating one or more intrinsic RB functions. If T or E1A binding led to a reproducible and specific change in the quality and/or dynamics of the RB DNA-binding function, one would be led to the speculation that DNA binding, either directly or indirectly, signals a physiologically important RB action.

It is also noteworthy that mutations affecting the T/E1A-binding domain occur relatively frequently in human tumors lacking normal RB function (23, 24, 44, 45, 56). Whether this is due to the presence of a "hot spot" for recombination (23) or arises because the presence of such a mutation confers a growth advantage upon cells which harbor it is unknown. In this regard, others have tested several spontaneous RB mutants and have found that they are uniformly defective in T/E1A binding (24, 24a, 44, 45).

The breadth of different viral proteins which all bind to RB by what seems to be a common route, the fact that a sizeable fraction of the RB sequence is required for this interaction to take place, and the knowledge that this region is frequently altered in naturally arising RB mutants together suggest that a cellular protein which binds to RB in a similar manner may exist. Such a protein might, for example, serve either to regulate RB function or as a substrate upon which RB acts or both. Conceivably the binding of this protein to RB might be cell cycle dependent, as was observed for the interaction of T and RB (35). Although the existence of such a cellular protein remains unproven, a computer-assisted search for proteins with a sequence structurally similar to the putative RB-binding region of T, E1A, and E7 identified both c-*myc* and the fission yeast *Saccharomyces pombe* cdc 25 product (10, 11). Experiments designed to test the potential of these two proteins as RB ligands are in progress.

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