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Two distinct and frequently mutated regions of retinoblastoma protein are required for binding to SV40 T antigen

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The retinoblastoma susceptibility gene (RB) encodes a phosphoprotein of 110 kd (pp110^{RB}) that forms specific complexes with SV40 T antigen and the transforming proteins of several other DNA tumor viruses. Interaction with RB is thought to contribute to transformation by these viruses as demonstrated by genetic analyses. To help understand the function of these interactions, the regions of RB that are involved in binding to T have been mapped. An *in vitro* protein synthesis system capable of producing full-length RB protein has been developed to facilitate the mapping study. A 5- to 10-fold increase in translational efficiency in the reticulocyte lysate was obtained when the 5′ non-coding region of RB mRNA was replaced with that of β-globin mRNA or a plant viral RNA, alfalfa mosaic virus (AMV) RNA4. A series of mutated RB polypeptides produced from this system were assayed for T binding. Two non-contiguous regions of the RB protein, amino acid residues 394–571 and 649–773, were found to be necessary for binding to T: mutations in either region abolished T–RB complex formation. These results are consistent with the finding that, in all the cases analyzed so far, mutated RB proteins in human tumor cells also failed to bind to T antigen due to deletions including at least one of the two required regions. Thus the regions of RB defined *in vitro* as necessary for interaction with T might be physiologically relevant as well, and might play a fundamental role in normal RB protein function.

Key words: retinoblastoma/RB binding/SV40 T antigen

Introduction

Retinoblastoma is a childhood tumor for which a genetic predisposition to cancer formation can be clearly shown (for review, see Vogel, 1979). Localization of the involved genetic element to chromosome 13q14 (Francke, 1976; Yunis and Ramsay, 1978; Balaban *et al.*, 1982) and evidence of its recessive nature (Knudson, 1971; Cavenee *et al.*, 1983; Dryja *et al.*, 1986) have led to the cloning of the retinoblastoma susceptibility (RB) gene (Friend *et al.*, 1986; Fung *et al.*, 1987; Lee *et al.*, 1987a). The RB gene encodes a nuclear phosphoprotein of 110 kd (pp110^{RB}) that is expressed in every tissue type examined (Lee *et al.*, 1987a,b). The upstream promoter of RB gene is confined to a 70 bp region and has similar features to those of other 'housekeeping' promoters (Hong *et al.*, 1989). Surveys of many different tumor cell types have revealed alterations of the RB gene not only in most retinoblastomas (Friend *et al.*, 1986; Fung *et al.*, 1987; Lee *et al.*, 1987a; Dunn *et al.*, 1988), but also in some osteosarcomas (Toguchida *et al.*, 1988; Shew *et al.*, 1989), soft tissue sarcomas (Friend *et al.*, 1987), breast cancers (Lee *et al.*, 1988a; T'Ang *et al.*, 1988; Varley *et al.*, 1989), small cell lung carcinomas (Harbour *et al.*, 1988; Yokota *et al.*, 1989; Hensel *et al.*, 1990), bladder carcinomas (Horowitz *et al.*, 1989), prostate carcinomas (Bookstein *et al.*, 1990) and leukemias (Cheng *et al.*, 1990). These results, taken together with the observation that patients with hereditary retinoblastomas have higher risk of developing second primary tumors (Abramson *et al.*, 1979; Draper *et al.*, 1986), suggest that RB may have a broad role in oncogenesis. The tumor suppression function of RB gene was recently demonstrated by the loss of tumorigenicity in nude mice of a retinoblastoma cell line infected with an amphotropic virus carrying the RB gene (Huang *et al.*, 1988). Expression of exogenous RB proteins in other RB deficient cell lines such as prostate carcinoma DU145 (Bookstein *et al.*, 1990) also exerts suppressive effects on their neoplastic phenotype. Thus the tumor suppression function of RB appears to operate in different cell types, which further suggests a common cellular activity of RB vital to many, if not all, cells (for review, see Lee *et al.*, 1988b).

The function of the RB gene product at the biochemical level is as yet poorly understood. Consistent with its nuclear localization, pp110^{RB} is associated with DNA-binding activity (Lee *et al.*, 1987b; Wang *et al.*, 1990). Several phosphorylated and unphosphorylated forms of RB co-exist in the cell (Ludlow *et al.*, 1989; Shew *et al.*, 1989). The phosphorylation status of pp110^{RB} oscillates during the cell cycle and hyperphosphorylation of pp110^{RB} is correlated with mitogenic stimulation (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989). These observations suggest that phosphorylation may play an important role in RB protein function. An interesting connection between tumor suppressor genes such as RB and some DNA tumor viral oncogenes was forged recently by the finding that RB protein forms complexes with oncoproteins such as adenoviral protein E1A, SV40 large T antigen and E7 protein of the human papilloma virus (DeCaprio *et al.*, 1988; Whyte *et al.*, 1988; Dyson *et al.*, 1989a). RB binds specifically to the transforming regions of these oncoproteins, suggesting that it might be one of the cellular targets for their action (DeCaprio *et al.*, 1988; Whyte *et al.*, 1989). In the case of T, only the underphosphorylated member of the RB product family was present in the complex (Ludlow *et al.*, 1989), implying a regulatory effect of RB phosphorylation on its ability to bind T.

To further understand the functional significance of these interactions, we examined the regions of pp110^{RB} involved

in binding to T. Previous work with several human tumor cell lines have identified mutated RB proteins that lost E1A or T binding activity (Horowitz *et al.*, 1989; Bookstein *et al.*, 1990; Shew *et al.*, 1990a,b). Analysis of RB gene expression in these cell lines showed that exon 16 or 21 was deleted from the RB mRNA transcript. These results suggest that sequences in exons 16 and 21 were probably required for binding to T *in vivo*. To define the regions involved in more detail, an *in vitro* systematic mapping study was undertaken by assaying, for binding to T, the various mutated RB polypeptides generated from *in vitro* transcription and translation. Consistent with the findings in mutant cells, two non-contiguous regions were identified as necessary for interaction with T, one within exons 12−18 and the other consisting of exons 19−22. As part of this analysis, we found an improved *in vitro* translation system to produce RB protein, which should benefit other studies that include synthesis of RB protein *in vitro*.

Results

Enhanced translation of chimeric RB mRNAs containing the 5′-non-coding region of alfalfa mosaic virus (AMV) RNA4 or β-globin mRNA

SP6/T7 *in vitro* transcription, followed by *in vitro* translation, offers a convenient system to manipulate the RB polypeptide, and therefore an ideal system for our *in vitro* mapping studies. A construct pGEM-Y79N containing the 4.7 kb RB cDNA inserted downstream of the T7 promoter (Figure 1a) was initially tested for RB expression. The plasmid DNA was linearized with *Bam*HI (pGEM-Y79N/*Bam*HI) and transcribed with T7 polymerase. The transcripts were then translated in reticulocyte lysates and [^{35}S]methionine labeled products were analyzed by SDS−PAGE. Only a small amount of full length polypeptide was synthesized (Figure 2, lane 2). The prominent bands were truncated ones due to internal initiation (see below). Similar results were obtained when translation was carried out in wheat germ extracts (data not shown). Having ruled out the possibility of RNA or protein degradation, we suspected that the transcript derived from pGEM-Y79N was inefficiently translated *in vitro*. Translational efficiency of an mRNA is mostly influenced by its 5′-non-coding region, and enhanced translation has been obtained with chimeric RNAs containing heterologous 5′-non-coding sequences (Jobling and Gehrke, 1987; Gallie *et al.*, 1987); therefore, the 5′-non-coding region from the RB cDNA might be partly responsible for the low yield of full length polypeptides synthesized *in vitro*.

Consequently, chimeric RB cDNAs were constructed using the 5′-non-coding sequences from two efficiently translated mRNAs: AMV RNA4 and β-globin mRNA. The 5′-non-coding sequence of RB cDNA was replaced with that of AMV RNA4 (pA9), with the chimeric RB cDNA under T7 promoter control (Figure 1b). Construct pB10 has, downstream of the SP6 promoter, a chimeric RB cDNA containing the 5′-non-coding sequence of β-globin mRNA (Figure 1c). When the same amount of linearized plasmid DNA templates from the three constructs pGEM-Y79N/*Bam*HI, pA9/*Hin*dIII and pB10/*Bam*HI were transcribed, and the resulting transcripts were translated in the reticulocyte lysate, both modified constructs produced more full length proteins than the original construct (Figure 2, lanes 2, 3 and 4). Quantitation of radioactivity in the full length protein band

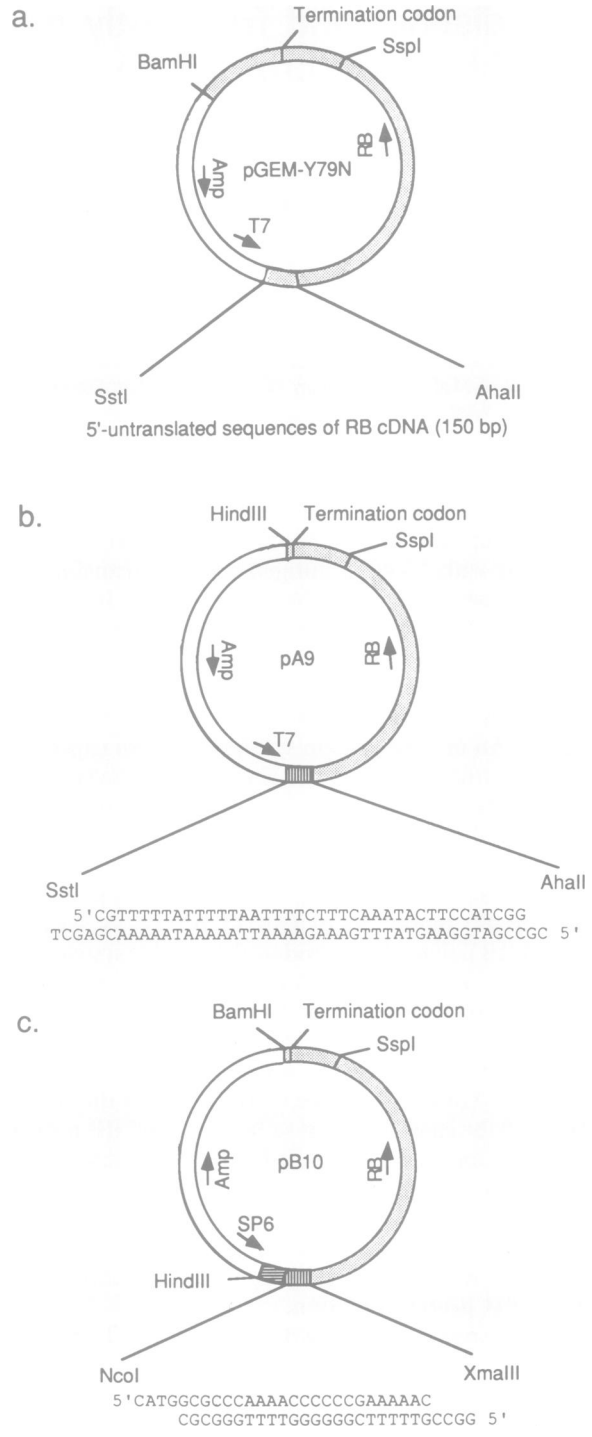

Fig. 1. Restriction maps of plasmids containing RB cDNA or chimeric RB cDNAs. Symbols: vector sequences are shown as open bars □; the RB cDNA fragment is shown as shaded bars ▨; the oligomers used for construction (Materials and methods) are represented as vertically lined bars ▥; the 5′-non-coding region of β-globin mRNA is represented as horizontally lined bars ▤. (**a**) Restriction map of plasmid pGEM-Y79N. (**b**) Restriction map of plasmid pA9. (**c**) Restriction map of plasmid pB10.

showed the relative enhancement in translation was 10-fold for pA9 and 5-fold for pB10 (data not shown). Immunoprecipitation with the anti-trpE-RB fusion protein antibody 0.47 (Materials and methods) confirmed that the polypeptides of 110 kd were RB proteins (Figure 2, lanes 8, 9 and 10). Some of the truncated polypeptides, such as that of 56 kd,

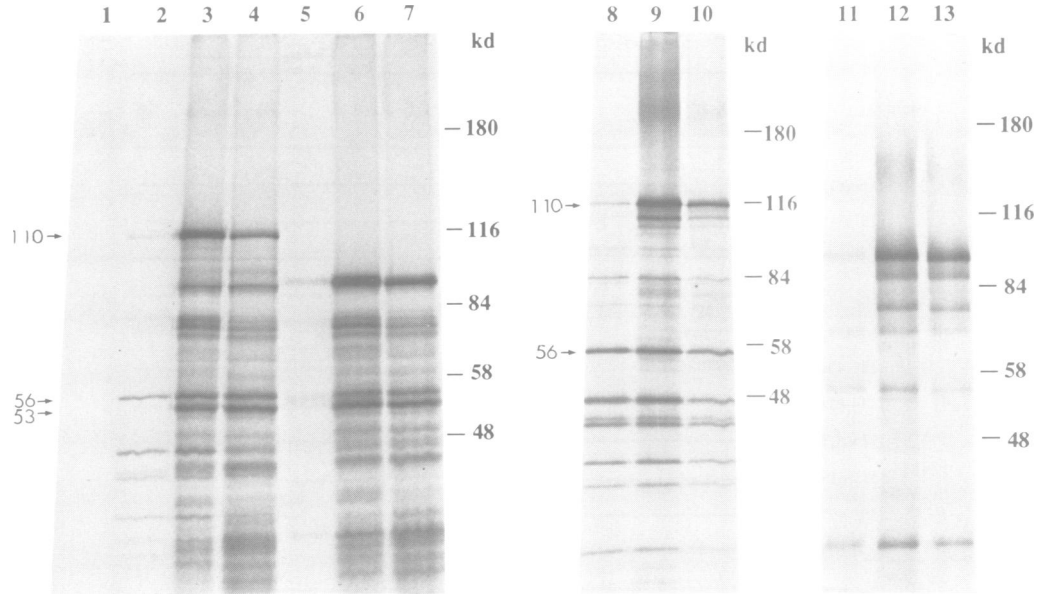

Fig. 2. Comparison of translational efficiency of RB cDNA transcripts versus chimeric RB transcripts containing 5′-non-coding sequences of AMV RNA4 or β-globin mRNA. Linearized plasmid DNA was transcribed by either T7 or SP6 RNA polymerase and the resulting capped-transcripts were translated in reticulocyte lysates. ^{35}S-labeled translation products were either directly analyzed by 10% SDS−PAGE or analyzed after immunoprecipitation by anti-trpE-RB fusion antibodies 0.47 or 0.495. **Lanes 1−7**: direct analysis of translation products. Lane 1, no RNA; lanes 2 and 5, RNAs from construct pGEM-Y79N digested with *Bam*HI and *Ssp*I, respectively; lanes 3 and 6, RNAs from construct pA9/*Hin*dIII and pA9/*Ssp*I, respectively; lanes 4 and 7, RNAs from construct pB10/*Bam*HI and pB10/*Ssp*I, respectively. **Lanes 8−13**: analysis of immunoprecipitated translation products. Lanes 8, 9 and 10 show immunoprecipitated polypeptides by antibody 0.47 of translation products from transcripts of pGEM-Y79N/*Bam*HI, pA9/*Hin*dIII and PB10/*Bam*HI, respectively; lanes 11, 12 and 13 show immunoprecipitated polypeptides by antibody 0.495 of translation products from transcripts of *Ssp*I digested pGEM-Y79N, pA9 and pB10, respectively. Mol. wt markers are shown on the right side of each autoradiogram. Numbers with arrows on the left represent mol. wts of the full length and truncated polypeptides mentioned in the text.

were also precipitated, indicating that they were translated from internal methionines because antibody 0.47 only recognized the carboxyl-terminal sequences within exons 23−27 of RB. We also noticed some premature termination events by the modified transcripts resulting in the truncated polypeptides (e.g. 53 kd) which were not precipitated by antibody 0.47. Despite the drawback of having some premature termination, modified chimeric transcripts increased the yield of full length polypeptides significantly.

To further demonstrate that the effect was due to 5′-non-coding sequences rather than 3′-non-coding sequences, *Ssp*I-linearized plasmids DNA were transcribed to generate truncated RB transcripts with the same 3′ end sequences among all three constructs (Figure 1). Translation of these truncated transcripts produced the expected polypeptides of 90 kd that could be immunoprecipitated by the anti-trpE-RB fusion antibody 0.495 against sequences encoded by exons 19−22 of RB (Figure 2, lanes 5−7 and 11−13). In keeping with the above-described results, transcripts from constructs pB10/*Ssp*I and pA9/*Ssp*I were translated 5- to 10-fold better than that from pGEM-Y79N/*Ssp*I. Thus, by simply modifying the 5′-non-coding sequences of RB transcripts, it was possible to produce more full length RB proteins *in vitro*. This facilitated our mapping study, and should provide a useful system for other studies using RB proteins translated *in vitro*.

RB protein synthesized in vitro forms complexes with T

Truncated RB proteins synthesized in the reticulocyte lysate have been shown to bind to adenoviral E1A proteins (Dyson *et al.*, 1989a). To examine whether full length

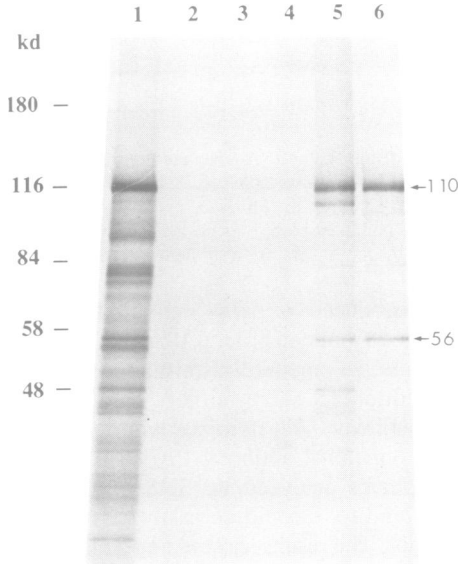

Fig. 3. RB polypeptides synthesized *in vitro* binds to SV40 large T antigen. T7 transcripts from construct pA9/*Hin*dIII were translated in reticulocyte lysates. The ^{35}S-labeled translation mixture was loaded onto 10% SDS−PAGE after treatment with one of the following: **lane 1**, no treatment; **lane 2**, lysate was immunoprecipitated with normal rabbit antiserum; **lane 3**, lysate was immunoprecipitated with anti-T monoclonal antibody PAB419; **lane 4**, lysate was mixed with purified T and precipitated with protein A−Sepharose without addition of PAB419; **lane 5**, lysate was immunoprecipitated with antibody 0.47; **lane 6**, lysate was mixed with T followed by PAB419 immunoprecipitation. Numbers with arrows on the right represent the two major polypeptides co-precipitated by PAB419.

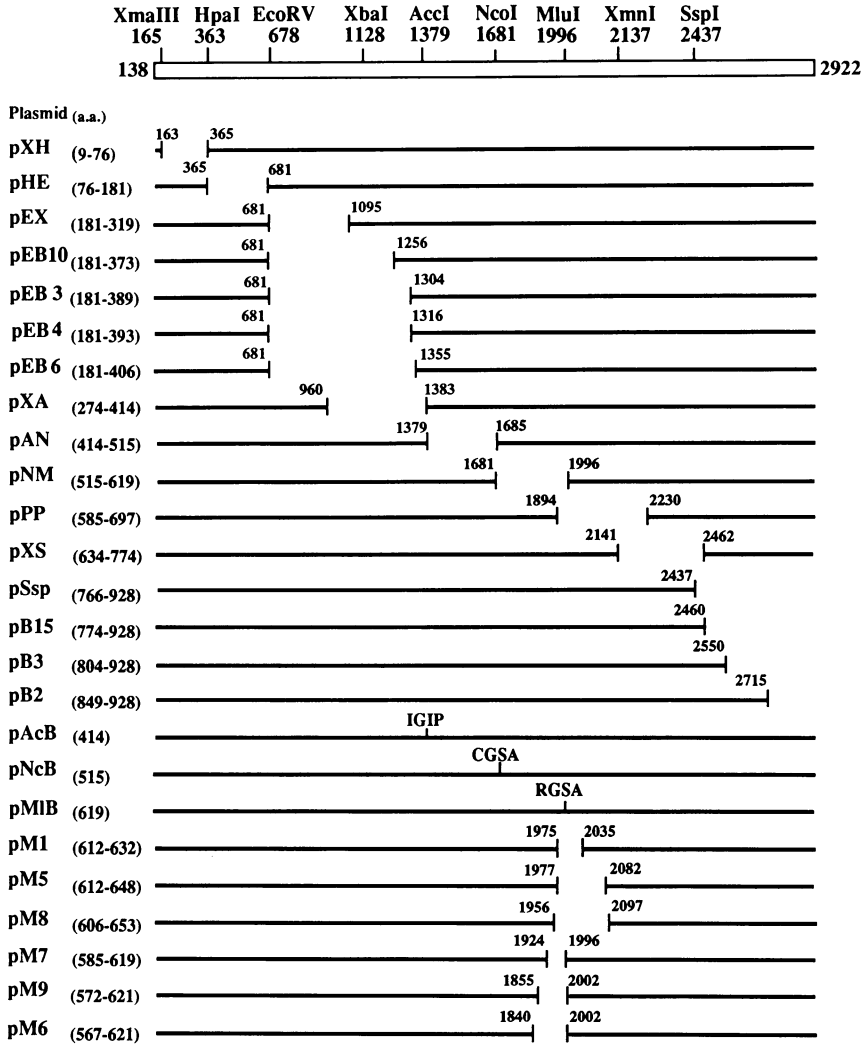

Fig. 4. Structure of RB mutants. A schematic representation of RB cDNA coding sequence as open bar is shown on top of the figure. The various unique restriction sites are also indicated. Schematic representation of each of the RB mutants is shown as straight black lines. Each mutant is given a plasmid name as listed on the left side. Numbers beside each name refer to the deleted amino acid residues or the position of the residue where a linker is inserted. Numbers placed above the schematic representations refer to the nucleotides at the boundaries of the deletions. Amino acid residues inserted by the linkers are also shown above the representations. No amino acid substitutions occurred with each of the deletion mutants.

RB proteins produced *in vitro* can bind T, translation products derived from construct pA9/*Hin*dIII were assayed for T binding. Equal amounts of translation mixture labeled with [^{35}S]methionine were either immunoprecipitated with anti-RB antibody 0.47, or mixed with T and precipitated with anti-T monoclonal antibody PAB419. By comparing immunoprecipitates analyzed by SDS−PAGE, the polypeptides that bind to T could be easily distinguished from those that do not. The results showed that addition of purified T to the translation mix resulted in the co-precipitation of full length plus some truncated forms of RB protein by antibody PAB419 (Figure 3). The fact that internally initiated polypeptides, such as that of 56 kd, could also bind T indicated that the amino-terminal sequences are dispensable for T binding (see below). The band right underneath the p110 band (Figure 3, lane 5) was due to protein degradation during the process of immunoprecipitation as it was not present in the translation mixture (Figure 3, lane 1). These results suggested that RB proteins synthesized *in vitro* can bind to T and this assay system can be used to map the regions of RB involved in T binding.

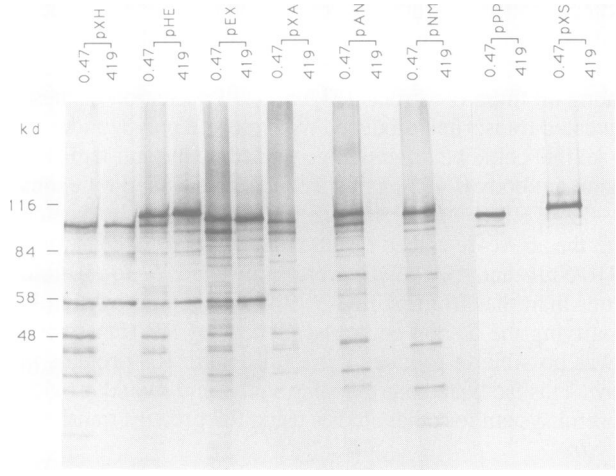

Fig. 5. Analysis of large internal deletion mutants. Immunoprecipitations of ^{35}S-labeled translation products of large internal deletion mutants were analyzed on 10% SDS−PAGE. The mol. wt markers are shown on the left. The mutated RB polypeptides were precipitated either by anti-RB antibody 0.47 or by anti-T antibody 419 (PAB419) after mixing with purified T.

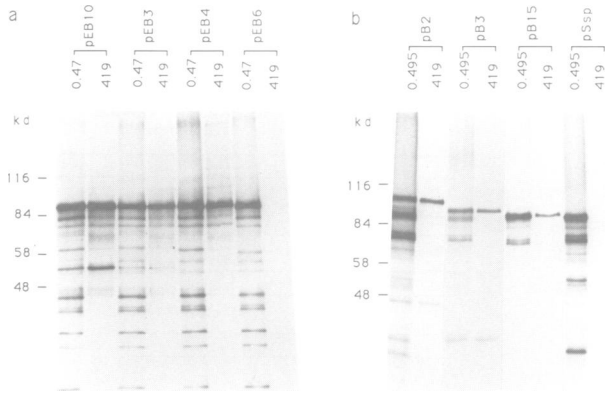

Fig. 6. Analysis of amino-terminal and carboxyl-terminal deletion mutants. *In vitro* synthesized, ^{35}S-labeled mutant RB polypeptides lacking the amino-terminal region (**a**) or carboxyl-terminal sequences (**b**) were immunoprecipitated either by anti-RB antibody 0.47 (a) or 0.495 (b) or by anti-T monoclonal antibody 419 (PAB419) after incubation with purified T. The immunoprecipitated polypeptides were run on 10% SDS−PAGE. Numbers on the left represent the mol. wt of standard size markers.

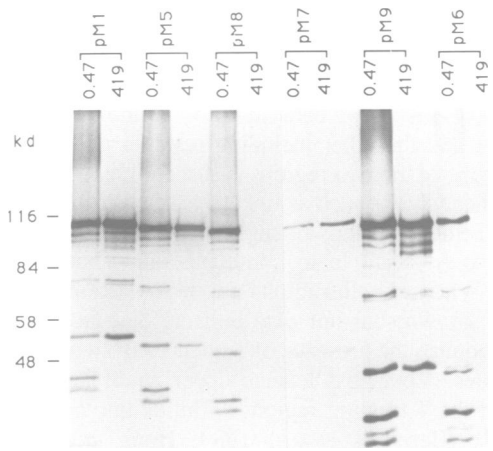

Fig. 7. Immunoprecipitations of ^{35}S-labeled RB polypeptides synthesized from small internal deletion mutants (the pM set of mutants). Immunoprecipitations of ^{35}S-labeled RB polypeptides translated from the pM set of mutants were run on 10% SDS−PAGE. The mutant RB proteins were immunoprecipitated either with antibody 0.47 or with antibody 419 (PAB419) in the presence of purified T antigens. Numbers on the left represent the mol. wts of standard size markers.

Mapping of the regions of RB protein required for binding to T

A series of deletion and linker insertion mutants of RB were derived from constructs pA9 and pB10 as described in Materials and methods and reported in Figure 4. These mutant RB polypeptides could all be immunoprecipitated by anti-RB antibodies (Figures 5−7). Each of the antibodies, either 0.47 or 0.495, bound to those mutant polypeptides containing its recognition epitopes with similar efficiency (results not shown). These mutated RB polypeptides appeared to have the predicted size on SDS−PAGE except for the one truncated at its amino-terminus (Figure 5, mutant pXH). Lack of an unusual amino-terminal poly-alanine and poly-proline sequence (Lee *et al.*, 1987a) may explain its apparent faster mobility.

To locate the regions of the RB polypeptide involved in binding to T, a set of relatively large internal deletion mutants were initially examined. The amino-terminal mutations deleting amino acid residues 9−319 did not affect T binding (Figure 5, mutants pXH, pHE and pEX), consistent with the aforementioned observation that internally initiated RB polypeptides could bind T. However, several large internal deletions including amino acid residues 319−774 eliminated binding to T (Figure 5, mutants pXA, pAN, pNM, pPP and pXS). These results suggested that one or more regions of the RB polypeptide between residues 319 and 774 were probably needed for association with T.

The amino-terminal boundary of this putative T binding region was necessarily located between residues 319 and 414 (Figure 5, mutants pEX and pXA). The smallest internally initiated polypeptide that could still bind T was ~56 kd (Figure 3, lane 6), and was therefore most likely initiated from one of three methionines at residues 379, 386 and 387. Taken together, these results suggested that the amino-terminal boundary was located within residues 379−414. To further define this boundary, a set of mutants was examined that contained small deletions of the sequences around these methionine residues (Figure 6a). Deletions extending to amino acid residue 393 did not affect T binding (Figure 6a, mutant pEB4). Therefore amino acid 394 appeared to represent the amino-terminal boundary of the sequences required for binding to T.

To address the question of where the carboxyl-terminal boundary was located, a truncated RB polypeptide ending at residue 766 was conveniently synthesized from construct pA9/*Ssp*I, and it failed to bind to T (Figure 6, mutant pSsp). This result led us to construct a set of *Bal*31 deletions starting from the extreme carboxyl-terminus. Two such deletion mutants retained T binding activity (Figure 6b, mutants pB2 and pB3) including one in which half of exon 23 was removed (Figure 6b, mutant pB3). Exon 23 has two striking features: it is proline rich (26% proline residues, Lee *et al.*, 1987a), and it is very highly conserved among human and murine RB proteins (Lee *et al.*, 1987a; Bernards *et al.*, 1989). Therefore, we assumed that the region encoded by exon 23 functioned as a single domain, and constructed a mutant (pB15) with deletion of exon 23 to the carboxyl-terminus (see Materials and methods). Its truncated RB polypeptide could still bind to T albeit less efficiently, indicating that some exon 23 sequences might participate in, but were not essential for, binding to T (Figure 6b, mutant pB15).

Results presented above defined a large region of the RB polypeptide, from amino acid residues 394−773, that was necessary for binding to T. Since the internal deletions examined so far were relatively large, it was possible that some short sequences within this region were actually unnecessary for binding to T. To test for the existence of such sequences, linker scanning was performed. One linker inserted at amino acid residue 619 did not affect T binding (data not shown). A set of mutants with small deletions around residue 619 were subsequently constructed. Analysis of T binding activities of their encoded polypeptides demonstrated that a 'gap' region between residues 572 and 648 (Figure 7, mutant pM9 and pM5) did indeed exist that was not required for T binding.

Discussion

This study has defined two non-contiguous regions of RB polypeptide that are both required for binding to SV40 T antigen *in vitro*, one consisting of amino acid residues 394−571 and the other including residues 649−773. The fact that one of the two regions is susceptible to inactivation by minor mutations such as linker insertions (mutants pAcB and pNcB, data not shown) suggests that T−RB interaction may be very sensitive to conformational changes in RB protein. Whether both regions are in physical contact with T is not known, but our data indicate that the interaction with T requires the presence of sequences from each of these two regions. A potential 'leucine zipper' motif has been noted in one of the T binding regions within amino acid residues 661−691 (Bernards *et al.*, 1989; Hong *et al.*, 1989). However, we consider it unlikely that the mechanism proposed for interaction between 'leucine zipper'-containing DNA-binding proteins would operate in T−RB interaction, because T lacks the obligatory 'leucine zipper' for 'zipping' with RB (Landschulz *et al.*, 1988).

It has been shown that T binds only to the underphosphorylated form of RB (Ludlow *et al.*, 1989; Dyson *et al.*, 1989a). Phosphorylation is considered an important modulator of RB function because RB phosphorylation oscillates during the cell cycle (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989). Preliminary data has indicated that RB protein might be a substrate of the human cdc2 kinase homolog (Lin *et al.*, unpublished). Indeed, eight consensus cdc2 phosphorylation sites are found scattered in the RB polypeptide chain (Shenoy *et al.*, 1989). However, none of them is located in the T binding regions. These observations, taken together with the exclusive binding of T to the underphosphorylated RB, suggest that T−RB complex formation could be perturbed by the phosphorylation-induced conformational alteration of RB protein. Alternatively, other kinases could phosphorylate RB within the T binding regions and thus exert a more direct effect on T−RB interaction.

This study indicated that transcripts derived from RB cDNA were poorly translated *in vitro* with few full-length polypeptides synthesized. This was apparently due to the 5′-non-coding region of RB transcripts because improved translational efficiency could be obtained with chimeric RB transcripts containing heterologous 5′-non-coding sequences. Since the 5′ end non-coding sequence of our RB cDNA clone is not complete (Lee *et al.*, 1987b), the transcript derived from this clone contained only 150 nucleotides of 5′ non-coding sequences, which was ~120 nucleotides shorter than that of the major RB mRNA species detected *in vivo* (Hong *et al.*, 1989). It is possible that full length RB mRNA could be translated more efficiently. While we have not tested this possibility either *in vivo* or *in vitro*, we consider it unlikely. The 5′ non-coding sequence of RB mRNA is highly GC rich (Hong *et al.*, 1989) which favors formation of stable secondary structure. Such structure, when present in front of the initiation codon, is thought to decrease translational efficiency (Pelletier and Sonenberg, 1985; Lawson *et al.*, 1986). Therefore RB mRNA is not likely to be translated efficiently. However, a firm conclusion may have to await the analysis of the polysomal profile of RB mRNA.

SV40 T antigen is a well-studied transforming oncoprotein. The region of the T polypeptide required for transformation coincides with the region involved in binding to RB (DeCaprio *et al.*, 1988). It has been suggested that T exerts its transforming action by altering the activities of RB and other potential tumor suppressor gene products through complex formation (Dyson *et al.*, 1989b; Ewen *et al.*, 1989). It is likely that the most efficient way for T to affect RB activity through physical contact is to interact with functional regions of RB protein. By so doing, T could conceivably compete with cellular components that normally interact with RB, and thus mask RB protein from functioning normally.

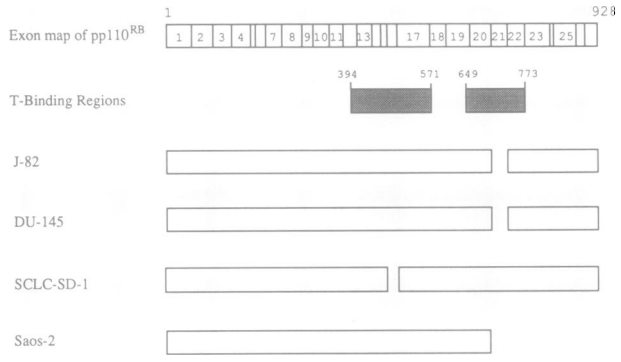

Fig. 8. Structure of mutated RB polypeptides detected in several human tumor cell lines. A schematic representation of RB protein consisting of 27 exons is shown above representations of mutated RB protein. RB polypeptide chains are shown as open bars, and gaps in between represent the deleted regions. Shaded bars represent the regions of RB required for T binding as defined *in vitro*, and the numbers above the bars represent amino acid residues located at the boundaries.

Therefore, the regions of RB polypeptide involved in binding to T may be the functional domains of RB. This is consistent with the observation that all the mutated, and presumably inactive, RB polypeptides detected so far in human tumor cell lines were unable to bind to T. For example, bladder carcinoma cell line J-82 had 'in-frame' deletion of exon 21 sequences, and the resulting mutated polypeptide failed to bind both T and adenoviral E1A proteins (Horowitz *et al.*, 1989; Shew *et al.*, unpublished). Prostate carcinoma cell line DU145 also expressed an exon 21 'in-frame' deleted 107 kd polypeptide that failed to bind to T (Bookstein *et al.*, 1990; Shew *et al.*, unpublished). SCLC-SD-1, a small cell lung carcinoma cell line, expressed a mutated RB polypeptide of 108 kd that had an 'in-frame' deletion of part of exon 15 plus all exon 16 sequences and was unable to bind to T (Shew *et al.*, 1990a). One osteosarcoma cell line, Saos-2, expressed a shortened 95 kd RB protein lacking sequences from exons 21−27 and unable to bind to T (Shew *et al.*, 1990b). Interestingly, the defects in these polypeptides, apparently abolishing T binding *in vivo*, all occurred in the regions defined *in vitro* as necessary for binding to T (Figure 8). Thus the regions of RB involved in binding to T *in vivo* appeared to coincide with the regions needed *in vitro*. While a fine mapping study *in vivo* has yet to be done, we suspect that the results would be similar to the data obtained *in vitro*.

The RB gene is conceived to be a tumor suppressor gene (for reviews, see Sager, 1986; Klein, 1987). Consistent with this notion, many tumor cell lines do not express, or express mutated, RB gene products. That mutated RB protein is

inactive in tumor suppression has been shown in the case of prostate carcinoma DU145 cells, in which expression of exogenous RB protein leads to loss of tumorigenicity in nude mice (Bookstein *et al.*, 1990). The fact that the abnormal RB polypeptide also had altered T binding regions suggests that these regions might play a role in the tumor suppression function of RB. It would be very interesting to see whether sequences involved in such functions of RB coincide with the regions required for binding to T. Experiments are now in progress to examine the tumor suppression activities of mutated RB genes by expressing them in tumor cell lines.

Materials and methods

In vitro transcription–translation and construction of chimeric RB RNA

The reagents for *in vitro* transcription–translation were from Promega Biotech. The unmodified RB transcript was synthesized from *Bam*HI linearized pGEM-Y79N (constructed by F.Hong), a plasmid containing the 4.7 kb RB cDNA (Lee *et al.*, 1987b) inserted in the vector pGEM-1.

To construct plasmid pA9 a double-stranded oligomer of 45 bp with *Sst*I and *Aha*II cohesive ends was synthesized using a DNA Assembler (Pharmacia). The sequence of the oligomer is from the 5′-non-coding region of the AMV RNA4 as shown in Figure 1. An *Aha*II–*Hin*dIII fragment of RB cDNA was derived from pGEM44-1, a plasmid containing the RB cDNA coding region inserted in the *Bam*HI–*Hin*dIII sites of the vector pGEM-1. These two DNA fragments were ligated into the *Sst*I and *Hin*dIII sites of the vector pGEM-1 to form the plasmid pA9 containing the chimeric RB cDNA downstream from the T7 promoter. The T7 run-off transcription was performed with *Hin*dIII linearized pA9.

To construct plasmid pB10, an *Nco*I–*Bam*HI fragment containing the pSP64 vector and the 5′-non-coding region of β-globin mRNA was derived from pSP64-xβm. This plasmid was obtained from Dr D.Melton at Harvard, and contains a full length *Xenopus* β-globin cDNA inserted downstream of the SP6 promoter in the *Hin*dIII–*Bam*HI sites of the vector pSP64 (Krieg and Melton, 1984). An *Xma*III–*Bam*HI fragment of RB cDNA was derived from pGEM44-2, a plasmid containing the RB cDNA coding region inserted into the *Bam*HI site of the vector pGEM-1. These two DNA fragments were then ligated together with a 27 bp oligomer with the cohesive ends of *Nco*I and *Xma*III. The sequence of the oligomer is from the initiation codon to the *Xma*III site in the RB cDNA as shown in Figure 1. The chimeric RNA was transcribed from *Bam*-linearized pB10.

Construction of mutants

Plasmid pA9 was used to derive most of the RB mutants described below. The reading frames of all mutants were confirmed by double-stranded DNA sequencing using Sequenase (US Biochemicals) and various oligo primers (synthesized with DNA Assembler from Pharmacia) covering every exon of RB cDNA. No amino acid substitutions occurred in each mutant. For internal deletions the basic strategy was to take advantage of a number of unique restriction sites located evenly in the RB cDNA as shown in Figure 4. The fragment to be deleted was cut out by two enzymes, the ends of the remaining fragment were modified by nuclease before ligation in order to obtain 'in-frame' deletion mutants. The nucleases used here were *Bal*31, Mung Bean Nuclease and Klenow fragment, and the protocols were as described (Maniatis *et al.*, 1982). Mutants constructed by this procedure include pXh, pHE, pAN and pNM.

Mutant pEX was derived from ligation of the 5 kb *Eco*RV–*Acc*I fragment and a fragment amplified by the polymerase chain reaction (PCR) from nucleotide 1095 to the *Acc*I site. PCR was carried out as described (Saiki *et al.*, 1988). To construct mutant pXA, pA9 was linearized with *Acc*I, treated with *Bal*31, deleting ~400 bp each end, and was digested with *Sst*I. The shorter fragment of ~1 kb was isolated and ligated with the 4.5 kb *Sst*I–*Acc*I fragment derived from pA9. Mutant pPP was from the self-ligated 5.3 kb *Pst*I fragment from pA9 with *Bal*31 blunted ends. Mutant pXS was derived from three piece ligation of fragments derived from pA9: the 3.8 kb *Xmn*I fragment, the 1.1 kb *Hin*dIII–*Xmn*I fragment and the fragment of ~0.40 kb isolated from the *Hin*dIII digestion of the *Bal*31-treated 1.80 kb *Xmn*I fragment (~0.3 kb deleted each end after *Bal*31 treatment).

To construct the pEB set of mutants, *Eco*RI (cut at nucleotide 1037)-digested pA9 was treated with *Bal*31 to delete ~300 bp each end, and was then cut with *Mlu*I. The shorter fragment of ~700 bp was isolated, and ligated to the 4.5 kb *Eco*RV–*Mlu*I fragment derived from pA9.

The carboxyl-terminal deletion mutants pB2 and pB3 were derived from the *Hin*dIII linearized pA9, which was religated after treatment with *Bal*31, deleting 150–400 bp each end and addition of an eight nucleotide *Bam*HI linker. The plasmids were linearized with *Bam*HI for *in vitro* transcription. The pSsp mutant was obtained simply from run off transcription of *Ssp*I digested pA9. pB15 was the only mutant derived from pB10 for the reason of convenience. A 4.8 kb *Mlu*I–*Sma*I fragment was isolated from pB10, and was ligated with a *Mlu*I-digested PCR fragment amplified from primers RB15 and RB17 with RB cDNA as the template. RB15 primer was from nucleotides 2460–2442. RB 17 primer was from nucleotides 1840–1860. The region of plasmid pB15 derived from the PCR fragment was verified by DNA sequencing. SP6 transcription was carried out with *Sst*I-linearized pB15.

Mutants pM1, 5 and 8 were from religation of *Mlu*I-linearized pA9 after *Bal*31 treatment deleting 20–100 bp each end. To construct mutants pM6, 7 and 9, plasmid pA9 was cut with *Mlu*I, treated with *Bal*31 to remove a few nucleotides and then cut with *Sst*I. The larger fragment of ~3.8 kb was isolated. To make the other fragment, the *Mlu*I-linearized pA9 was treated with *Bal*31 for a longer time to remove 50–150 nucleotides each end, and was then cut with *Sst*I. The shorter fragment of ~1.7 kb was isolated and ligated with the aforementioned 3.8 kb fragment.

For linker insertion mutants pNcB and pMlB, an eight nucleotide *Bam*HI linker was ligated with Klenow-treated, *Nco*I- or *Mlu*I-linearized pA9. For pAcB, a 10 nucleotide *Bam*HI linker was ligated with Klenow-treated, *Acc*I-linearized pA9.

Large T antigen binding and immunoprecipitation assays

The large T antigen was purified by an affinity column procedure described previously (Simanis and Lane, 1985). The anti-T monoclonal antibody PAB419 was from Oncogene Inc. The complex formation assay was as described previously (DeCarpio *et al.*, 1988) with minor modifications. Briefly, 10 μl of translation lysate was mixed with 500 μl of EBC buffer (50 mM Tris–HCl, pH 8.0, 120 mM NaCl, 0.5% NP-40) containing 200 U/ml of aprotinin. Purified T (150–200 ng) was added to the mix and the mixture was incubated on ice for 90 min. A 20 μl aliquot of anti-T antibody (100 μg/ml) was then added and the mixture was further incubated on ice for 1 h. Then, 80 μl of a 1:1 mixture of freshly washed and suspended protein A–Sepharose (Pharmacia) in TBS–BSA (25 mM Tris–HCl, pH 8.0, 120 mM NaCl) containing 10% bovine serum albumin (Miles Laboratories) was added. The mixture was rocked at 4°C for 40 min. The protein A–Sepharose beads were then washed 5 times with 1 ml of NET-N (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) at 4°C. The beads were suspended in SDS gel sample buffer and SDS–PAGE was followed by fluorography at −70°C after impregnation of the gel with acetic-acid-based 2,5-diphenyloxazole.

In vitro translated proteins were immunoprecipitated by two antibodies: one of them, 0.495, against the trpE-RB fusion protein consisting of sequences from exons 19–22; the other, 0.47, against the trpE-RB fusion protein containing sequences from exons 23–27 of RB. Rabbit anti-RB IgG was prepared as described (Lee *et al.*, 1987b). A 10 μl aliquot of translation mixture was resuspended in 0.5 ml EBC buffer, and mixed with 10–20 μl of antibodies (100–200 μg/ml) on ice for 1 h. Then, 80 μl of protein A–Sepharose beads in TBS–BSA was added and the mixure was rocked at 4°C for 1 h. The beads were subsequently washed with lysis buffer (25 mM Tris–HCl, pH 7.4, 50 mM NaCl, 0.2% NP-40, 0.5% deoxycholate, 200 U/ml of aprotinin, 0.02% SDS), then with 1 M NaCl in lysis buffer, then with 0.15 M NaCl in lysis buffer and, finally, with lysis buffer. The immunoprecipitated proteins were analyzed by 10% SDS–PAGE and fluorography. For quantitation of radioactivity, the bands of interest were then cut out of the gel and dissolved in Tissue Solubilizer (Amersham), 10% SDS (9:1, v/v) for 10 h at 62°C. After additional shaking for 48 h at 37°C, 9 volumes of Aquasol were added and radioactivity was measured by liquid scintillation counting.

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