The Transcription Factor E2F-1 Mediates the Autoregulation of RB Gene Expression

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The retinoblastoma (RB) gene is the prototype tumor suppressor gene. Mutations in this gene are often associated with the occurrence of various tumors. Several mutations have been found in the promoter region of the gene, suggesting that inappropriate transcriptional regulation of the RB gene contributes to tumorigenesis. Sequence analysis of the RB promoter has revealed a potential E2F recognition site within a region critical for RB gene transcription. By using the cloned E2F-1 gene, here we report that (i) RB expression is negatively regulated by its own gene product, (ii) E2F-1 binds specifically to an E2F recognition sequence in the RB promoter and transactivates the RB promoter, (iii) overexpression of RB suppresses E2F-1-mediated stimulation of RB promoter activity, and (iv) the expression of the RB gene is paralleled by the expression of the E2F-1 gene during cell cycle progression. These results demonstrate that expression of RB is negatively autoregulated through E2F-1.

The retinoblastoma (RB) gene encodes a nuclear phosphoprotein that is constitutively expressed in most normal vertebrate cells (41; for a review, see reference 18). Structural analysis of the RB gene has revealed a complicated genomic organization containing 27 exons (3, 29). The major promoter activity for the RB gene is contained within a 71-bp G+C-rich region between nucleotides –264 and –194 relative to the translation initiation codon (29). A naturally occurring tumor that contains a 103-bp deletion including this region fails to express detectable Rb protein (4). In addition, two other naturally occurring mutations in this region or next to this region have been found in retinoblastoma patients (52), further suggesting that inappropriate transcriptional regulation of the RB gene contributes to tumor formation.

The RB promoter has characteristics similar to many housekeeping genes that are ubiquitously expressed (29). As a consequence, the amount of Rb protein expressed is relatively constant in a given cell type. When additional Rb protein is introduced into cells, either by transfection or by injection, cells frequently arrest in the G₁ phase of the cell cycle (19, 28, 48, 50). Transgenic mice containing extra copies of the human RB gene are smaller than their non-transgenic littermates, demonstrating that RB overexpression causes growth retardation at the level of the organism (1). Conversely, inactivation of the endogenous Rb protein is tightly associated with neoplastic growth (for a review, see reference 2). Recently, we and others have observed that mice lacking normal Rb protein die during embryogenesis (10, 34, 39). Crossing RB-deficient mice heterozygous for the normal RB gene with human RB-transgenic mice can rescue embryos from embryonic death, and the rescue is dependent on the amount of Rb protein expressed from the transgene (7). These results suggest that the amount of Rb protein in the cell is critical for normal cell growth and differentiation.

Numerous studies have focused on the roles that Rb

protein plays in regulating cell growth. The mechanism by which RB executes its regulation is, however, largely unknown. One hypothesis is, by analogy to the viral oncoproteins of DNA tumor viruses, that Rb protein interacts with several cellular proteins and regulates cell growth through these complexes. Indeed, a number of cellular proteins that interact with Rb have been identified, including RBp1 and RBp2 (14), a 46-kDa protein (32), c-Myc and N-Myc (51), ATF-2 (38), cdc2 kinase (30), phosphatase type 1 (16), transcription factor E2F-1 (27, 36, 55), myogenic factors (21), D cyclins (15, 17, 45), RbAp48 (49), and other proteins (22, 37, 40, 55). Among these associated proteins, E2F-1 is the best characterized and has been shown to interact with Rb both in vitro and in vivo.

E2F was originally identified as a cellular factor mediating the transcription of the adenovirus E2 gene (for a review, see reference 47). The cloned E2F-1 gene product binds to the E2F DNA recognition sequence in the E2 promoter and transcriptionally activates promoters with E2F binding sites (27, 55). This E2F-1-mediated transactivation can be inhibited by Rb through direct protein-protein interaction (26). Overexpression of E2F-1 induces quiescent cells to enter S phase (35), in agreement with the observations that the promoters of several growth-related genes, including *c-myc*, *N-myc*, *cdc2*, DNA polymerase α , thymidine kinase, and dihydrofolate reductase, contain the E2F recognition sequence (for a review, see reference 25). This finding is particularly interesting since one of the cellular functions of RB appears to be to restrict entry of cells into S phase of the cell cycle. E2F may be a partner for Rb protein in regulating the genes involved in G₁-S progression, since E2F is known to associate with RB in a cell cycle-dependent manner (6, 56).

Sequence analysis of the RB promoter has revealed a potential E2F recognition site within the region critical for promoter activity, which raises the possibilities that the RB promoter itself is a target for E2F and that RB regulates its own gene expression through E2F. The E2F-Rb interaction, therefore, may participate in a feedback regulatory pathway in controlling expression of the RB gene. Here we test this hypothesis by using the cloned E2F-1 and demonstrate that

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the transcription of RB is down-regulated by its own gene product through E2F-1.

MATERIALS AND METHODS

Plasmid constructions. The DNA fragments containing the RB promoter sequence and the series of 5' deletions were cloned upstream of the chloramphenicol acetyltransferase (CAT) gene as described previously (29), and the endpoint numbers of deletion constructs were changed to numbers relative to the translation initiation codon. The RB promoter with a 103-bp internal deletion was reported previously (4). Constructs for glutathione *S*-transferase (GST)-E2F fusion proteins GST-SH5, GST-BB8, GST-P3, and GST-BH7 were described in a previous report (55). GST-D4 was constructed by subcloning a *DdeI*-*HindIII* fragment of E2F-1 in frame into GST-2T (Promega, Madison, Wis.); GST-HX5 was derived from GST-P3 by deleting the 3' sequence of an *XhoI* site (at position 1251). pCMVE2F-1 (previously named pCMVAp12*Stu*) and pCMVE2F-DS (previously named pCMVAp12-DS) were described previously (55); pCMVE2F-D was constructed by digestion of pCMVE2F-1 with *DraIII* (at position 227) and by introduction of a stop codon; pCMVE2FBH was constructed by digestion of pCMVE2F-1 with *BglII* (at position 847) and *BamHI*, and 3' sequence of the *BglII* site was removed; and pCMVE2F-X was generated by digestion of pCMVE2F-1 with *XhoI*, and the downstream sequence was deleted. All E2F-1 mutant constructs were confirmed by sequencing. pRb-3HA/SVE was kindly provided by Dennis Templeton (Case Western Reserve University, Cleveland, Ohio); the *c-jun* promoter (1.2 kb)-CAT construct was the gift of Micheal Karin (University of California at San Diego, San Diego, Calif.); the *c-myc* promoter (1.4 kb) was kindly provided by David Hall (Thomas Jefferson University, Philadelphia, Pa.); the EGR-1 promoter (1.0 kb)-CAT construct was the gift of Vikas Sukhatme (University of Chicago, Chicago, Ill.), and pCMV-CAT was purchased from Invitrogen (San Diego, Calif.).

DNA gel mobility shift assay. The *EcoRI*-*HindIII* DNA fragment containing nucleotides -290 to -90 of the human RB promoter from pRBCAT 3-9 (29) was end labeled with Klenow fragment and [α - 32 P]dCTP and then purified by gel electrophoresis by standard methods (53). The conditions for the assay were similar to those described previously (55). The 32 P-end-labeled fragment (1 ng) was incubated with the diluted GST-Ap12 bacterial lysates in 12.5 μ l of binding buffer [20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.6), 1 mM MgCl₂, 0.1 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 40 mM KCl, 10% glycerol, 0.1% Nonidet P-40, 1 μ g of salmon sperm DNA] at room temperature for 30 min. The DNA fragments (70 ng) containing either the wild-type E2F recognition sequence (TTTCGC GC---GCGCGAAA) or the mutated E2F site (TTTAGCG C---GCGCTAAA) were added as competitors. Protein-DNA complexes were separated on a 4% polyacrylamide gel in 0.25 $\times$ Tris-borate-EDTA, and the gel was dried and autoradiographed.

Immunoblot analysis. The purified bacterially expressed GST-E2F fusion proteins (0.1 μ g) or transfected Saos2 cells (2×10^5) were lysed directly in sodium dodecyl sulfate loading buffer and were analyzed by polyacrylamide gel electrophoresis. Gels were transferred to Immobilon membranes (Millipore Corp., Bedford, Mass.). The blots were incubated with a monoclonal anti-E2F-1 antibody 17E2 and

then followed by using either the ProtoBlot Western Blot AP System (Promega), for detecting GST-E2F fusion proteins or the ECL Western Blotting Detection System (Amersham, Arlington Heights, Ill.) for detecting transfected E2F gene products.

Site-directed mutagenesis. Mutagenesis was carried out by a PCR-based method. The primers to generate point mutations in the E2F recognition site of the RB promoter were as follows: A mutation, ACCGGAATTCGAGCTCGCCAGT GACGTTTACCGCGGTTGGA; AA mutation, ACCGGAATTCGAGCTCGCCAGTGACGTTTACCGCGATTGGA; and AAA mutation, ACCGGAATTCGAGCTCGCCAGT GACGTTTACCGCGATTGGA. The template for the mutagenesis was RB-P-CAT (-194), which was generated by 5' deletion of pRbCAT2 (29). The sequences in boldface were from the RB promoter including ATF and E2F sites, and the rest were from the pGEM-1 vector including an *EcoRI* site. The nucleotides underlined were those which had been changed. The other primer used for PCR was a 23-mer oligonucleotide from the CAT gene (ATACGGAATTCGG GATGAGCATT) which also contained an *EcoRI* site. The PCR products were digested with *EcoRI* and were subsequently used to replace the wild-type *EcoRI* fragment of RB-P-CAT. The mutations were confirmed by sequencing.

Cell synchronization and Northern (RNA) blot analysis. Normal monkey kidney CV1 cells were cultured in Dulbecco's modified Eagle medium with 10% fetal calf serum in the presence of Lovastatin (50 μ M) for 36 h and then released for 8 h or in the presence of hydroxyurea (0.5 mM) for 16 h and then released for 5 h or were incubated in the presence of nacodazole (0.4 μ g/ml) for another 16 h. Total RNAs were then extracted from the cells synchronized at these stages of the cell cycle. In serum stimulation experiments, CV1 cells, cultured in 0.5% fetal calf serum for 4 days followed by addition of 10% serum for an appropriate period, were harvested for RNA extraction. Ten micrograms of the RNAs was denatured and analyzed by formaldehyde gel electrophoresis. Three sets of RNA blots were hybridized with 32 P-labeled cDNAs of full-length E2F-1, RB, or the G β -like gene (55).

Transient-transfection assay. Transfection assays were performed in Saos2 cells by the calcium phosphate precipitation method (53). Briefly, purified plasmid DNAs in 0.1 $\times$ Tris-EDTA buffer with 250 mM of CaCl₂ were added dropwise to 2 $\times$ HEPES-buffered saline solution (280 mM NaCl, 10 mM KCl, 1.5 mM Na₂HPO₄, 12 mM glucose, 50 mM HEPES), and the mixtures were incubated at room temperature for 20 min. Each transfection was carried out in duplicate. In experiments described in the legend to Fig. 1, 5 μ g of reporter plasmid (RB-P-CAT or c-JUN-P-CAT) DNA was cotransfected with 5 μ g of control plasmid DNA (Rous sarcoma virus long terminal repeat driving a *lacZ* gene). In Fig. 3, 4, and 7, 5 μ g of CAT reporter plasmid DNAs and 5 μ g of cytomegalovirus (CMV) vector alone (an expression plasmid driven by the cytomegalovirus immediate-early gene promoter), CMVE2F-1, or E2F derivatives were transfected. In Fig. 5, 1 μ g of RB-P-CAT DNAs with point mutations and 5 μ g of CMVE2F-1 were transfected. In Fig. 8 and 9, 5 μ g of RB-P-CAT DNAs were cotransfected with 5 μ g of CMVE2F-1 (Fig. 8) or CMVE2F-X (Fig. 9), 5 or 10 μ g of RB-3HA/SVE, or 5 or 10 μ g of CMVE2F-BH. In most of the transfections, vector plasmid DNAs were added to adjust the total amount of DNAs to 20 μ g. The precipitates (1 ml) were added to the cells with 2 ml of Dulbecco's modified Eagle's medium in 10-cm dishes. Four hours after transfection, the cells were washed once with phosphate-

FIG. 1. The promoter activity of the RB gene is reduced in reconstituted RB⁺ cells. An RB promoter (1.6 kb)-CAT construct (RB-P) or 1.6 kb of *c-jun* promoter segment fused to a CAT reporter gene (c-JUN-P) was transfected into retinoblastoma cell line WERI-Rb27 or its reconstituted derivative WR2E3 cells. CAT activity was measured 40 h after transfections and was quantitated by using a PhosphorImager. A *lacZ* reporter gene driven by an RSV promoter was included in the transfections to serve as an internal control. The histograms represent the conversion rate of acetylation conducted by the CAT enzyme, reflecting the promoter activity in given cells.

buffered saline and incubated in 8 ml of fresh medium for 40 h. Cell extracts containing the same amount of proteins were used for CAT assays as previously described (20), and CAT activities were measured quantitatively by a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). Most experiments were repeated at least four times, and one of each of these (in duplicate) is presented in this report.

RESULTS

Transcription of RB is down-regulated in reconstituted RB⁺ cells. Several RB-reconstituted retinoblastoma cell lines have been generated by introducing a wild-type RB gene via retrovirus-mediated gene transfer (8, 31). Comparing these RB⁺ cells with parental or revertant RB⁻ cells, little difference has been found in their growth rates in culture. However, RB⁺ cells invariably lose their tumorigenicity in nude mice assays (8). These syngeneic cell lines provide us with a useful system to study whether RB has any effect on transcription of genes of interest, since the level of the Rb protein expressed in RB⁺ cells is close to the physiological level. To determine whether RB regulates its own transcription, the promoter sequence of RB was inserted 5' of the bacterial CAT reporter gene, and the resultant construct was transfected into RB⁺ and RB⁻ retinoblastoma cells. As shown in Fig. 1, RB promoter activity is significantly decreased in RB⁺ cells (WR2E3) compared with that in parental RB⁻ cells (WERI-Rb27), whereas the transcription of other promoters such as the *c-jun* promoter is unaffected in these cells. Since the reconstituted RB⁺ cells and parental RB⁻ cells appear to be genetically indistinguishable except for the reintroduced RB gene, these results indicate that the

FIG. 2. E2F-1 forms a specific complex with the RB promoter. **A** ³²P-labeled DNA fragment of the RB promoter containing the E2F recognition site was mixed with GST-E2F fusion proteins, and the DNA-protein complex was assayed by polyacrylamide gel electrophoresis. Lane 1, P3, a 115-amino-acid C-terminal portion of E2F-1, fused to GST, was used for the binding assay; lane 2, no protein was added to the DNA probe; lanes 3 to 5, BH7, a 102-amino-acid stretch of the protein containing the DNA-binding domain of E2F-1, was added to the probe for the assay; lane 4, in the presence of an unlabeled DNA fragment containing the wild-type E2F binding sequence; lane 5, an unlabeled DNA fragment containing a single nucleotide change in the E2F binding site.

transcription of RB is down-regulated by its own gene product.

The transcription factor E2F-1 binds to an E2F recognition sequence in the RB promoter. To study the mechanism of RB down-regulation, we have reexamined the RB promoter sequence and found a typical E2F recognition site, TTTC CCGC, from nucleotides -186 to -179. To test whether this E2F recognition site can be recognized by the cloned E2F-1 gene product, an in vitro DNA gel mobility shift assay was performed. A DNA fragment containing the RB promoter sequence from nucleotides -290 to -90 was radiolabeled and mixed with a bacterially expressed protein containing the DNA-binding domain of E2F-1 fused to GST. As shown in Fig. 2, this GST-E2F-1 fusion protein (BH7) binds to the ³²P-labeled DNA fragment. The formation of the DNA-protein complex is apparently specific, since the labeled complex is abolished by adding an unlabeled oligonucleotide containing the E2F cognate sequence but not a mutated E2F recognition sequence (55, 58). The multiple bands observed could be due either to the multimerization of the fusion protein product or to more than one E2F-binding site in the RB promoter. An additional control, GST-P3, a C-terminal portion of the GST-E2F-1 fusion protein (55), also fails to bind the labeled DNA fragment.

E2F-1 transactivates the RB promoter. To test the effect of E2F-1 on RB promoter activity, a plasmid expressing E2F-1 under the control of the CMV immediate-early gene pro-

FIG. 3. E2F-1 stimulates the transcriptional activity of the RB gene. (A) CAT activities for the effects of E2F-1 on various promoters, which were measured quantitatively by a Phosphor-Imager shown in panel B. The promoter-CAT constructs were transfected with either CMV vector DNA or CMVE2F-1 plasmid DNA, and CAT assays were performed. RB-P, RB promoter (1.6 kb)-CAT; RB-P-HS, RB promoter-CAT with a 103-bp internal (–248 to –146) deletion in the RB promoter; c-JUN-P, *c-jun* promoter (1.6 kb) driving CAT; EGR-P, EGR-1 promoter (1.0 kb) fused to CAT; c-MYC-P, *c-myc* promoter (1.4 kb) fused to CAT; CMV-P, CMV promoter directly fused to CAT. Each transfection was carried out in duplicate. (B) The fold stimulation of these promoter activities mediated by E2F-1 is shown at the bottom of the histograms.

motor was cotransfected with the RB promoter-CAT reporter construct into Saos2 cells, an osteosarcoma cell line which contains no functional Rb protein. As shown in Fig. 3, the expression of E2F-1 strongly stimulates RB promoter activity (by 10-fold), whereas E2F-1 has little or no effect on several growth-related gene promoters (*c-jun*, *c-myc*, and EGR-1, an early growth response gene), as well as the CMV promoter. We also tested the transcriptional activity of a naturally occurring RB promoter mutant (RB-P-HS) which fails to express a detectable level of Rb protein (4). This promoter is derived from a prostate tumor and contains a 103-bp deletion including the E2F site. Our results show that the internal deletion of this RB-P-HS promoter not only abolishes the basal level of transcriptional activity of RB but also fails to respond to E2F stimulation (Fig. 3), suggesting that the E2F site in the RB promoter participates in the transcriptional regulation of RB gene expression.

To identify the elements responsible for the E2F-mediated stimulation in the RB promoter, a series of 5' deletion mutants of the RB promoter sequence fused to the CAT

FIG. 4. A 40-bp promoter element of RB mediates the stimulation by E2F-1. (A) A series of 5' deletions of the RB promoter were fused to the CAT reporter gene, and the constructs were cotransfected in duplicate with either CMV vector DNA or CMVE2F-1 plasmid DNA. The numbers (–155, –194, –264, –290, –319, –612, –791, and –1241) indicate the 5' end of the promoter fragment relative to the translation initiation site. (B) The results from panel A were quantitated as the conversion rate of the acetylation and presented as histograms. (C) Schematic diagram of the serial deletions of the RB promoter. The numbers at the right ends of the horizontal bars indicate the 5' boundary relative to the translation initiation site. The detailed sequence from –206 to –173 containing the E2F site is also included. The numbers in the right column indicate the fold stimulation by E2F-1, calculated by dividing the percentage of acetylation in cells transfected with CMVE2F-1 by the percentage in cells transfected with CMV plasmid alone.

reporter gene were cotransfected with the E2F expression plasmid into cells and CAT activities were measured. As shown in Fig. 4, the minimal promoter sequence responsible for the E2F-1-mediated stimulation resides within a region from nucleotide –194 to –155. Not surprisingly, the E2F site TTTCCCGC is contained within this 40-nucleotide region. The promoter constructs containing the longer 5'

upstream sequence have no obvious additional effects on E2F-mediated stimulation, suggesting that it is the E2F site that mediates stimulation. To pinpoint the sequence responsible for the stimulation, point mutations of the E2F recognition site in the RB promoter were generated by site-directed mutagenesis. The E2F site plus its 3' adjacent sequence TTTCCCGCGGTT was changed to either TTTACCGCGGTT (A mutant, underlined), TTTACCGCGATT (AA mutant), or TTTACCGCGATT (AAA mutant). The overall strategy for the mutagenesis was to change a G or C to an A, since previous experiments with the E2 promoter indicated that a G-to-A conversion abolished the protein-DNA interaction (55, 58). These promoter-mutated CAT constructs were cotransfected with CMVE2F-1, and the results are shown in Fig. 5. While the RB promoter activities with one or two point mutations (A or AA mutant) are similar to that of the wild type, the triple point mutation which disrupts the GC stretch of the E2F site (AAA mutant) results in a substantial decrease in activity. These observations are different from those obtained with the E2 promoter. One possibility is that the number of G's or C's in the E2F site is critical for the E2F-1-mediated stimulation, since the E2 promoter contains five GCs after T's, whereas the RB promoter contains seven GCs. The A or AA mutants still retain near-wild-type activities, because there are still five GCs in the middle even though two GCs at the ends have been changed to A's. Another possibility is that there are two overlapping E2F sites which share the middle seven GCs, and each of these two sites is sufficient for conducting E2F-1-mediated transactivation of the RB promoter. Further detailed mutagenesis is needed to test these hypotheses.

E2F-1-mediated stimulation requires both the E2F DNA-binding domain and transactivation domain. Like most other transcription factors, E2F-1 contains a DNA-binding domain and a strong transcriptional activation domain. In addition, E2F also contains an Rb-binding domain in its C terminus (27, 55). To demonstrate that transcriptional stimulation for the RB promoter is indeed due to expression of a functionally active E2F-1, several deletion mutants of E2F-1, including deletions of its DNA-binding domain, Rb-binding domain, or transactivation domain, were cloned into the CMV expression plasmid. We first examined whether the transfected genes were expressed in Saos2 cells. An anti-E2F-1 monoclonal antibody, 17E2, was raised by injecting a purified, bacterially expressed fusion protein, GST-SH5, which contained the C-terminal 315 amino acids of E2F-1, into mice. To determine the specificity of the antibody, we constructed a series of GST-SH5 deletion mutants. As shown in Fig. 6A and B, the antibody binds to GST-SH5, GST-BB8, GST-P3, and GST-HX5 but not to GST-D4 and GST-BH7, indicating that the antibody specifically recognizes a C-terminal region of E2F-1 containing 58 amino acids (323 to 380). This antibody was then used to examine the expression of transfected genes in Saos2 cells, including E2F-1 (the wild type), E2F-X (a mutant deleting the Rb-binding domain), and E2F-DS (a mutant deleting the DNA-binding domain). As shown in Fig. 6C, transfected cells readily express detectable E2F proteins and the molecular weights for the wild type and mutants are as predicted. Next, we assayed the effects of these mutants on the RB promoter activity. As shown in Fig. 7, when the mutant DNAs containing either the N-terminal region of the protein (E2F-D) or the N-terminal region plus the DNA-binding domain (E2F-BH) are cotransfected with the RB promoter-CAT construct, no stimulation of transcription is observed. Similarly, the construct containing a deletion in the DNA-

FIG. 5. Point mutations in the E2F site of the RB promoter abolish the E2F-1-mediated stimulation. Point mutations were generated by site-directed mutagenesis. The minimal promoter-CAT construct (-194) containing the E2F site was used as a template. The E2F site plus its 3' adjacent sequence TTTCCCGCGGTT was changed to TTTACCGCGGTT (A mutant; underlined), TTTACCGCGATT (AA mutant), or TTTACCGCGATT (AAA mutant). The minimal promoter (-194) was then replaced by the PCR products containing the mutated E2F sequences. (A) The promoter mutants were cotransfected with CMVE2F-1 in duplicate, and CAT activities were measured. (B) The results from panel A were quantitated by a PhosphorImager and presented as histograms. (C) Detailed sequence of the E2F site in the RB promoter and resultant point mutations. The numbers in the right-hand column indicate the relative CAT activity, setting wild-type (WT) promoter activity as 1.00.

binding domain (E2F-DS) also shows no activity. Thus, we conclude that the stimulation of RB transcription requires both the E2F-1 DNA-binding domain and the transactivation domain of the protein, suggesting that E2F-1 specifically mediates the transcriptional activation of the RB gene. Interestingly, the mutant containing the truncated Rb-binding domain (E2F-X) retains some stimulatory activity, although the activity is reduced, consistent with our previous observation made with a GAL4-E2F-X fusion protein (55).

Overexpression of RB suppresses the E2F-mediated stimulation of the RB promoter. As shown in Fig. 1, RB promoter

FIG. 6. The transfected E2F-1 and its derivatives were expressed in Saos2 cells. (A and B) Monoclonal anti-E2F-1 antibody 17E2 specifically recognized a region of E2F-1 containing amino acids 323 to 380. Purified GST-SH5 and its derivatives were analyzed by Western blot with 17E2. (C) Extracts from the transfected cells were analyzed by Western blot with 17E2. The arrow indicates a nonspecific species with which the antibody cross-reacts. Lane 1, cell extracts transfected with CMVE2F-1; lane 2, extracts transfected with CMVE2F-X; lane 3, extracts transfected with CMVE2F-DS; lane 4, extracts transfected with CMV.

activity is down-regulated in Rb⁺ cells. To determine whether this down-regulation is mediated through E2F, we cotransfected an RB expression vector driven by simian virus 40 early promoter (Rb-3HA/SVE), with CMVE2F-1, together with the minimal RB promoter-CAT (Fig. 4C, -194) containing the E2F site and then assayed for CAT activity. As shown in Fig. 8, overexpression of RB suppresses the E2F-1-mediated transactivation of the RB promoter. To ensure that this repression is not due to the effect of Rb on the CMV promoter which is used for transcribing E2F-1, the RB expression plasmid was cotransfected with a DMV-CAT construct and was shown to have no effect on CMV promoter activity (data not shown). To determine whether transactivation of the RB promoter is mediated specifically through E2F-1, one additional experiment was performed: overexpression of a truncated E2F-1 containing only the E2F DNA-binding domain, which would compete

FIG. 7. E2F-1 mutants fail to stimulate RB promoter activity. (A) Transfections of E2F-1 or E2F-1 mutants with RB promoter-CAT (-194) were carried out in duplicate, and CAT activities were measured; (B) quantitation by a PhosphorImager; (C) schematic diagram of E2F-1 and its mutants. E2F-D encodes amino acids 1 to 75; E2F-BH encodes amino acids 1 to 282; E2F-X encodes amino acids 1 to 417, and E2F-DS contains an internal deletion of 75 amino acids (75 to 150). The numbers in the right-hand column indicate the fold stimulation of CAT activity by expression of these constructs.

with the wild-type E2F-1 for the E2F-binding site in the RB promoter. A plasmid expressing only the DNA-binding domain of E2F-1 (E2F-BH) was cotransfected with the wild-type E2F-1. Our results show that the wild-type E2F-1 activity is reduced by the addition of the mutant E2F-BH, indicating that the stimulation of the RB promoter activity is an E2F-1-specific effect.

To further determine whether the interaction between E2F-1 and Rb is necessary for the Rb-mediated repression, the Rb-binding domain deletion mutant of E2F-1, E2F-X, was cotransfected with the RB expression plasmid and transcriptional activity on the RB promoter was tested. As shown in Fig. 9, while this mutant alone is still able to stimulate the Rb promoter activity, overexpression of the RB gene is no longer able to suppress the stimulation, indicating that the interaction between Rb and E2F-1 is necessary for the repression. As an E2F specificity control for E2F-X, cotransfection of E2F-BH, the DNA-binding domain of E2F-1, is able to compete with E2F-X activity, similar to the effect seen with the wild-type E2F-1.

FIG. 8. RB suppresses the stimulation of its own promoter mediated by E2F-1. (A) An RB expression plasmid (Rb-3HA/SVE) or a plasmid expressing a C-terminal truncated E2F-1 lacking its transactivation domain (CMVE2F-BH) was cotransfected with CMVE2F-1 at a ratio (wt/wt) of 1:1 or 2:1, along with the RB promoter (−194), into Saos2 cells. The experiments were carried out in duplicate. (B) CAT activities were measured quantitatively as histograms. The effects of RB repression on E2F-mediated stimulation were measured as a percentage of the stimulation by E2F-1 in cells transfected with RB relative to that of cells transfected without RB, as indicated at the bottom of the histograms.

The expression of endogenous RB gene is cell cycle regulated. Given that the expression of the E2F-1 gene is under cell cycle control (36, 55), one would expect to see a change in the expression of the RB gene at the G₁/S boundary. To determine whether the expression of the RB gene is cell cycle regulated, Northern blot analyses were performed with two sets of RNAs prepared from normal monkey kidney CV1 cells synchronized at different stages of the cell cycle either by drug arrest or by serum stimulation. As shown in Fig. 10, the accumulation of the mRNA level of RB is increased by fivefold at the G₁/S boundary (hydroxyurea arrest) or by threefold at 18 h after release from serum starvation of CV1 cells. The expression of the RB gene during cell cycle progression is paralleled by the cell cycle-dependent expression of the E2F-1 gene, suggesting that E2F-1 is capable of regulating expression of the RB gene under physiological conditions. As a control, the expression of the G β -like gene is rather constant during the cell cycle (55). These observations for the first time provide clear evidence that the expression of the RB gene is also subjected to cell cycle regulation and that E2F-1 is probably a major player in this process.

DISCUSSION

There are a number of observations which suggest the level of Rb protein is tightly regulated. The most striking

ones come from animal studies. In RB transgenic mice, although the RB gene copy number could vary from one to seven, the level of protein produced has an upper limit (1). Moreover, the high-copy-number strains are unstable, losing gene copies after several generations, suggesting that only a certain amount of Rb protein can be tolerated by cells in the animals. Notably, these transgenic mice were generated by expressing human RB driven by its own promoter (1). Furthermore, studies from crossing RB transgenic mice with RB-deficient mice (7) have shown that phenotypic differences in the rescued mice are determined by how much Rb protein is actually expressed. This dosage effect of the Rb protein is most evident when one notes that no Rb protein causes embryonic lethality and too much Rb protein results in undersized mice. Such data support the notion that the dosage of Rb protein plays a key role in normal animal development. An Rb dosage effect has also been observed in microinjection studies with purified Rb protein, causing G₁ arrest (19). All of these observations strongly suggest that the level of Rb protein is critical for its normal cellular functions and that expression of RB must be tightly controlled.

Most studies of regulation of Rb have so far been focused on how phosphorylation regulates functions of the Rb protein. Rb phosphorylation oscillates during the cell cycle (5, 9, 12, 13), and several putative Rb kinases involved in this process have been identified (17, 28, 42, 43, 45). However, it

FIG. 9. Rb-E2F-1 interaction is necessary for Rb repression of E2F-mediated stimulation of the RB promoter. (A) An RB expression plasmid (Rb-3HA/SVE) or a plasmid expressing a C-terminal and transactivation domain-truncated E2F-1 (E2F-BH) was cotransfected with CMVE2F-X at a ratio (wt/wt) of 1:1 or 2:1, along with the RB promoter (–194), into Saos2 cells. The experiments were carried out in duplicate. (B) CAT activities were measured quantitatively as histograms. The effects of RB repression on E2F-mediated stimulation were measured as a percentage of the stimulation by E2F-1 in the presence of RB relative to stimulation in the absence of RB, as indicated at the bottom of the histograms.

has also been suggested that expression of the RB gene may be transcriptionally regulated. (i) In three cell lineages, namely, erythroid, muscle, and B cells, late stages of differentiation were associated with increased amounts of RB mRNA (11). (ii) A good correlation was observed between RB gene expression and differentiation in testicular germ cell tumors (57). (iii) Naturally occurring mutations in the promoter region of the gene have been found in three patients with tumors (4, 52). These evidences suggested that the transcriptional regulation of the RB gene may be crucial for participating tumor suppression.

The results presented in this report indicate that the expression of RB is autoregulated by its own gene product and that this negative autoregulation is mediated by the transcription factor E2F-1. These observations were made not only with Saos2, an osteosarcoma cell line, but also with normal monkey kidney CV1 cells (54). In addition, the expression of the RB gene is paralleled by the expression of E2F-1 in the cell, and expression of both genes is increased gradually during the G₁ transition and peaks at the G₁/S boundary. These observations are in agreement with our

previous finding that Rb exerts its growth inhibitory function in early G₁ (19). The presence of an appropriate amount of the Rb protein during this time window is apparently tightly controlled. If for any reason this kind of tight control is lost, the cell may have too much Rb protein and cell division will be arrested. On the basis of these results, a possible mechanism of how an appropriate amount of the Rb protein is modulated during cell cycle progression can be suggested. The Rb protein is constitutively synthesized in general during the cell cycle. However, in early G₁, when Rb is demanded for its growth inhibitory function, the expression of RB is up-regulated by E2F-1, whose expression is activated a little earlier than that of RB. When the Rb protein is in excess, it binds to E2F-1 and inactivates it, thus appropriately controlling its own production. In addition, at about the same time or earlier, Rb becomes gradually phosphorylated. It is believed that the phosphorylation of the Rb protein is one of the key mechanisms for regulating its own activities, and only the hypophosphorylated form of Rb protein is able to interact with viral oncoproteins and cellular proteins, including E2F-1 (27, 44, 55). As a consequence, the

FIG. 10. The expression of RB and E2F-1 during cell cycle progression. Total RNAs from synchronized CV1 cells were hybridized either with 32 P-labeled RB, E2F-1, or G β -like cDNAs. (A) Cells were treated with lovastatin for 36 h to arrest cells in early G₁ phase (G₀/G₁ [lane 1]), followed by an 8-h release to allow cells to enter early mid-G₁ phase (G8 [lane 2]); with hydroxyurea to arrest cells at the G₁/S boundary (G₁/S [lane 3]), followed by a 5-h release to allow entry into S phase (S [lane 4]); or with nocodazole to arrest cells in the mitotic phase (M [lane 5]). The levels of RB and E2F-1 mRNAs from different stages of the cell cycle were quantitated by the PhosphorImager and were normalized by the level of expression of the G β -like gene, represented as relative mRNA levels in panel C. (B) Cells were cultured in 0.5% serum for 4 days, and then 10% serum was added, and cells were harvested at appropriate times. Lane 1, mRNA prepared from starved cells; lane 2, 2 h; lanes 3, 4 h; lane 4, 6 h; lane 5, 8 h; lane 6, 12 h; lane 7, 18 h; lane 8, 24 h after serum addition, respectively. (D) The mRNA levels of RB and E2F-1 were normalized to that of G β -like.

phosphorylation of Rb releases its binding to E2F-1 and probably results in further increased expression of the RB gene. At the G₁/S boundary, Rb is hyperphosphorylated, thus adjusting its own activity.

However, since RB expression does not precisely oscillate in synchrony with E2F-1, other factors may be important in regulating RB transcription. There are several binding sites in the RB promoter for other transcription factors such as ATF (23) and one similar to Sp1 (46), implicating the complexity of the promoter. Our preliminary results indicate that the overexpression of ATF-2 only slightly increases RB promoter activity; coexpression of both E2F-1 and ATF-2 results in no apparently synergistic effect on the RB promoter. Another interesting molecule that might regulate RB gene expression is RBF-1 (52), a nuclear protein that binds to the Sp1-like sequence in the RB promoter. In addition, there seem to be multiple E2Fs (25), and they can form heterodimers between family members (33), which may also modulate RB expression. On the basis of our results from deletion analysis of the RB promoter sequence, it is likely that there are two types of transcription factors for the regulation of RB gene expression. One is for controlling the basal level of transcription that contributes to the constitutive expression of the Rb protein. RBF-1 may belong to this

group, since its binding site is absolutely required for the basal level of RB transcription (29, 52, 59). The other one is for regulating the transcription in response to environmental changes such as signals for cell growth and differentiation. E2F-1 (and other E2Fs) and ATF may be included in this group, since basal activity of RB transcription is very low, even when both E2F and ATF sites are intact. In the presence of an extra amount of E2F-1, the low-activity promoter can be converted into a high-activity one, causing RB gene expression to increase to a level much higher than the basal level.

One of the fundamental questions in gene regulation is to address how a housekeeping promoter such as RB is regulated. One way is through autoregulation. The expression of the gene can be regulated by its own gene product, either by directly participating in a protein-DNA complex in the promoter region, or by a protein-protein interaction to regulate the activity of transcription factors for the gene. The results presented in this paper suggest that the transcription of the RB gene can be regulated by the transcription factor E2F-1, in response to demands for Rb protein during cell cycle progression. The Rb protein, in turn, can adjust its expression level by inactivating the activity of its activator, here, E2F-1.

The presence of E2F recognition sites in promoters of a number of growth-related genes implies that E2F is important in promoting cell entry into S phase and that Rb may regulate cell cycle progression by interaction with E2F. The discovery of multiple E2Fs makes this regulation process more complex. Our results have shown that *c-myc*, which contains a typical E2F site in its promoter, is probably not a target for E2F-1, consistent with the observation that the expression of E2F-1 occurs much later after serum stimulation than that of *c-myc* (55). Interestingly, the E2F binding site in the *c-myc* promoter was found to be responsible for RB repression (24). This finding suggests that other E2Fs may mediate RB repression of the *c-myc* promoter and that the surrounding sequence of the E2F site in the promoter may determine the specificity of different E2Fs. This E2F-Rb regulation mode may be applicable to many other growth-related genes, the promoters of which contain E2F recognition sites. Although the precise nature of the E2F-Rb relationship needs further exploration, the results reported here clearly indicate that E2F-1 can mediate the regulation of RB gene expression by its own gene product. Thus, in addition to phosphorylation and possible interactions with other cellular proteins, Rb regulates itself.

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