MOLECULAR AND CELLULAR BIOLOGY, Oct. 1993, p. 6501–6508
0270-7306/93/106501-08$02.00/0

Vol. 13, No. 10

Inhibition of E2F-1 Transactivation by Direct Binding of the Retinoblastoma Protein

KRISTIAN HELIN,* ED HARLOW, AND ALI FATTAEY

Massachusetts General Hospital Cancer Center, Building 149, 13th Street, Charlestown, Massachusetts 02129

Received 26 May 1993/Returned for modification 21 July 1993/Accepted 22 July 1993

Loss of a functional retinoblastoma tumor suppressor gene product, pRB, is a key step in the development of many human tumors. pRB is a negative regulator of cell proliferation and appears to participate in control of entry into the S phase of the cell cycle. The recent demonstration that pRB binds to transcription factor E2F has provided a model for the mechanism of pRB-mediated growth regulation. Since adenovirus E1A proteins dissociate the pRB-E2F complexes and stimulate E2F-dependent transcription, it has been suggested that pRB inhibits E2F transactivation. Although some evidence for this hypothesis has been provided, it has not been possible to determine the mechanism of pRB-mediated inhibition of E2F transactivation. In this study, we constructed mutants of E2F-1 that do not bind to pRB yet retain the ability to transactivate the adenovirus E2 promoter through E2F DNA-binding sites. We demonstrated that transactivation mediated by the wild-type E2F-1 protein was inhibited by overexpression of wild-type pRB but not by a naturally occurring mutant of pRB. Transactivation mediated by mutants of E2F-1 which do not bind to pRB was not affected by overexpression of wild-type pRB. Furthermore, when the E2F-1 transactivation domain was fused to the GAL4 DNA-binding domain, pRB inhibited GAL4–E2F-1 transactivation through GAL4 sites. Expression of pRB did not inhibit transactivation mediated by GAL4–E2F-1 mutant constructs which were devoid of pRB binding. In conclusion, these data demonstrate that pRB inhibits E2F-dependent transactivation by direct protein-protein interaction.

Retinoblastoma is a rare childhood cancer which is initiated by two genetic lesions (for a review, see reference 54). In several retinoblastomas, the lesions were mapped to a common region of human chromosome 13, and the corresponding gene, *RB-1*, was the first mammalian tumor suppressor to be characterized at the molecular level (54). Analysis of *RB-1* has demonstrated that nearly all retinoblastomas carry mutations in each allele of the *RB-1* gene. Mutations in the *RB-1* gene are also observed in a subset of other human cancers, including osteosarcomas, bladder carcinomas, small-cell lung carcinomas, prostate carcinomas, and cervical carcinomas (54). The wide variety of tumors carrying mutated *RB-1* genes suggests not only that pRB is important for growth regulation of retina cells but that this protein has a more global role in the regulation of normal cell proliferation.

The notion that pRB is a regulator of cell proliferation is further supported by several avenues of research. (i) Gene transfer experiments with cell lines with a nonfunctional pRB have demonstrated that the transformed phenotype of retinoblastomas (27), osteosarcomas (27), prostate carcinomas (6), bladder carcinomas (51), and breast carcinomas (53) is reversed by introduction of a retroviral vector that expresses pRB. (ii) The transforming proteins of small DNA tumor viruses, such as adenovirus E1A, simian virus 40 large T, and human papillomavirus E7, all bind to pRB (12, 14, 55), and the transforming ability of these viral proteins is dependent on pRB binding (38, 41, 56). (iii) Expression of pRB in osteosarcoma cells, which lack a functional pRB, prevents progression into the S phase of the cell cycle (15, 22, 42). This pRB-mediated block in the cell cycle can be overcome by overexpression of cyclin A or cyclin E (22), supporting the initial suggestion that un(der)phosphorylated pRB is an active repressor in the G_1 phase of the cell cycle (7, 9, 13). (iv) pRB has been found in stable protein complexes with the transcription factor E2F (2, 4, 8, 10). Since E2F is important for the regulated transcription of several genes essential for growth and DNA synthesis, regulation of this transcription factor appears to be crucial for normal cell proliferation (reviewed in references 19 and 40).

The transcription factor E2F was originally characterized as a sequence-specific DNA-binding factor bound to the adenovirus E2A promoter (32). E2F-binding sites have now been identified in several cellular promoters, including promoters which control the expression of growth regulators such as c-Myc, N-Myc, Cdc2, and cyclin A, as well as promoters for genes whose products are required for DNA synthesis and replication, such as DNA polymerase α, thymidine kinase, thymidylate synthase, and dihydrofolate reductase (19, 40). Transcription of these cellular genes is temporally regulated through the cell cycle, and at least for the c-*myc* (52), *DHFR* (5, 49), and *cdc2* genes (11) the E2F-binding sites contribute to this regulation.

Two findings support the notion that pRB inhibits E2F activity. (i) The presence of adenovirus E1A stimulates E2F-dependent transcription (32, 43), and E2F does not form stable complexes with pRB in the presence of E1A (1, 8). This suggests that E1A alleviates the inhibitory effects of pRB by binding to this protein and thus indirectly stimulating E2F transcription. (ii) Overexpression of pRB abrogates the ability of endogenous E2F to stimulate transcription (17, 21, 58). However, the pleiotropic effects of overexpressing pRB were not addressed in these experiments. Recent reports have, in fact, demonstrated that expression of pRB in certain pRB-negative cell lines can lead to cell cycle arrest (15, 22, 42, 59). Therefore, the mechanism by which pRB inhibits

* Corresponding author.

E2F-dependent transcription has remained speculative. In this study, we took advantage of the recent cloning of *E2F-1* (20, 30, 47) to generate mutants whose functional properties clearly demonstrate that pRB inhibits E2F-dependent transactivation through direct protein-protein interaction.

MATERIALS AND METHODS

Cell culture. Saos-2 (a human osteosarcoma cell line), C-33A (a human cervical cell line), J82 (a human bladder carcinoma cell line), and T98G (a human glioblastoma cell line) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Plasmid constructions. The plasmids that encode GST–E2F-1 amino acids 386 to 437, 386 to 426, 386 to 417, and 409 to 437 have all been described before (20). The plasmid that expresses glutathione *S*-transferase (GST)–E2F-1 (amino acids 409 to 437 [Y-411→C]) was constructed by polymerase chain reaction amplification of the sequence from amino acids 409 to 437 with E2F-1 cDNA as the template. The forward primer carried a mutation which would change the predicted tyrosine residue at position 411 to a cysteine. The amplified fragment was then cloned into pGEX30X (kind gift of J. Armstrong and R. Treisman), and the mutation was confirmed by DNA sequencing.

To construct a mammalian expression plasmid that encodes amino acids 1 to 437 of the E2F-1 protein (pCMVE2F-1), E2F-1 cDNA was cloned into pCMVneoBam (3) by use of *Bam*HI linkers. Δ417-437 was constructed by first cloning full-length E2F-1 cDNA into pGEX20T (kind gift of J. Armstrong and R. Treisman), resulting in pGEXE2F-1 (amino acids 1 to 437). An *Xho*I fragment (amino acids 417 to 437) was then deleted from pGEXE2F-1 (amino acids 1 to 437), resulting in pGEXE2F-1 (amino acids 1 to 417), and finally a *Bam*HI-*Xba*I fragment that encodes amino acids 1 to 417 and an in-frame stop codon after amino acid 417 was excised from pGEXE2F-1 (amino acids 1 to 417), blunt ended, and cloned into blunt-ended pCMVneoBam, resulting in pCMVE2F-1Δ417-437. The expression plasmid that encodes E2F-1 amino acids 1 to 437 (Y-411→C) was constructed by polymerase chain reaction amplification of amino acids 151 to 417 with a mutation in the reverse primer changing codon 411 to encode a cysteine residue. The amplified fragment was cloned into pBSKE2F-1, replacing a similar fragment, and the insert was sequenced to confirm that no other mutations had occurred. From the resulting plasmid, pBSKE2F-1(Y411C), a *Bam*HI fragment containing the entire open reading frame of E2F-1 was cloned into pCMVneoBam, giving rise to pCMVE2F-1(Y411C).

Expression plasmids that encode GAL4–E2F-1 and GAL4–E2F-1 Δ417-437 fusion proteins were constructed by inserting a blunt-ended *Bgl*II-*Hin*dIII fragment or a blunt-ended *Bgl*II-*Xho*I fragment from full-length E2F-1 cDNA into the *Sma*I site in the pSG424 (45) expression vector, respectively. pGAL4/E2F-1(Y411C) was constructed by inserting a blunt-ended *Bgl*II-*Bam*HI fragment from plasmid pBSKE2F-1(Y411C) into the *Sma*I site of pSG424. These three constructs were digested with *Hin*dIII and *Xba*I, releasing the corresponding GAL4–E2F-1 fragment, and cloned into plasmid pBSK for in vitro transcription and translation.

The pRB expression plasmids were a kind gift of W. G. Kaelin (42). GST-pRB and GST-pRB(C706F) have been previously described (31). Plasmid pSGVP, which expresses GAL4-VP16, was the kind gift of M. Ptashne (44). Reporter constructs for chloramphenicol acetyltransferase (CAT) assays have been previously described (20, 33).

Purification of GST fusion proteins. Production and purification of GST fusion proteins were done essentially as previously described (20). After binding to glutathione agarose (Sigma), the proteins were eluted with reduced glutathione (Sigma). The purity and size of the eluted proteins were then evaluated by Coomassie blue staining of sodium dodecyl sulfate (SDS)-polyacrylamide gels.

In vitro binding experiments. In vitro binding experiments were performed as previously described (20). Briefly, in vitro-translated proteins (either full-length pRB or GAL4–E2F-1 fusion proteins) were mixed with GST fusion proteins for 2 h on ice, and complexes were recovered with glutathione-agarose (Sigma) and resolved on SDS–8 or 10% polyacrylamide gels.

Transient transfections and Western blotting (immunoblotting). Transfections were performed essentially as previously described (16). To evaluate the expression of proteins, cells grown in 9-cm-diameter tissue culture dishes were transfected with 10 or 20 μg of an expression plasmid and lysed at 36 h after transfection as previously described (20). A 50-μg sample of the total cell lysate was separated on 8 or 10% polyacrylamide gels and transferred to an Immobilon membrane (Millipore) by standard techniques (18). Transferred proteins were detected by using the indicated antibodies and developed by the ECL system (Amersham). The primary antibodies used in Western blotting were a mouse polyclonal antibody to E2F-1 (20); a monoclonal antibody to pRB, XZ77 (26); and a polyclonal antibody to GAL4 (kind gift of I. Sadowski and M. Ptashne).

Transactivation assays. For transactivation assays, transfection was done as described above. Reporter plasmids pE2(−80/−70)CAT and pE2(−64/−60, −45/−36)CAT (35) were used to measure E2F-1-dependent transactivation through E2F DNA-binding sites. Reporter plasmids E1bCAT and 5xGAL4E1bCAT (33) were used to measure transactivation through GAL4 DNA-binding sites. Cells were transfected with 200 ng of the respective expression plasmids, the pRB expression plasmid, 4 μg of the reporter construct, 3 μg of an internal control luciferase expression plasmid (57), and carrier DNA to a total of 20 μg/9-cm dish. CAT and luciferase activities were measured by standard procedures as previously described (20). Fold activation refers to units of CAT activity normalized to the luciferase activity for each cell extract. The basal level of the reporter plasmid without cotransfection of an expression plasmid was set to unity. The experimental results shown in the figures are representative of a minimum of three independent duplicate transfections for each cell line.

RESULTS

The E2F-1 protein contains a DNA-binding domain, a transactivation domain, and a specific region for binding to pRB (20, 30, 47). We have previously demonstrated that 18 amino acids near the C terminus of E2F-1 are necessary and sufficient for binding to pRB. This pRB-binding domain lies entirely within the E2F-1 transactivation domain. These data suggested that pRB might act as a transcriptional inhibitor by hindering E2F-1-mediated transactivation through direct binding. To test this hypothesis, we generated different E2F-1 constructs containing mutations in the pRB-binding domain. These mutant constructs were expressed as GST fusion proteins and tested for binding to in vitro-translated pRB. As previously described, a mutant E2F-1 lacking

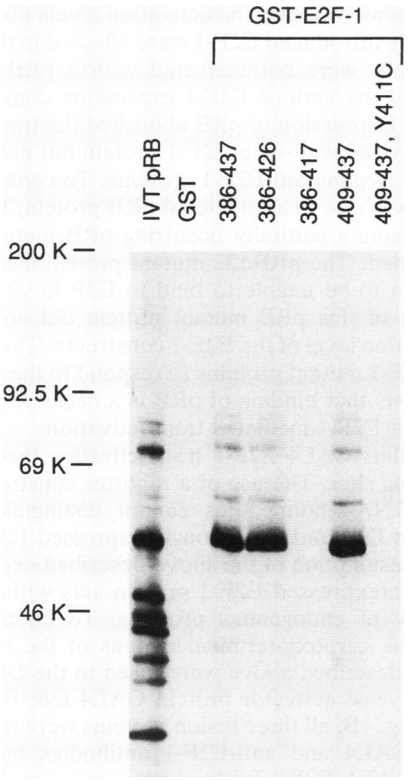

FIG. 1. Binding of E2F-1 proteins to in vitro-translated (IVT) pRB. Ten microliters of in vitro-translated pRB was tested for binding to 5 μg of GST–E2F-1 fusion proteins as described in Materials and Methods. The E2F-1 amino acids fused in frame with GST are indicated above the lanes. Binding to GST alone was used as a negative control. A 1-μl volume of the pRB in vitro-translated reaction was run as a control for binding efficiency. Molecular mass markers are given at the left in kilodaltons (K).

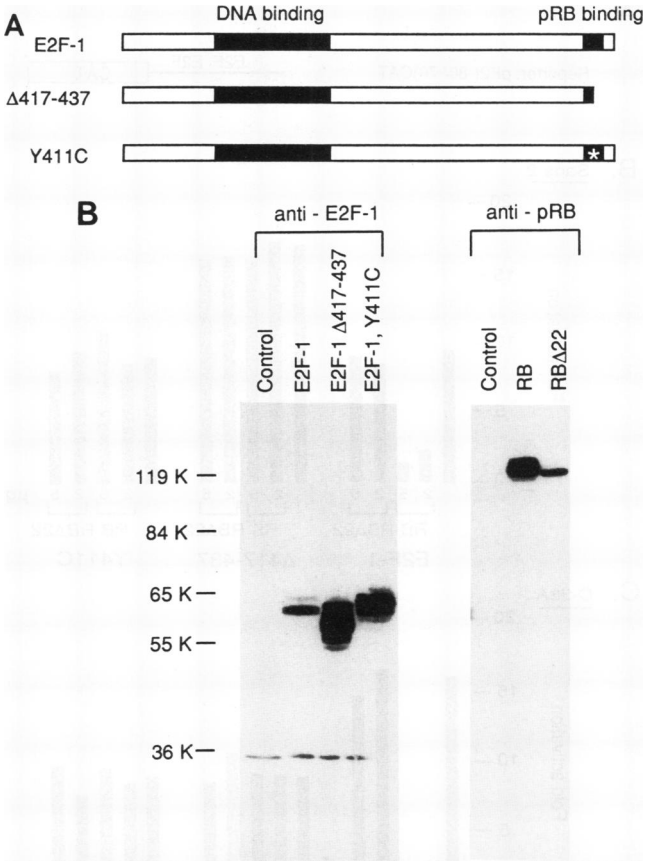

FIG. 2. Expression of E2F-1 proteins. (A) Diagram of wild-type and mutant E2F-1 proteins. The wild-type E2F-1 protein contains a specific DNA-binding domain (amino acids 89 to 191) and a specific pRB-binding domain (amino acids 409 to 426). The last 20 amino acids of E2F-1 were deleted in Δ417-437. Y411C contains a point mutation that changes tyrosine residue 411 to a cysteine residue. (B) Expression of E2F-1 and pRB proteins in the cervical carcinoma cell line C-33A. Expression of proteins was detected by Western blotting with a polyclonal mouse antibody to E2F-1 (20) or mouse monoclonal antibody XZ77, which is specific for pRB (26). Cells transfected with the expression vector alone were used as a control. Molecular mass markers are given at the left in kilodaltons (K).

amino acids 417 to 437 (Δ417-437) did not bind to pRB (Fig. 1 and reference 20). Additional mutant E2F-1 proteins containing more subtle alterations within the pRB-binding domain were also tested. A mutant protein in which the tyrosine residue at position 411 was substituted for a cysteine (Y411C) was no longer able to bind pRB (Fig. 1). In the converse experiment, GST-pRB bound to in vitro-translated wild-type E2F-1 but not in vitro-translated mutant proteins Δ417-437 and Y411C (data not shown).

pRB inhibits E2F-1 transactivation through E2F DNA-binding sites. To determine whether the wild-type and mutant E2F-1 proteins were able to transactivate a reporter construct containing E2F DNA-binding sites, the corresponding DNA fragments were cloned into a mammalian expression vector (Fig. 2A). These expression constructs were then transfected into cell lines lacking a functional pRB (Saos-2, an osteosarcoma cell line [48], and C-33A, a cervical carcinoma cell line [46]) and analyzed for expression of the respective proteins (Fig. 2B and data not shown). Transient transfection of the different E2F-1 constructs into Saos-2 and C-33A cells resulted in high-level expression of the predicted-size proteins for the wild-type and two mutant forms of E2F-1. The E2F-1 protein has previously been reported to migrate as a doublet with molecular masses of approximately 60 kDa (20, 30). The E2F-1 proteins detected after transfection of the E2F-1 expression plasmids reproducibly migrate in a position similar to that of endogenous E2F-1 under SDS-polyacrylamide gel electrophoresis conditions. Since the E2F-1 protein was highly overexpressed when cells were transfected with 10 μg of the expression plasmid, the endogenous level of E2F-1 protein is not visible in the short exposure of the Western blot shown in Fig. 2B. The E2F-1 Δ417-437 proteins migrated as multiple bands slightly smaller than wild-type E2F-1. The reason for these multiple proteins is unclear, but they may be due to abnormal posttranslational modification or degradation of the protein after removal of the C-terminal 20 amino acids. Transient transfection of expression plasmids containing either the wild-type *RB-1* gene or a naturally occurring mutated form of the *RB-1* gene (RBΔ22) in which exon 22 has been deleted (24) resulted in high-level expression of pRB (Fig. 2B). Consistent with a previous report (42), pRBΔ22 was not phosphorylated after transfection into the pRB-negative cell lines, whereas the wild-type pRB protein was phosphorylated in C-33A cells and not phosphorylated in

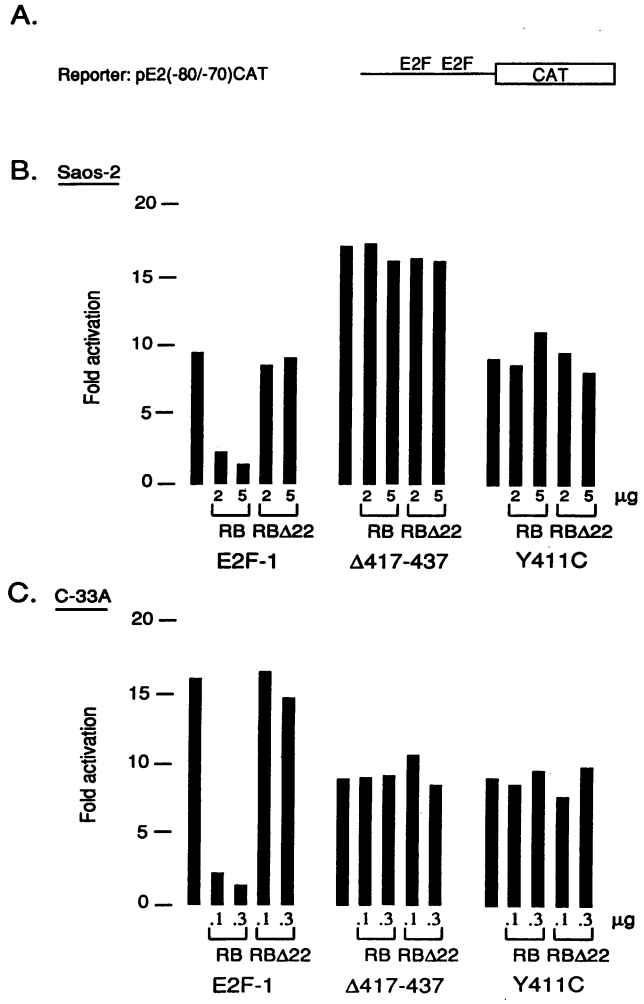

FIG. 3. Transactivation by E2F-1 through E2F DNA-binding sites. (A) Diagram of the reporter plasmid, E2(−80/−70)CAT, used in the transactivation experiments. (B) Transactivation of the reporter plasmid after transfection of the indicated E2F-1 constructs into Saos-2 cells as described in Materials and Methods. The effects of pRB were examined by cotransfection of the indicated amount of expression plasmid pCMVRB or pCMVRBΔ22. The data presented are representative of at least three independent transfection experiments. Fold activation refers to units of CAT activity normalized to the luciferase activity for each cell extract. The basal level of E2(−80/−70)CAT was set to unity. (C) Same as panel B, except that transfections were performed with C-33A cells.

Saos-2 cells, as judged by its mobility in SDS gels (Fig. 2B and data not shown).

To examine the transactivation functions of the E2F-1 constructs, these expression plasmids were cotransfected with a reporter construct containing two E2F DNA-binding sites (Fig. 3A) into Saos-2 and C-33A cells. Expression of the three different E2F-1 constructs resulted in 8- to 17-fold-increased activation of the reporter construct compared with the endogenous level of E2F activity (Fig. 3B and C). This transactivation was dependent on the presence of the E2F-binding sites in the reporter plasmid, since reporter constructs lacking these binding sites were not activated by the E2F-1 proteins (data not shown). From these results, we conclude that mutations within the pRB-binding domain of E2F-1 do not significantly alter its transactivating functions.

To determine whether the activation levels observed with exogenously introduced E2F-1 were affected in the presence of pRB, cells were cotransfected with a pRB expression plasmid and the various E2F-1 expression constructs (Fig. 3B and C). Expression of pRB abolished the transactivation mediated by the wild-type E2F-1 protein but not that mediated by the two mutant E2F-1 proteins. To confirm that this inhibition was due to a functional pRB protein, the effect of overexpressing a naturally occurring pRB mutant (RBΔ22) was examined. The pRBΔ22 mutant protein has previously been shown to be unable to bind to E2F in vitro (42, 58). Expression of this pRB mutant protein did not affect the transactivation level of the E2F-1 constructs. The inability of the two E2F-1 mutant proteins to respond to the presence of pRB suggests that binding of pRB is a necessary step in its inhibition of E2F-1-mediated transactivation.

pRB inhibits GAL4–E2F-1 transactivation through GAL4 DNA-binding sites. The use of a reporter construct containing E2F DNA-binding sites cannot distinguish between endogenous E2F and exogenously expressed E2F-1. Moreover, one assumption of the above-described experiments is that the overexpressed E2F-1 protein acts without altering the activity of endogenous proteins. To circumvent this problem, the carboxy-terminal regions of the three E2F-1 constructs described above were fused to the DNA-binding domain of yeast activator protein GAL4 (36; Fig. 4A). As shown in Fig. 4B, all three fusion proteins were reactive with both anti-GAL4 and anti-E2F-1 antibodies but only the wild-type GAL4–E2F-1 fusion protein was capable of binding to pRB. To examine the activity of these fusion products, the constructs were cloned into a mammalian expression vector and shown to produce equivalent protein levels in transfected cells (Fig. 4C). In these experiments, an expression construct containing the GAL4 DNA-binding domain fused to the acidic activation domain of the herpes simplex virus VP16 protein was used for comparison (44).

The ability of these fusion proteins to activate a promoter containing five GAL4 DNA-binding sites was tested (Fig. 5). Expression of the three GAL4–E2F-1 fusion proteins resulted in activation of the reporter construct at levels similar to that of GAL4-VP16. This transactivation was dependent on the presence of the GAL4 DNA-binding sites, and transfection of the reporter plasmid with a construct that encodes GAL4 amino acids 1 to 147 alone did not activate the reporter plasmid (data not shown). Coexpression of wild-type but not mutant pRB in the three pRB-negative cell lines (C-33A, Saos-2, and J82, a bladder carcinoma cell line [25]) led to a decrease in the transactivation obtained by the wild-type GAL4–E2F-1 fusion protein. This pRB-mediated inhibition was dependent on an intact pRB-binding domain, since the activity of the GAL4–E2F-1 mutants was not affected by pRB expression. Moreover, pRB had no inhibitory effect on the transactivating functions of the GAL4-VP16 protein in any of the cell lines examined.

It has previously been found that expression of exogenous pRB in cells containing wild-type pRB has little effect on the transactivating capacity of cellular E2F and no effect on cell cycle progression (59). Consistent with this finding, we detected no significant decrease in the transactivation levels of wild-type GAL4–E2F-1 protein in a cell line with a normal pRB (Fig. 5D, T98G glioblastoma cells). These results, obtained with the GAL4–E2F-1 transactivation assay, are consistent with the data obtained with the E2F-1 protein itself.

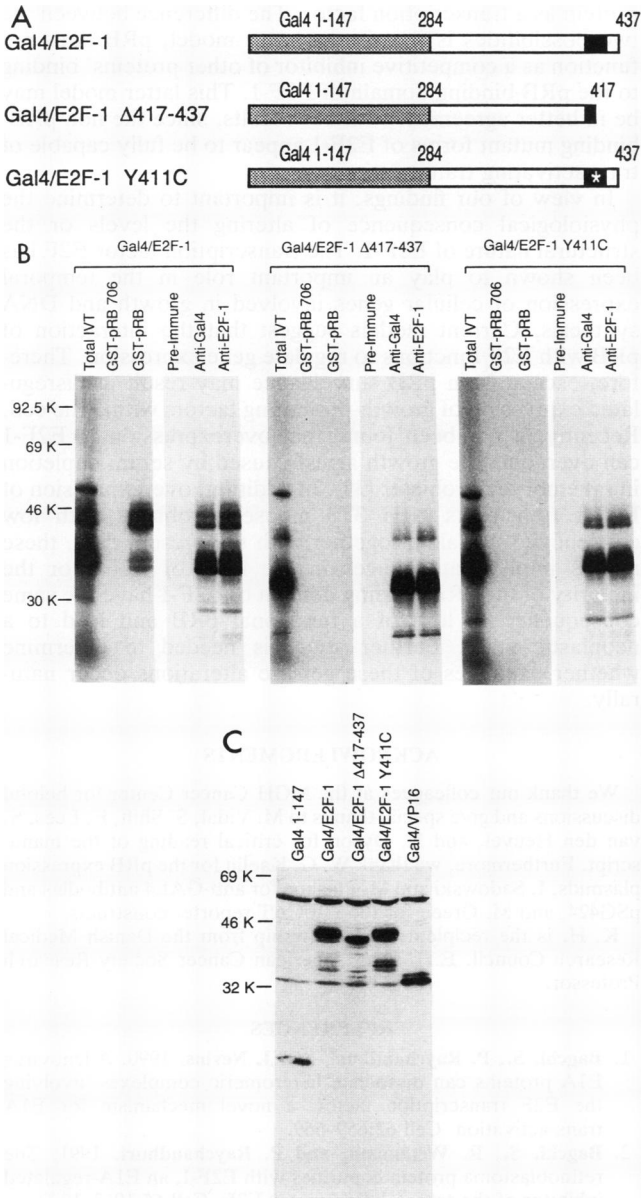

FIG. 4. Expression of GAL4–E2F-1 fusion proteins. (A) Diagram of wild-type and mutant GAL4–E2F-1 fusion proteins expressed either as in vitro-translated (IVT) products or in tissue culture cells from a mammalian expression vector. All three proteins are fusions between GAL4 (amino acids 1 to 147) and E2F-1 (amino acids 284 to 437 for GAL4–E2F-1, 284 to 417 for GAL4–E2F-1 Δ417-437, or 284 to 437 with the substitution of tyrosine for cysteine at position 411 for GAL4–E2F-1 Y411C). (B) Binding of in vitro-translated GAL4–E2F-1 proteins to pRB. In vitro-translated GAL4–E2F-1 fusion proteins were examined for binding to GST-pRB or GST-pRB with the single amino acid mutation of C to F at position 706 (31). The translated products were also immunoprecipitated with preimmune serum and anti-GAL4 (kind gift of I. Sadowski and M. Ptashne) and anti-E2F-1 (20) antibodies. (C) Detection of GAL4 fusion proteins in transfected C-33A cells. Anti-GAL4 antibodies were used in Western blotting experiments to examine the expression of GAL4 or the GAL4–E2F-1 fusion proteins. The numbers to the left of panels B and C are molecular sizes in kilodaltons (K).

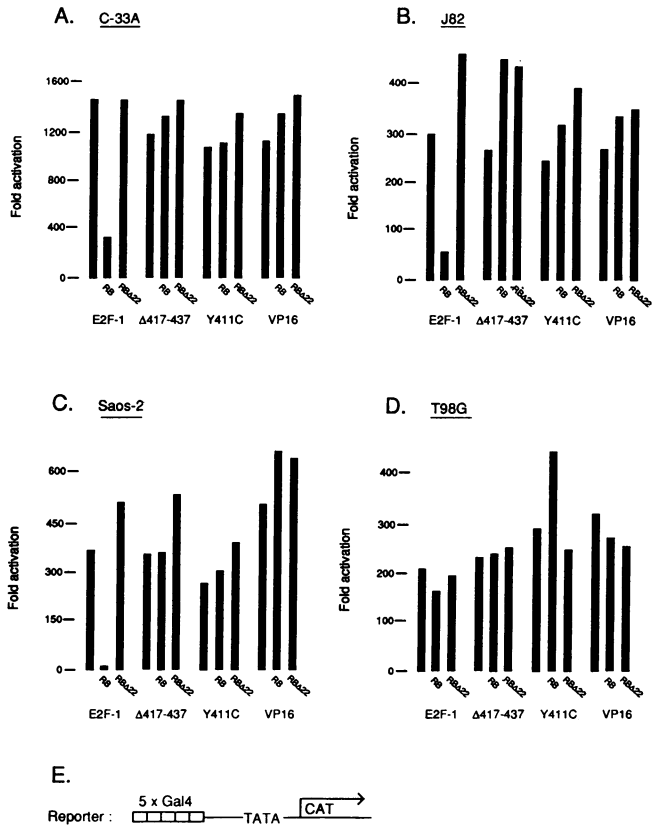

FIG. 5. Transactivation by the GAL4–E2F-1 constructs through GAL4 DNA-binding sites. The reporter construct shown in panel E, containing five GAL4 DNA-binding sites, has been described previously (33). The corresponding GAL4–E2F-1 or the GAL4-VP16 expression constructs were transfected into C-33A (A), J82 (B), Saos-2 (C), or T98G (D) cells in the presence or absence of a pRB or pRBΔ22 expression plasmid as described in Materials and Methods. The data presented are representative of at least three independent transfection experiments. Fold activation was measured as described in the legend to Fig. 3, by setting the level of CAT activity from the reporter plasmid transfected alone as unity.

DISCUSSION

Two main lines of evidence support the notion that pRB negatively regulates cellular growth. (i) Loss or mutations within both alleles of the *RB-1* gene are characteristic of nearly all retinoblastomas and a number of other human cancers. (ii) The oncoprotein products of several DNA tumor viruses, such as adenovirus E1A, tightly bind to pRB, and the transforming ability of these oncoproteins is dependent on pRB binding. These genetic and biological results strongly suggest that loss of pRB function provides a growth advantage to affected cells.

The finding that pRB binds to the transcription factor E2F has suggested a possible mechanism of pRB function. E2F was originally defined as a cellular factor that bound to specific sequences within the adenovirus E2A gene promoter and whose activity was enhanced in the presence of the E1A proteins. Furthermore, the same regions of E1A were shown to be essential for binding to pRB and cellular transformation, as well as enhancement of E2F transactivating functions. These findings led to the demonstration that pRB binds to E2F and that the viral oncoproteins dissociate

pRB-E2F complexes and, hence, result in active E2F. In addition, expression of pRB in cells lacking this tumor suppressor protein led to a decrease in the levels of E2F activity. The recent cloning of the *E2F-1* gene based on the ability of its product to bind directly to pRB has allowed us to demonstrate that E2F-1 is negatively regulated by pRB and elucidate the mechanism of this regulation.

We have previously determined that as few as 18 amino acids (amino acids 409 to 426) in the carboxy-terminal region of E2F-1 are sufficient for direct binding to pRB (20). In addition, it has been demonstrated that the carboxy-terminal region of E2F-1 mediates its transactivation functions (30). To understand the relationship between pRB binding and E2F-mediated transactivation, it was essential to determine whether these two functions of E2F-1 were separable or overlapping. Our approach to this question was to generate and analyze mutant E2F-1 proteins which no longer bound to pRB. Since our goal was to specifically analyze the transactivation functions of E2F-1, we took advantage of the modular structural nature of this protein and constructed hybrid fusions between the transactivation domain of E2F-1 and the DNA-binding domain of yeast activator GAL4. This would allow us to monitor specific E2F-1-mediated transactivation through an independent (foreign) DNA-binding sequence. The region of E2F-1 used to generate such hybrid proteins was chosen to lie outside the DNA-binding (20, 30, 47) and dimerization (28, 29) domains of E2F-1.

One of the E2F-1 mutant proteins that we generated contained a point mutation within the pRB-binding domain of E2F-1 (Y411C) which led to a non-pRB-binding E2F-1 protein. In addition, we had previously demonstrated that deletion of the carboxy-terminal 21 amino acids of E2F-1 (Δ417-437) also abolishes E2F-1's ability to bind to pRB. Our results show that the integrity of the pRB-binding domain is not necessary for E2F-1-mediated transactivation as assayed by using either a full-length or a GAL4 fusion E2F-1 construct. These results are consistent with those of others (47) which also indicated that alteration of the pRB-binding domain did not impair E2F transactivation.

Our second goal was to determine the effect of pRB on the transactivation functions of E2F-1. For this purpose, we took advantage of cell lines lacking a functional pRB to determine the effects of its coexpression with the E2F-1 constructs. Transactivation by wild-type E2F-1 (both full length and in a GAL4 fusion) was inhibited when a functional pRB was present. However, neither the point mutant nor the carboxy-terminal deletion forms of E2F-1 were affected by the exogenous expression of pRB in these cells. These results, combined with the fact that E2F-1 binds directly to pRB, demonstrate that pRB inhibits E2F-1-mediated transactivation through direct binding.

How does the binding of pRB to E2F-1 inhibit the activity of this transcription factor? A likely mechanism for this inhibition may be that pRB blocks the binding of E2F-1 to other proteins. Considering that the pRB-binding domain of E2F-1 lies within its transactivation domain, such proteins may be components of the basal transcription machinery. Therefore, identification of cellular proteins which specifically interact with the transactivation domain of E2F-1 may provide insight into the mechanism of pRB-mediated inhibition of transcription. In this context, components of the basal transcription machinery have been shown to interact with the activation domains of certain transcription factors (23, 34, 50). It is possible that E2F-1 can also interact with such cellular proteins to mediate E2F-1-dependent transactivation.

Alternatively, pRB may induce local allosteric changes in the E2F-1 structure which would, in turn, incapacitate the protein as a transcription factor. The difference between the two possibilities is that in the latter model, pRB does not function as a competitive inhibitor of other proteins' binding to the pRB-binding domain of E2F-1. This latter model may be in better agreement with our results, since the non-pRB-binding mutant forms of E2F-1 appear to be fully capable of transactivating transcription.

In view of our findings, it is important to determine the physiological consequence of altering the levels or the structural nature of E2F-1. The transcription factor E2F has been shown to play an important role in the temporal expression of cellular genes involved in growth and DNA synthesis. Current models suggest that the interaction of pRB with E2F functions to regulate gene expression. Therefore, escape from pRB surveillance may result in disregulated expression of growth-promoting factors within the cell. Recently, it has been found that overexpression of E2F-1 can overcome the growth arrest caused by serum depletion in rat embryo fibroblasts (39). In addition, overexpression of E2F-1 transforms NIH 3T3 mouse fibroblasts with low efficiency (37). Taken together with the present data, these results imply that changes in the level of E2F-1 or the integrity of the pRB-binding domain of E2F-1 have the same consequence as loss of a functional pRB and lead to a neoplastic state. Further study is needed to determine whether examples of these genetic alterations occur naturally.

ACKNOWLEDGMENTS

We thank our colleagues at the MGH Cancer Center for helpful discussions and give special thanks to M. Vidal, S. Shiff, E. Lees, S. van den Heuvel, and N. Dyson for critical reading of the manuscript. Furthermore, we thank W. G. Kaelin for the pRB expression plasmids, I. Sadowski and M. Ptashne for anti-GAL4 antibodies and pSG424, and M. Green for the E1bCAT reporter constructs.

K. H. is the recipient of a fellowship from the Danish Medical Research Council. E.H. is an American Cancer Society Research Professor.

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