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Retinoblastoma-repression of E2F-dependent transcription depends on the ability of the retinoblastoma protein to interact with E2F and is abrogated by the adenovirus E1A oncoprotein

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ABSTRACT

The product of the retinoblastoma tumor suppressor gene interacts with the transcription factor E2F. Two distinct types of interactions can be detected between the retinoblastoma gene product (Rb) and E2F. The first type involves an Rb-binding protein, RBP60. The Rb/E2F complex formed in the presence of RBP60 is able to bind DNA and migrates with a distinct mobility in gel retardation assays. The second type of Rb/E2F complex is seen in the absence of RBP60. This second type of Rb/E2F complex does not form a band-shift complex in gel retardation assays and its formation results in an apparent inhibition or loss of the DNA binding activity of E2F. Using a series of Rb-mutants we show that these two types of Rb/E2F complexes depend on common domains of the Rb protein. The T/E1A-binding region as well as the carboxyl-terminus of the Rb protein are critical for these two types of Rb/E2F interactions. We also show that the retinoblastoma protein represses the E2F-dependent transcription, and this Rb-repression of the E2F-dependent transcription depends on the ability of Rb to interact with E2F. Moreover, the adenovirus E1A gene product, which binds Rb, counteracts the Rb-repression and restores E2F-dependent transcription.

INTRODUCTION

The product of the retinoblastoma tumor suppressor gene (Rb) binds to several DNA tumor virus oncoproteins including E1A, T antigen and E7 (1–7). The ability to interact with the Rb protein plays a key role in the tumorigenic potential of these viral oncoproteins. Mutations in the E1A and the T antigen genes that impair the ability of E1A and T antigen to bind Rb also significantly reduce their transforming ability (2,7). Similarly, it has also been shown that all of the naturally occurring loss-of-function mutations of the retinoblastoma gene, which produce tumors, cluster in the region of the Rb protein that is important for binding to T antigen and E1A (8,9). These observation suggest that the T/E1A binding region is critical for the tumor suppression function of Rb. The amino acid sequences of the Rb protein that are involved in binding to T antigen and E1A have been defined (8,9) and are recognized as the 'pocket region' of Rb protein. One of the major interests is to understand the normal cellular function of the pocket region of Rb.

Several lines of evidence now indicate that the Rb protein is a regulator of the cellular transcription machinery and influences transcription of several cellular as well as viral genes. Transcription from the adenovirus E2 promoter (10) and from the c-fos promoter (11) are inhibited by the Rb protein. Transforming growth factor (TGF)-β promoter is regulated by Rb in a cell type dependent manner (12). Rb inhibits transcription from the TGF-β promoter in NIH-3T3 and AKR-2B mouse cell line but stimulates expression from this promoter in CCL-64 mink lung epithelial cells and A-549 human lung adenocarcinoma cells. These experiments with the TGF-β promoter indicate that depending on the cellular environment Rb can influence the cellular transcription machinery in multiple ways.

The retinoblastoma protein has been shown to interact with the transcription factor E2F (13–18) and can be found in complex with this transcription factor in the extracts of U937 promonocytes (16,18) and human T cells (18). Interest on Rb/E2F interaction also arises from the fact that E2F is involved in the transcription of several proliferation associated genes including c-myc (19) and dihydrofolate reductase (20). Thus, it is quite likely, that Rb would regulate expression of these cell-proliferation genes by inactivating E2F-function. Our recent work, using in vitro binding assays, indicates that Rb forms two types of complexes with E2F (21). One of these two types involves a binary complex of Rb and E2F that does not bind DNA in a gel retardation assay, and the second type of Rb/E2F complex involves another factor RBP60 which allows Rb/E2F complex to bind DNA and produce a distinct complex in gel retadation assay. Although significance of these two types of complexes is not clear, one attractive

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possibility is that Rb might be regulating the DNA-binding as well as the transcription activation function of E2F.

To investigate a link between the transcription regulatory and tumor suppression functions of Rb, we have analyzed several mutants of Rb in transient transfection experiments. Clearly, while the wild type Rb interacts with E2F and inhibits E2F-dependent transcription, the loss-of-function mutants are unable to bind E2F and inhibit the E2F-dependent transcription. Moreover, we also see a requirement for the carboxyl-terminus of the Rb protein in the Rb/E2F interaction as well as in the regulation of E2F-dependent transcription.

METHODS

Cells and extracts

HeLa cells were grown in spinner culture using S-MEM (GIBCO, BRL) and 5% calf serum. Extracts were prepared following a previously described procedure (22). Monolayer cultures of HeLa cells and NIH-3T3 cells were maintained in Dulbecco modified Eagle media containing 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml).

Glutathione-S-transferase (GST)-Rb fusion proteins

pGEX-2T clones containing Rb-cDNA and various mutants at the BamH1 site were obtained from Bill Kaelin and D.M.Livingston (Dana Farber Cancer Institute). The clones were transformed in to E.coli DH5α. The induction of GST-Rb fusion protein production and the purification of GST-Rb proteins were carried out following a previously published procedure (9). The purified preparations were analyzed in SDS-gels followed by staining with Coomassie brilliant blue and the concentration of Rb protein was estimated by comparing intensities of bands with known standards.

Plasmids

To obtain the eukaryotic expression vectors for Rb, the RB sequences from the various pGEX-2T constructs were recovered by PCR. GGGAAGCTTAGCAGGTATGATCCAACAATTA-ATGATGATT was used as the upstream primer and GGCGG-ATCCTCATTTCTCTTCCTTGTTTGAGGTATCCAT was used as the downstream primer. For the construct pBC-RB(379−792), the downstream primer was GGCGGATCCT-CAAGGGCTTCGAGGAATGTGAGG. The PCR products were digested with Hind III and Bam H1 and cloned into the Hind III/Bam H1 sites of pBC-12SE1A (23,24) from which the 12SE1A sequences were removed by Hind III/Bam H1 digestion. The plasmid pFCΔ94/53 was described by Fisch et al. (25). To obtain pFCΔ94/53/E2F, an oligonucleotide corresponding to sequences between −30 and −70 of the adenovirus E2 promoter

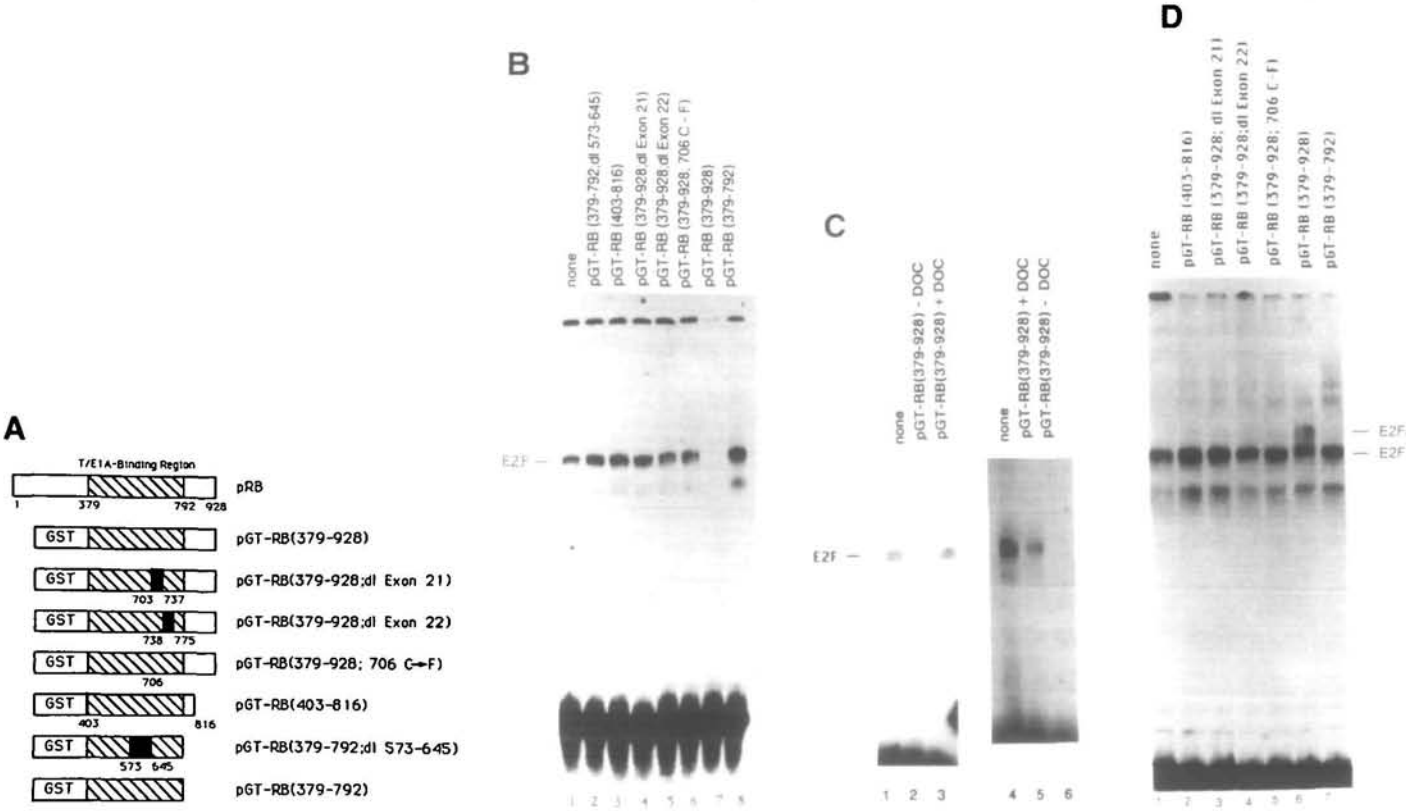

Figure 1. The two types of Rb/E2F interactions depend on common domains of the retinoblastoma protein. **A.** Structure of the GST-Rb fusion proteins (for details see ref.9). The hatched region indicates the T antigen/E1A-binding region, and deletions within this region are indicated by black box. **B.** 10 ng of the indicated GST-Rb fusion proteins were mixed with purified E2F (approximately, 5 ng protein) in the presence of components required for the sequence specific DNA binding of E2F (Methods) in a total reaction volume of 30 ul. At the end of a 30 min. incubation at 25°C, aliquots of 7 μl were analyzed by gel retardation assays as described before (28). The faster migrating band represents partially proteolyzed E2F. **C.** Affinity purified E2F was incubated alone (lanes 1 and 4) or the indicated GST-Rb fusion protein (lanes 2, 3, 5 and 6) at 4° for 20 min. At the end of this incubation, sodium deoxycholate was added to reaction mixtures in lanes 3 and 5 to a final concentration of 0.9% and the incubation was continued for another 20 min followed by addition of NP40 (final concentration 1%) to all of the reaction mixtures. Equivalent aliquots were assayed for E2F DNA binding activity as before. **D.** Heparin agarose purified HeLa cell E2F (2 μg) were incubated with the indicated GST-Rb fusion proteins (10 ng) in the presence of components required for the DNA binding of E2F. After an incubation for 30 min. aliquots of the reaction mixtures were analyzed in gel retardation assays (as described in Methods). E2F indicates E2F-DNA complex and E2Fr indicates Rb/E2F-DNA complex.

was cloned into the Bgl II site of the plasmid pFCΔ94/53. The plasmid pFCΔ94/53/E2F contains two E2F binding sites at position −53, and that was confirmed by sequencing.

Transfections

DNA transfections for transient expression assays were performed by calcium phosphate coprecipitation method (26). HeLa cells or NIH-3T3 cells were plated and grown to 50% confluence in Dulbecco modified Eagle media containing 10% fetal calf serum. Each 100-mm dish was transfected with 5 μg of the target CAT gene construct and various levels of the RB-expressing plasmid. The concentrations of DNA were monitored by measuring absorbance at 260nm and by comparing intensities in ethidium bromide stained agarose gels. The total concentration of the DNA for transfection was maintained at 20 μg/100-mm dish by adding sonicated salmon sperm DNA. DNA precipitates were removed 12h after the transfection, and the cells were replenished with fresh media containing 0.05% calf serum. Cells were harvested 36h later and CAT assays were performed as described previously (27).

Assay of E2F

E2F is assayed based on its ability to bind DNA in a sequence specific manner as previously described (28,29).

RESULTS

Rb/E2F interaction involves the T/E1A-binding domain and the C-terminus

Recently, we showed that the retinoblastoma protein interacts with E2F in two different ways. First, when purified E2F is used, the Rb protein inhibits the DNA binding activity of E2F. The second type of interaction is observed with cruder preparations of E2F. Such preparations allow formation of an Rb/E2F complex that can bind DNA. We also showed that the formation of the latter complex involves an Rb-binding protein, RBP60 (21). To investigate the details of these two types of Rb/E2F interaction, we have analyzed a series of mutant Rb proteins (Fig. 1A).

The Rb fusion protein and various mutants including pGT-RB(379−792;dl 573−645), pGT-RB(403−816), pGT-RB(379−928;dl exon 21), pGT-RB(379−928;dl exon 22), pGT-RB(379−928; 706 C-F), pGT-RB(379−928) and pGT-RB(379−792) were expressed and purified using pGEX-2T clones (generous gift of Bill Kaelin and David Livingston, Harvard Medical School). The mutants were described before by Kaelin et al.(9); briefly, the numbers in parenthesis indicate the number of amino acid from the N-terminus. For instance, pGT-RB(379−792;dl 573−645) represents a GST-Rb fusion protein that contains an Rb sequence corresponding to amino acid 379 to amino acid 792 from which sequences between 573 and 645 have been deleted. Equal amounts of these proteins were added to E2F purified from HeLa cells (30,21). Figure 1B shows an autoradiogram of a gel retardation assay for E2F after the addition of Rb. The Rb fusion protein, pGT-RB(379−928), containing an intact T/E1A-binding domain (also known as the pocket region of Rb, sequences between amino acid 379 and 792; see ref.9) and C-terminus, inhibited the DNA binding activity of E2F. However, the mutants pGT-RB(379−792;dl 573−645), pGT-RB(403−816), pGT-RB(379−928;dl exon 21), pGT-RB(379−928;dl exon 22) and pGT-RB(379−928; 706 C-F) were unable to inhibit E2F. These mutants are also defective in the binding of SV40 T antigen and adenovirus E1A (8,9). Moreover, a fusion protein with a C-terminal truncation, pGT-RB(379−792) that binds adenovirus E1A and SV40 T antigen was also unable to block the DNA binding activity of E2F. These results are consistent with a recent work (31) which showed that the retinoblastoma protein inhibited the DNA binding activity of E2F, purified from rabbit reticulocyte lysates, depending on the pocket region as well as the C-terminus. This inhibition of the DNA binding activity of E2F most likely involves a direct interaction between E2F and Rb because the addition of sodium deoxycholate, a detergent that is known to disrupt protein-protein interactions, reversed the inhibitory effect of Rb (Fig. 1C).

When the GST-Rb fusion proteins were added to a partially purified preparation of E2F from HeLa cell extracts, no inhibition of E2F-DNA binding activity was observed in gel retardation assays (Fig 1D). On the other hand, a GST-Rb fusion protein

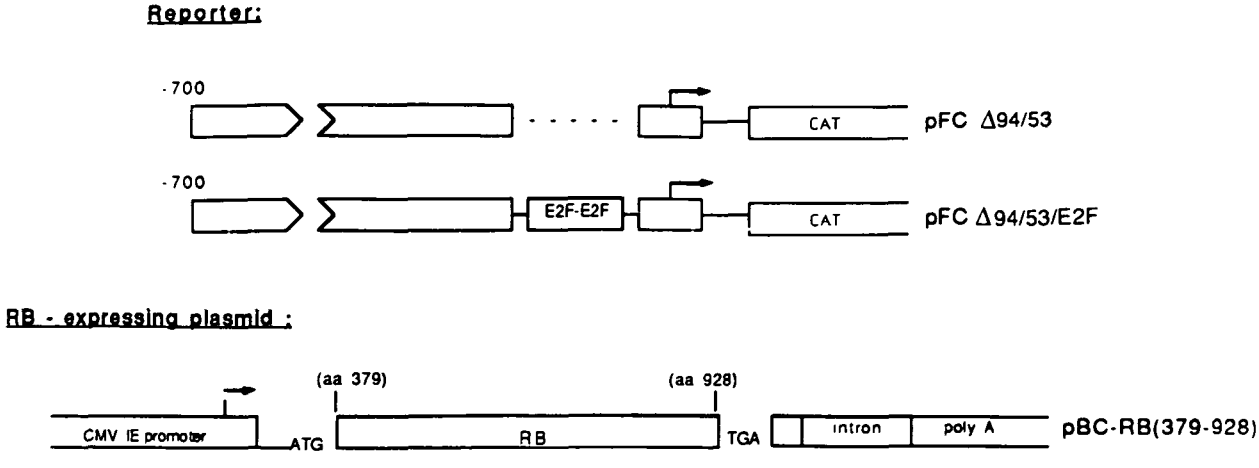

Figure 2. Schematics of the reporter CAT gene constructs and the RB-expression construct. The CAT-reporter plasmid pFCΔ94/53 contains human c-fos promoter sequences from −700 to + 42 from which sequences between −94 and −53 have been deleted as described (25). The CAT-reporter plasmid pFCΔ94/53/E2F contains two copies of the E2F binding site at position −53, and the construction of this plasmid is described in Materials and Methods. The construction of the RB-expressing vector is described in Methods. The transcription start sites are indicated by arrows.

that contained an intact T/E1A-binding domain along with the C-terminus induced formation of a new complex, E2Fr (Figs. 1D lane 6), at the expense of the E2F-DNA complex. The induction of the E2Fr complex has the same Rb-sequence requirement as that required for the inhibition. Moreover, the E2Fr complex contains the Rb protein as it is recognized by Rb-antibodies (21). These data are also in agreement with previous reports (15,16,18) that identified an Rb/E2F complex in the extracts of U937 promonocytic cell line that specifically bound the E2F cognate element. In experiment 1D, we see a partial conversion of E2F to the E2Fr complex. This may be due to instability of the E2Fr complex which breaks down to generate free E2F. We have recently shown that the formation of the E2Fr complex requires another cellular factor, RBP60 (21). It is also possible that this partial conversion is due to limiting amounts of RBP60 in these partially purified preparations of E2F.

The experiment described above suggests that the T/E1A-binding domain or the pocket region of the Rb protein is critical for the regulation of E2F in in vitro binding assays. It has been shown that all naturally occuring loss-of-function mutation of RB regularly map in the pocket region of the Rb protein (8,9 also see references in there). Thus, there is a clear overlap between the tumor suppressor function of Rb and its ability to interact with E2F. The experiment described in fig.1 also suggest a role for the C-terminus of Rb protein in the recognition of E2F. The significance of this result is not obvious; however, this would suggest that Rb/E2F interaction is more complex than the Rb/T or Rb/E1A interactions, which involve the pocket region of Rb and do not require the C-terminus (8,9).

The retinoblastoma protein represses transcription from an E2F-site containing promoter

We have analyzed the effect of the retinoblastoma protein on E2F-dependent transcription using co-transfection assays. The RB-cDNA sequences corresponding to amino acid residues between 379 and 928 as well as various mutants were recovered from the pGEX-2T constructs (described above) by PCR and cloned into an eukaryotic expression vector as described in the Materials and Methods and in figure 4. The upstream primer contained an initiating ATG with Kozak's consensus sequence and the downstream primer contained a stop codon. Cotransfection of the Rb-expressing plasmid, pBC-RB(379−928), with a previously described E2F-site containing reporter construct, pA10CAT-E2F, resulted in a 10−20 fold inhibition of CAT gene expression; however, a reporter construct without the E2F binding site, pA10CAT, was also inhibited by Rb in similar experiments (data not shown). This result suggests the possibility that SV40 early promoter, which drives the expression of CAT gene from the pA10CAT construct, contains element that can be regulated by Rb. We then decided to use a CAT gene reporter construct, pFCΔ94/53 (25),that is driven by a part of the human c-fos promoter from which most of the Rb-regulatory element (11), as well as the ATF site, have been deleted. Expression of the CAT gene from this construct is only marginally effected by Rb in several different cell lines (see figure 3).

To assay E2F dependent transcription, we introduced an oligonucleotide corresponding to sequences between −70 and −30 of the adenovirus E2 promoter at the BglII site of pFCΔ94/53. The resulting plasmid, pFCΔ94/53/E2F, contained two copies of E2F binding sites, in the same orientation as it is found in the adenovirus E2 promoter, at position −53 of the c-fos promoter. The construct pFCΔ94/53 had a relatively low

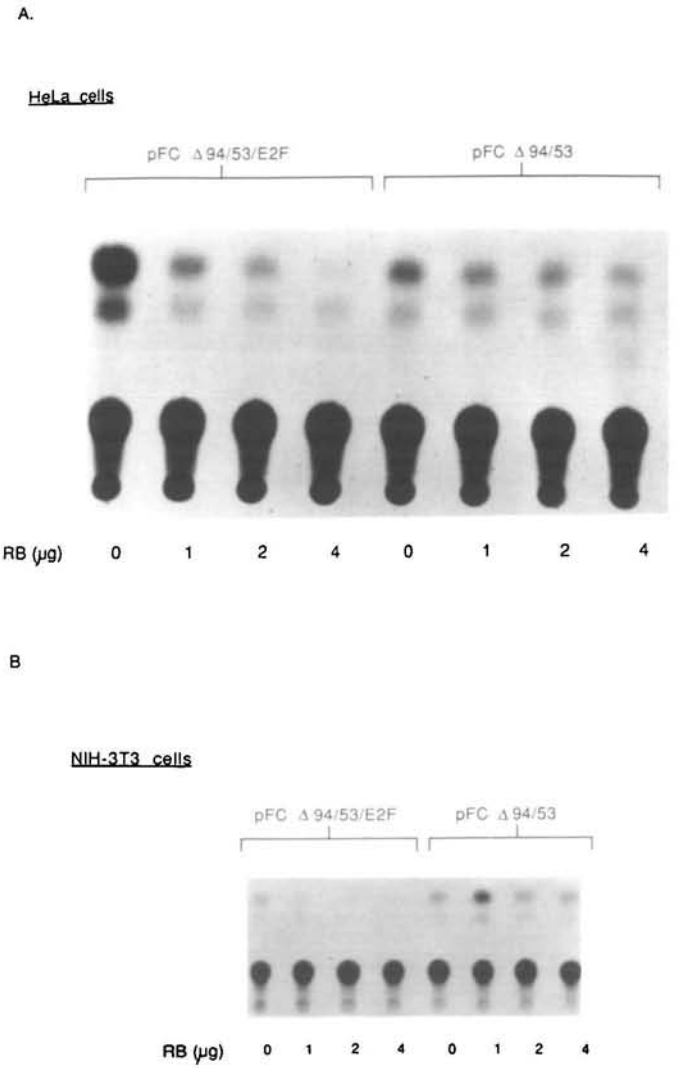

Figure 3. Effect of Rb on E2F-dependent transcription. HeLa cells (**A**) or NIH-3T3 cells (**B**) were transfected with 5ug of pFCΔ94/53 or pFCΔ94/53/E2F in combination with the indicated amounts of the RB-expressing plasmid as described in Methods. Cell extracts were assayed for CAT activity. The stimulation of CAT activity in figure 3B was not reproducible.

basal level of transcription in HeLa cells under these assay conditions, and the introduction of E2F sites resulted in a six fold stimulation of the promoter activity in HeLa cells (see figure 3A). Furthermore, when the cells were cotransfected with the Rb expressing plasmid, the construct with the E2F binding sites exhibited a significant inhibition of the CAT gene expression. HeLa cells express papillomavirus E7 protein which binds and inactivates Rb. However, by expressing Rb using CMV promoter, we still see an effect of Rb (at least 10 to 20 fold inhibition of E2F-dependent transcription) in this cell line. The CMV promoter allows very high level expression of Rb protein as judged by western blots of the transfected cell-extracts (Data not shown). We believe that this high level of Rb titrates out the E7 protein in Hela cell and allows us to see an effect.

As can be seen in fig.3B, we also assayed the effect of Rb on the E2F-dependent transcription in NIH-3T3 cells. Here again, Rb inhibited the expression of CAT gene from the E2F-sites

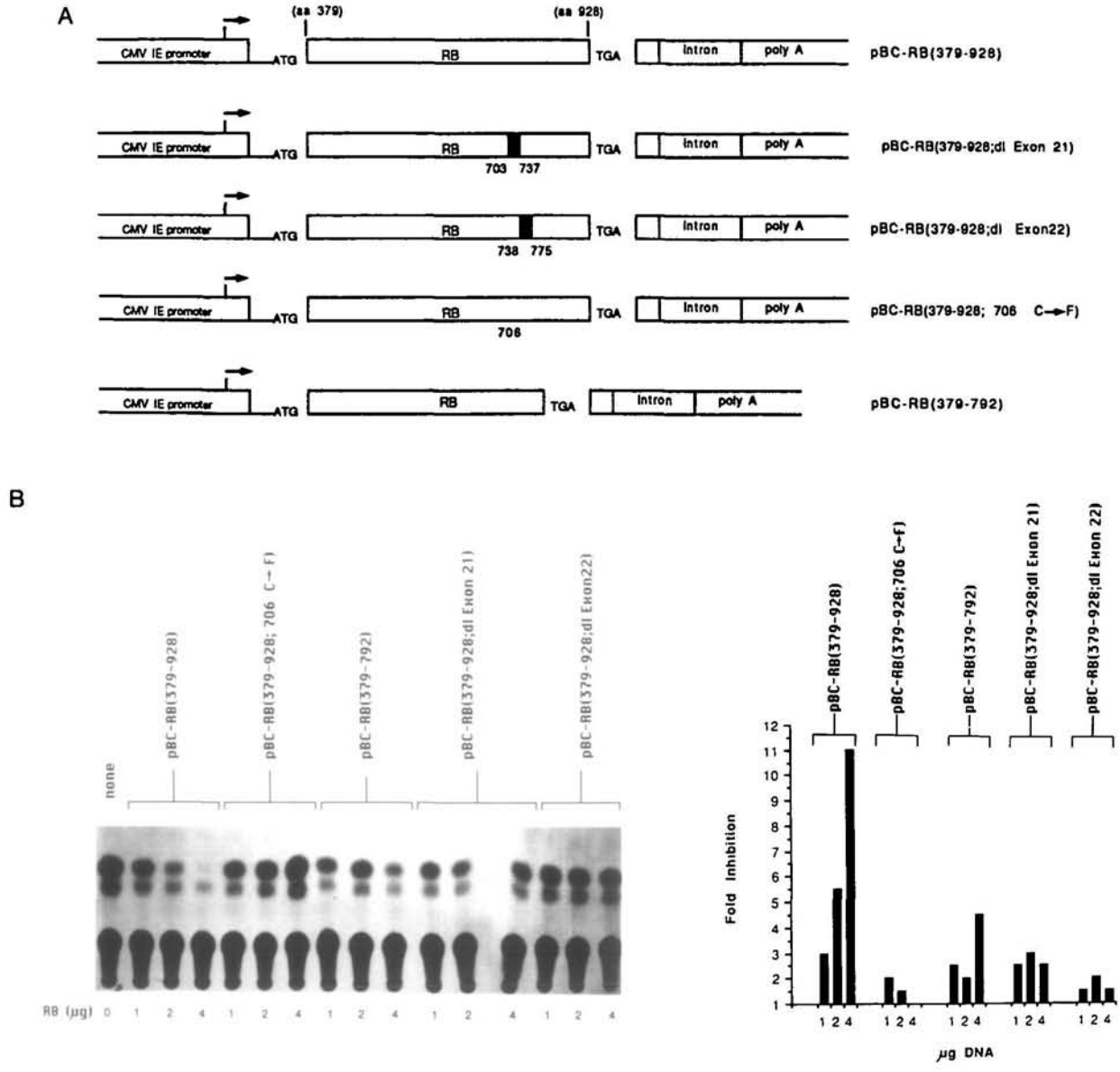

Figure 4. Rb-repression of E2F-dependent transcription in HeLa cells correlates with Rb/E2F interaction. **A.** The various Rb-mutant constructs used in the cotransfection assay. The black boxes indicate region deleted and the arrow indicates the transcription start site. **B.** HeLa cells were transfected with 5 μg of the plasmid pFCΔ94/53/E2F along with three levels (1 μg, 2 μg and 4 μg) of the indicated RB-expression plasmids. The transfections were carried out as described in Methods, and cell extracts were assayed for CAT activity. The panel on the left shows an autoradiogram of the assay, and the panel on the right shows fold inhibition obtained with increasing amounts of the wild type or the mutant retinoblastoma proteins

containing construct, while the expression of CAT gene from the pFCΔ94/53 construct was only marginally effected by Rb. It is also interesting to note that, unlike HeLa cells, in the NIH-3T3 cells the presence or the absence of the E2F-binding sites had little effect on the basal levels of transcription. This is most likely a reflection of the status of E2F in this cell line. In NIH-3T3 cells E2F is largely associated with other cellular proteins (19,23) whereas in HeLa cells the majority of this transcription factor exist in an apparently unbound form (23).

Overall, the results described above clearly show that Rb is a potent inhibitor of E2F-dependent transcription. This is also in agreement with the results obtained by Hiebert et al. (10) who showed that Rb inhibited transcription from adenovirus E2 promoter in cell line that did not express a functionally active Rb protein.

The Rb-repression of E2F-dependent transcription correlates with Rb/E2F interaction

With the assay system described above, we then asked whether the Rb-mutants that are unable to interact with E2F in vitro are also impaired in vivo in regulating E2F-dependent transcription. Vectors expressing several of the mutant Rb proteins (Fig. 4A) were constructed as described in the Materials and Methods. These vectors upon transfection into HeLa cells produced high levels of the mutant proteins as judged by western blot assays of the nuclear extracts obtained from the transfected cells (data not shown). Three different levels of these mutant Rb constructs were transfected into HeLa cells in combination with a fixed level of the reporter plasmid pFCΔ94/53/E2F. The results of such a transfection experiment is shown in figure 4B. Clearly, the

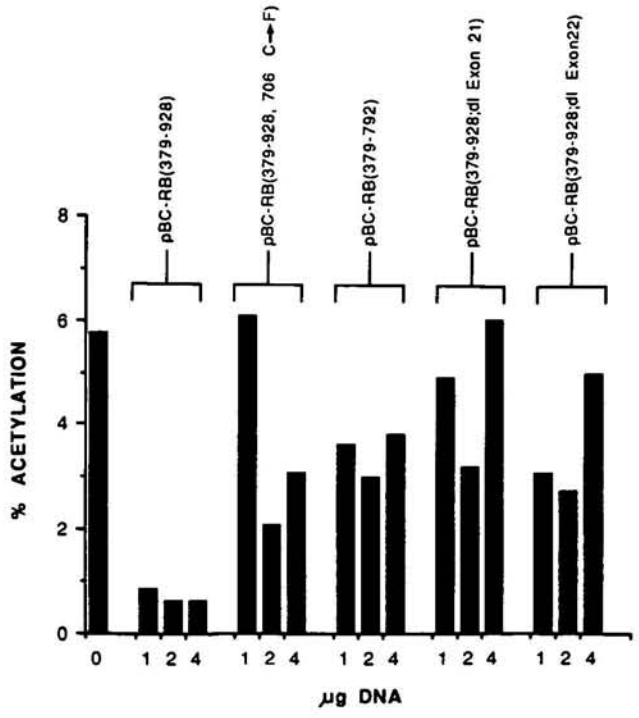

Figure 5. Rb-control of E2F-activity in NIH-3T3 cells has similar requirement as in the HeLa cells. Transfection and CAT assay were carried out as described in the legend to fig. 4 except that NIH-3T3 cells were used instead of HeLa cells. The numbers at the bottom indicate the microgram amounts of the RB-plasmids used in transfection. CAT activity was quantitated by scintillation counting of appropriate regions of the thin-layer chromatography plate.

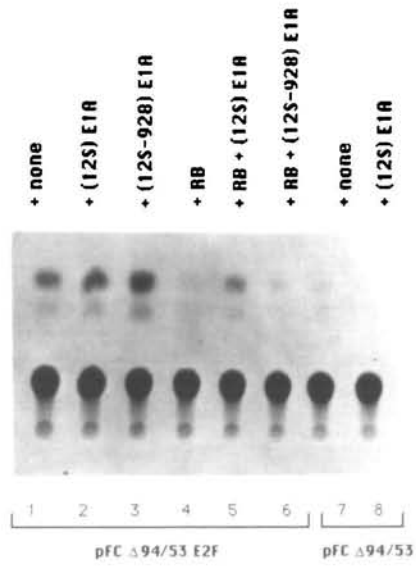

Figure 6. The adenovirus E1A gene product (12S) blocks the Rb-repression of E2F-dependent transcription. For experiments 1 to 6, 5 μg of the plsmid pFCΔ94/53/E2F were transfected alone, or with 10 μg of 12S E1A-expressing plasmid, or with 12S-928 E1A-expressing plasmid, or with 5 μg of RB-expressing plasmid, or a combination of RB and E1A expression plasmids into HeLa cells. For experiments 7 and 8, 5 μg of the plasmid pFCΔ94/53 were used either alone or in combination with 10 μg of the 12S E1A-expressing plasmid. The CAT activity was measured as described in Methods.

mutants that are defective in interacting with E2F are also impaired in their ability to repress the E2F-dependent transcription. Again, it appears that the T/E1A-binding region of Rb is important for regulating E2F-dependent transcription. The mutants pBC-RB(379−928, dl exon 21), pBC-RB(379−928, dl exon 22) and pBC-RB(379−928, 706 C-F) are loss-of-function mutants, and are also unable to bind DNA tumor virus oncoproteins. As shown in fig. 5 these mutants are also significantly impaired in regulating E2F-dependent transcription. We analyzed one c-terminus mutant, RB(379−792), that lacked amino acid residues between 792 and 928 and still bound T antigen and E1A. This mutant in in vitro binding assay was unable to interact with E2F (Fig.1). At the highest level of assay (4 μg Fig. 4B), this mutant inhibited the E2F-dependent transcription by 4.5 fold. However, this plasmid did not inhibit in a dose dependent manner, and increasing the amount of the plasmid actually lowered the extent of inhibition (Data not shown). Thus, we conclude that RB(379−792) is also impaired in its ability to inhibit E2F-dependent transcription.

We have also analyzed these mutants in NIH-3T3 cells, and the results are presented in figure 5. Once again we see the same pattern. The Rb-construct that contained an intact T/E1A-binding domain along with the carboxyl-terminus inhibited E2F-dependent transcription and the mutants that were unable to interact with E2F were also largely impaired in their ability to inhibit E2F-dependent transcription. A similar experiment with mouse L cells (LMTK-) produced essentially the same result (data not shown).

The adenovirus E1A gene product counteracts the Rb-repression

The adenovirus E1A gene product binds the retinoblastoma protein, and this binding depends on the sequences in the conserved regions 1 and 2 of E1A protein (7). The 12S E1A product binds Rb. However, a point mutant of 12S E1A (12S-928) that contains a substitution of the amino acid residue 124 (Cys to Gly) in the conserved region 2 does not bind Rb (32). We used plasmids that expressed the 12S E1A or the point mutant (12S-928) in transfection experiments to determine whether E1A, by binding to Rb, could block the Rb-repression of E2F-dependent transcription. HeLa cells were used in this set of experiments because we showed that the 12S E1A product had no effect on the E2F-dependent transcription in this cell line (Ref.23, and also see experiment in figure 6). The result of this experiment is shown in figure 6. Clearly, the retinoblastoma protein inhibits E2F-dependent transcription and the E1A gene product (12S) counteracts the transcription inhibitory function of Rb. Moreover, a mutant (12S-928), that is unable to bind Rb, is impaired in blocking Rb-repression.

DISCUSSION

Several loss-of-function mutations of the RB gene have been characterized (see ref. in 8). All of these mutations cluster in the region that is also critical for the binding of the Rb protein to DNA tumor virus oncoproteins including T antigen and E1A. This observation suggests that the recognition site for E1A and T antigen maps in a functional domain of Rb, and that by forming a complex with this domain the viral oncoproteins disrupts the

tumor suppressor function of Rb. More recently, it has been shown that the retinoblastoma protein interacts with the transcription factor E2F (13–18). And the notion that this Rb/E2F interaction might be related to the tumor suppression function of Rb is also supported by a recent analysis which show that the loss-of-function mutants of Rb can not interact with E2F (10). Thus, the interaction of the retinoblastoma protein with E2F offers at least one biochemical assay for the tumor suppression function of Rb.

While analyzing the details of the Rb/E2F interaction, we identified two types of interactions between Rb and E2F (21 and fig.1). One of these two interactions involves cruder preparations of E2F that are contaminated with an Rb-binding protein, RBP60 (21). The presence of RBP60 allows the formation of an Rb/E2F complex that is able to bind DNA which in a gel retardation assay migrates as a distinct complex (E2Fr in figure 1D). The second type of interaction is observed in the absence of RBP60. Affinity purified preparation of E2F forms a complex with Rb, which has a low affinity for DNA, and that is reflected by an apparent loss of the DNA binding activity of E2F in gel retardation assays (figure 1B and ref.21). Availability of the mutant Rb proteins have allowed us to determine what regions of Rb protein are involved in these two types of interactions with E2F. Experiments described in figure 1 clearly show that these two types of interactions between Rb and E2F depend on common sequences of the Rb protein. Moreover, we find that the loss-of-function mutants of Rb are also impaired in producing the E2Fr complex or the inhibition of the DNA binding activity of E2F. In agreement with Hiebert et al. (10), we also see a requirement for the carboxyl terminus outside the T/E1A-binding domain of the retinoblastoma protein in these Rb/E2F interactions. The significance of the involvement of the Rb-carboxyl terminus is not obvious as no naturally occurring loss-of-function mutations, identified so far, map in this region of the Rb protein.

To determine the consequence of Rb/E2F interaction, we have also analyzed the effect of Rb on the E2F-dependent transcription. To do this, we have utilized the well studied human c-fos gene promoter. It has been reported that c-fos promoter is regulated by the retinoblastoma protein and the site of regulation on the promoter has been localized between −102 and −71 relative to the transcription start site (11). We have chosen a CAT gene construct, pFCΔ94/53, which is driven by the fos promoter and lacks most of the Rb-regulatory element. In several different cell lines expression of the CAT gene from this promoter is only marginally effected by the coexpression of the Rb protein. Introduction of two copies of the E2F binding sites into this promoter resulted in a construct, pFCΔ94/53/E2F, that reproducibly showed higher basal level of transcription in HeLa cells compared to the construct, pFCΔ94/53, without the E2F binding site. This is consistent with the notion that E2F is a positively acting transcription factor which in HeLa cell largely exists in an apparently unbound and active form (23). However, this is not the case in mouse L cells or NIH-3T3 cells. In these cell lines we did not see an increase in the basal level of transcription in the presence of E2F binding sites. This is also consistent with the observation that in these cells the majority of E2F exists in complexes with other cellular proteins and such complexes of E2F have been suggested to be functionally inactive with respect to E2F-dependent transcription (33).

We have employed a plasmid that expresses a c-terminal 56 kd part of the Rb protein in transfection assays. This 56 kd Rb protein contains an intact T/E1A-binding domain along with the c-terminus of the Rb protein, and recent experiments (34) have shown that such a fragment of Rb is functionally active in arresting cell proliferation. Moreover, as shown in figure 1, we also find that a GST-Rb fusion protein which contains the 56 kd c-terminal part of Rb is also able to interact with E2F. Cotransfection of this Rb-expressing plasmid clearly resulted in an inhibition of the E2F-dependent transcription in the different cell lines used in this study. Transcription from a construct devoid of the E2F binding site was only marginally effected by the Rb protein.

We have analyzed three loss-of-function mutants of Rb in cotransfection assays. These mutants include a point muation at amino acid position 706, a deletion of the exon 21 and a deletion of the exon 22. These mutations map in the T/E1A-binding domain, and the corresponding Rb proteins are unable to interact with E2F. Data presented here clearly show that these mutants are also impaired in their ability to inhibit E2F-dependent transcription. We have analyzed one of the three c-terminus mutants described in figure 1A and 4. This c-terminus mutant, which expresses an Rb protein corresponding to amino acid sequence between 379 to 792, contains an intact T/E1A-binding domain but is unable to interact with E2F. Again, we see that this mutant is significantly impaired in its ability to inhibit E2F-dependent transcription. Thus, there is a strong correlation between ability of Rb to interact with E2F and the inhibition of E2F-dependent transcription.

Inhibition of the E2F-dependent transcription by the retinoblastoma protein is significant as the binding site for E2F is found in several proliferation associated genes, including c-myc (19), dhfr (20), DNA Polymerase α (35) and thymidylate synthetase (36). Data presented here may suggest that the retinoblastoma protein could be expected to inhibit expression of these genes. Inhibition of these proliferation associated genes would have a negative effect on cell proliferation and result in growth arrest. Thus, the Rb-control of E2F-dependent transcription also provides an insight into the biochemistry of growth suppression by the retinoblastoma protein. Moreover, the reversal of the Rb-inhibition by the adenovirus E1A protein also confirms the notion that the viral oncoproteins neutralize important biological function of this tumor suppressor protein.

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