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The Retinoblastoma Gene Product Regulates Sp1-Mediated Transcription

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We have demonstrated that the retinoblastoma gene product (Rb) can positively regulate transcription from the fourth promoter of the insulinlike growth factor II gene. Two copies of a motif (the retinoblastoma control element) similar to that found in the human c-*fos*, transforming growth factor β1, and c-*myc* promoters are responsible for conferring Rb regulation to the fourth promoter of the insulinlike growth factor II gene. We have shown that the transcription factor Sp1 can bind to and stimulate transcription from the retinoblastoma control element motif. Moreover, by using a GAL4-Sp1 fusion protein, we have directly demonstrated that Rb positively regulates Sp1 transcriptional activity in vivo. These results indicate that Rb can function as a positive regulator of transcription and that Sp1 is one potential target, either directly or indirectly, for transcriptional regulation by Rb.

Inactivation of the retinoblastoma gene product (Rb) has been associated with the etiology of a subset of human tumors (4, 10–13, 15, 16, 23, 24, 33, 34, 36, 37), thus classifying the Rb gene as an anti-oncogene or tumor suppressor gene. Although the mechanism through which Rb functions to suppress tumorigenesis and to constrain cell proliferation is unknown, recent work has shown that Rb is involved in regulating transcription of certain genes (21, 29, 31). In particular, we have demonstrated previously that Rb is able to regulate transcription of the c-*fos*, c-*myc*, and transforming growth factor β1 (TGF-β1) promoters in transient assays in either a positive or negative manner, dependent upon the cell type (21). Analysis of the sequences involved in regulating human c-*fos* and TGF-β1 expression have identified a common motif (GCCACCC) that has been termed the RCE, for retinoblastoma control element (21, 31). A similar sequence is also present in the c-*myc* promoter (28), as well as in several other Rb-regulated promoters (31), within the region responsible for conferring Rb-mediated regulation.

The contribution of the RCE to the regulation of the *fos*, *myc*, and TGF-β1 promoters by Rb is difficult to ascertain since a multitude of other regulatory factors, including the transcription factor E2F, bind to these complex promoters. E2F is of particular interest since Rb has been recently demonstrated to exist in a complex that contains E2F (1, 2, 5, 6). The interaction of Rb with the E2F complex apparently leads to an inhibition of E2F transcriptional activity in vivo (1, 5, 22). Rb also has been shown to bind to c-*myc* and N-*myc* proteins in vitro (32), although the effect of the interaction with Rb on Myc-mediated transcription is unknown. The observation that Rb associates with several transcription factors has led to the speculation that Rb functions to constrain cell proliferation solely by inhibiting the activity of certain transcription factors through protein-protein interaction (27, 35). However, this model does not readily explain the observation that Rb can positively regulate transcription from specific promoters (21).

To examine the ability of Rb to regulate transcription in a positive and negative manner in further detail, we sought first to implicate the RCE in conferring Rb-mediated regulation. Furthermore, we sought to identify the factor(s) that interacts with the RCE and that might be involved in conferring transcriptional regulation by Rb. In this report, we demonstrate that transcription from the fourth promoter of the insulinlike growth factor II (IGF-II) gene is significantly stimulated by Rb. Moreover, we have identified one predominant factor, termed RCF-1, that interacts with the consensus RCE sequence. On the basis of several binding criteria, we demonstrate that RCF-1 is indistinguishable from the transcription factor Sp1. We further demonstrate that a functional Sp1 binding site is required for Rb-mediated induction of transcription. Finally, using a GAL4-Sp1 fusion protein, we directly demonstrate that Sp1 is able to confer transcriptional regulation by Rb. These results suggest that Rb is able to regulate transcription in a positive manner through a specific transcription factor. The ability of Rb to confer positive transcriptional regulation suggests that Rb does not function solely by binding to and inactivating specific transcription factors but instead may play an active role in regulating transcriptional initiation.

MATERIALS AND METHODS

Cell culture. NIH 3T3 cells and CCL-64 mink lung epithelial cells were maintained in Dulbecco modified Eagle medium plus 10% calf serum and 10% fetal bovine serum, respectively. For transfection of 3T3 cells, a DEAE-dextran procedure was used (31); a calcium phosphate precipitation procedure was used for transfection of CCL-64 cells. The *Drosophila* Schneider cells were maintained in Dulbecco modified Eagle medium plus 10% fetal bovine serum. *Drosophila* Schneider cells were transfected by the calcium phosphate coprecipitation method, using 10 μg of the appropriate plasmids with either 100 ng of a control plasmid, pPac0, or

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100 ng of the Sp1 expression plasmid pPacSp1 (7). Plasmids pPac0 (no insert) and pPacSp1 contain the *Drosophila* actin promoter and polyadenylation signal. Cells were harvested 48 h after addition of the DNA, and extracts were assayed for chloramphenicol acetyltransferase (CAT) activity. All transfections were repeated at least three times. For normalization of transfection efficiencies in 3T3 and CCL-64 cells, a human growth hormone expression plasmid (pSVGH) was included in the cotransfections. The level of growth hormone expression was determined by using a growth hormone detection kit (Nichols Institute).

Plasmid constructs. The fourth (P4) promoter of the IGF-II gene–CAT fusion construct was generated as follows. Plasmid pHIGFII-14-6 was digested with *Nru*I and *Bst*EII (−413 to +124). After the ends were filled in, the resulting fragment was inserted into the *Sma*I site of the multiple cloning site of pGEM4-SV0CAT. Plasmids I5, I7, I8, I9, I7M1, I7M2, I7M3, and I7M4 were created by inserting fragments of the 5′ flanking region sequence of the P4 promoter of the human IGF-II gene, generated by polymerase chain reaction amplification into the pGEM4-SV0CAT vector. The polymerase chain reaction products were generated so that they have *Hin*dIII and *Xba*I restriction sites for subcloning into the pGEM4-SV0CAT vector. The actin-Sp1 construct and the Sp1 antibody were provided by E. Pascal and R. Tjian. The GAL4-Sp1N fusion plasmid was constructed and provided by Grace Gill. The G5BCAT reporter construct (26) and the human Rb expression vector (31) have been previously described.

Analysis of Sp1 binding. Sp1 binding was analyzed by using a buffer containing 50 mM Tris (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, and 100 μM $ZnCl_2$. The oligonucleotide probes used in the gel shift analysis were prepared by annealing the complementary strands and end labeling using the large fragment DNA polymerase I; 10,000 cpm was used for each binding reaction. Binding complexes were analyzed by electrophoresis on a 5% 0.5× Tris-borate-EDTA-polyacrylamide gel. For analysis of the effect of zinc on binding, the extracts were treated with 1,10-phenanthroline for 10 min prior to addition of the labeled probe. $ZnCl_2$ was added to the appropriate extracts just prior to addition of the probe. The purified Sp1 was generously provided by Mark Knuth and Richard Burgess. The purification of the two Sp1–β-galactosidase (β-Gal) proteins (778C and 168C [19]) from *Escherichia coli* was performed as described previously (18). For gel shift analysis using the I5, I7, and I8 probes, plasmids I5 and I7 were digested with *Hin*dIII, dephosphorylated, labeled with ^{32}P, digested with *Sma*I, and gel purified. The oligonucleotides for the I8 probes (−36 to −17) were synthesized, annealed, and end labeled. For the analysis of Sp1 binding using Sp1 antibody, 3 μl of Sp1 immune serum (2873) or preimmune control serum was incubated with the nuclear extract for 15 min prior to addition of the probe.

RESULTS

Mapping of the Rb-responsive element in the P4 promoter of the IGF-II gene. We have previously demonstrated that the TGF-β1, c-*fos*, and c-*myc* promoters can be positively and negatively regulated by Rb, dependent upon the cell type. A common motif, termed the RCE, was identified in the regions of these promoters responsible for conferring Rb regulation. By computer analysis, we have identified two consensus RCE sequences (CCACCC) within the P4 promoter of the IGF-II gene (for a review, see reference 30).

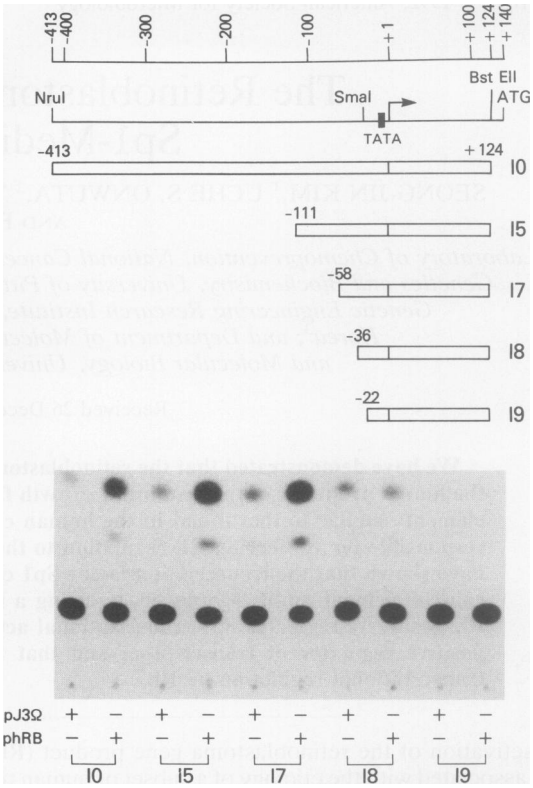

FIG. 1. Mapping of the Rb-responsive element in the P4 promoter of the IGF-II gene. The diagram shows the P4 promoter of the IGF-II gene (−413–+140) fused to the CAT gene (I0) and the 5′ deletion mutants (I5, nucleotide −111; I7, nucleotide −58; I8, nucleotide −36; I9, nucleotide −22). The different IGF-II–CAT plasmids were cotransfected into CCL-64 mink lung epithelial cells with either a control vector (pJ3Ω, labeled −) or a human Rb expression vector (phRB, labeled +). A human growth hormone expression plasmid (pSVGH) was included in the transfection as an internal control.

This promoter is found in the untranslated exons 4 and 4b of the IGF-II gene (9, 14) and is responsible for high levels of IGF-II RNA that are found in fetal liver and in certain human tumors. In particular, high levels of IGF-II RNA, expressed from the P4 promoter, have been found in Wilms' tumors, rhabdomyosarcomas, hepatoblastomas, and certain breast and colon cancers.

To determine whether Rb regulates the IGF-II P4 promoter in a manner similar to that of the c-*fos*, c-*myc*, and TGF-β1 promoters, a construct containing the P4 promoter fused to the bacterial CAT was used (I0; Fig. 1). Moreover, to examine the role of the two consensus RCE-like sequences in the P4 promoter, 5′ deletions of the promoter were constructed, and these plasmids were used in cotransfection experiments into CCL-64 mink lung epithelial cells. The plasmids were cotransfected with either a control plasmid (pJ3Ω) or the human Rb expression vector (phRB [31, 21]), and the level of CAT activity was determined 48 h posttransfection (Fig. 1). The activities of constructs I0 (−423), I5 (−111), and I7 (−58) were induced 12-, 15-, and 17-fold, respectively, by Rb (Fig. 1). However, the induction by Rb dropped to the basal level when the deletion reached −36 (deletion I8). These results suggest that sequences between −58 and −36, including the two RCE-like se-

quences, are important for positively regulating IGF-II expression in the presence of high levels of the human Rb protein. Moreover, the results are consistent with our previous observation that the c-*fos*, c-*myc*, and TGF-β1 promoters can be stimulated by Rb in CCL-64 cells.

Identification of factors that bind to the RCE. To identify the factors that interact with the RCE and might be responsible for conferring Rb-mediated transcriptional regulation, we performed a gel shift assay using several of the RCE-like sequences (Fig. 2A) as probes and the *fos* RCE as a competitor. A nuclear extract from F9 cells was used in the initial analysis since F9 cells have a high level of endogenous Rb protein (3). As shown in Fig. 2B, several specific complexes are observed binding to the Fos, Myc, and JunB RCE oligonucleotides, but only one predominant complex (RCF-1) and a minor complex (RCF-2) are competed for with the Fos RCE (lanes 1 to 10). The G+C-rich EGR-1 binding site does not bind RCF-1, nor does it compete for binding of RCF-1 to the Fos oligonucleotide. When a nuclear extract from a retinoblastoma cell line (Y79) was used for the gel shift analysis, RCF-1 and RCF-2 were observed binding to the Fos and TGF-β-A probes and could be competed for with the Fos, Myc, TGF-β-A and TGF-β-B oligonucleotides (lanes 11 to 19). The complex labeled RCF-3 appears not to be a specific binding factor since it can be partially competed for with many nonspecific oligonucleotides, including poly(dG-dC) (data not shown). Since Y79 cells are deficient for Rb, neither the RCF-1 nor the RCF-2 complex can be due to binding of Rb to the RCE.

To determine the recognition sequence for the binding of RCF-1, single and double base changes (Fig. 3A) were introduced into the c-*fos* RCE. These mutant RCE oligonucleotides were used as competitors in the gel shift assay for RCF-1 binding using the *fos* RCE as a probe (Fig. 3B). A single base change in positions 3 and 7 of the GCCACCC sequence leads to a significant reduction in the ability of the oligonucleotide to compete for RCF-1 binding. Similarly, a double base change at positions 1 and 5 also reduces the ability of the oligonucleotide to compete for binding. In contrast, the G-to-C change at position 1 and the C-to-G change at position 6 do not appear to significantly affect competition compared with the RCE, whereas the double 1/6 mutant is able to partially compete for RCF-1 binding. The similarity of RCF-1 and RCF-2 binding to the RCE mutant oligonucleotides suggests that RCF-2 is either a modified form of RCF-1 or a factor that binds to the same sequence as does RCF-1.

Sp1 can bind to the RCE. During the characterization of RCF-1, we noted a dependence upon zinc for high-affinity binding. The addition of the chelator 1,10-phenanthroline to both the F9 and Y79 nuclear extracts reduces binding of RCF-1 and RCF-2 (Fig. 2C, lanes 2 and 5), whereas the subsequent addition of $ZnCl_2$ to the binding reaction mixture stimulates binding (lanes 3 and 6). This finding suggests that RCF-1 may be a zinc finger DNA-binding protein. Given that RCF-1 binds to a G+C-rich sequence (4G) similar or identical to the binding site for the transcription factor Sp1 (18) and that it requires zinc for binding, we wanted to determine whether RCF-1 might be identical to the transcription factor Sp1. Therefore, Sp1 protein purified from HeLa cells as well as two Sp1–β-Gal fusion proteins, 778C and 168C, purified from bacteria were used in a gel shift analysis. The 778C and 168C proteins both contain Sp1, including the zinc finger DNA-binding domain, fused to β-Gal and have been shown to bind with high affinity to an Sp1 binding site (18). The migration of the Sp1 protein purified from HeLa cells bound

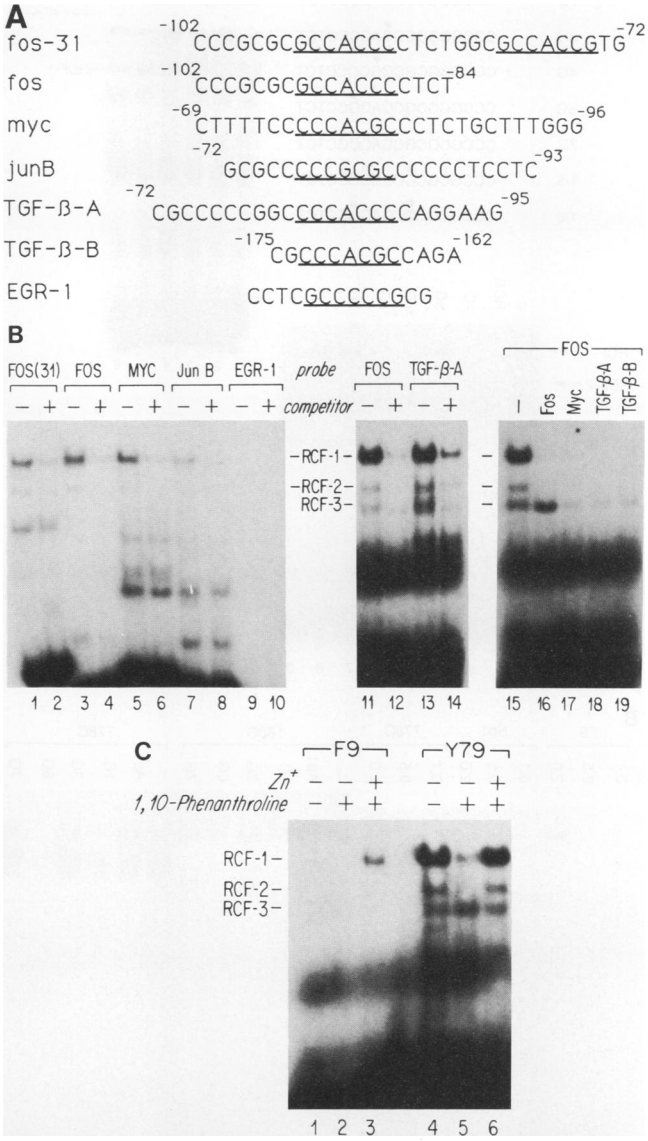

FIG. 2. Identification of factors interacting with the RCE. (A) Sequence of the oligonucleotides used as probes and/or competitors in a gel shift analysis. The region of homology to the Fos RCE sequence is underlined. (B) Gel shift analysis of factors binding to the RCE. For lanes 1 to 10, the indicated oligonucleotides were labeled to similar specific activities and then used in a gel shift analysis using a F9 nuclear extract. The Fos oligonucleotide (10 ng) was used as a competitor in the lanes marked +. A nuclear extract from Y79 cells, a retinoblastoma cell line, was used for the gel shift analysis in lanes 11 to 19. The Fos oligonucleotide was used as a probe in lanes 11, 12, and 15 to 19, whereas the TGF-β-A oligonucleotide was used as a probe in lanes 13 and 14. The Fos oligonucleotide was used as a competitor in lanes 12, 14, and 16, whereas the Myc and TGF-β-A and -B oligonucleotides were used as competitors in lanes 17, 18, and 19, respectively. (C) Effect of zinc on RCF binding. 1,10-Phenanthroline (10 μM) was added to F9 (lane 2) and Y79 (lane 5) nuclear extracts prior to the addition of the RCE probe; 100 μM $ZnCl_2$ was added to the 1,10-phenanthroline-treated extracts prior to the addition of the probe in lanes 3 and 6. Lanes 1 and 4 contain untreated nuclear extracts.

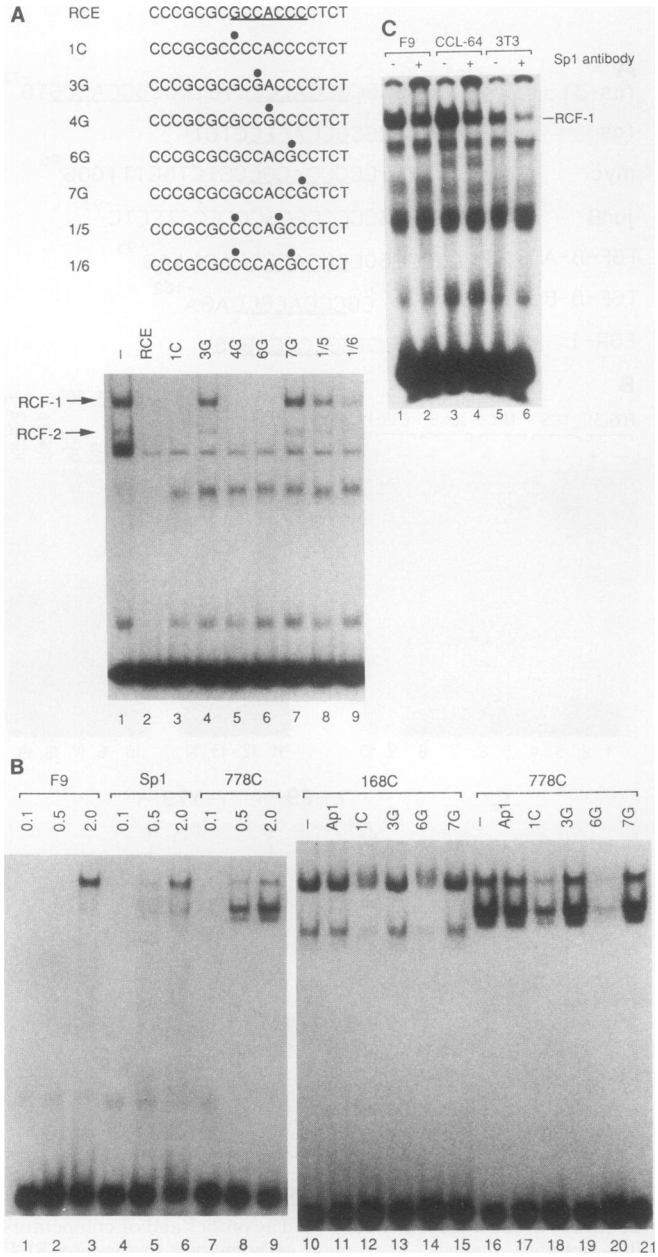

FIG. 3. (A) Characterization of the RCE. Single and double base changes were introduced into the Fos RCE sequence. The base alterations are marked for each oligonucleotide, and the consensus RCE is underlined. The different mutant oligonucleotides (10 ng) were used as probes in a gel shift analysis using a WERI-27 retinoblastoma cell line nuclear extract with the Fos RCE used as a probe. (B) Binding of Sp1 to the RCE. An F9 nuclear extract (lanes 1 to 3), Sp1 purified from HeLa cells (lanes 4 to 6), or bacterially purified Sp1–β-Gal fusion proteins (778C [lanes 7 to 9 and 16 to 21] and 168C [lanes 10 to 15]) were used in a gel shift analysis using the Fos RCE as a probe; 10 ng of a consensus AP1 binding site oligonucleotide (lanes 11 and 17) and the 1C (lanes 12 and 18), 3G (lanes 13 and 19), 6G (lanes 14 and 20), and 7G (lanes 15 and 21) oligonucleotides were used as competitors. Increasing amounts (0.1, 0.5, and 2.0 μg) of a 14-amino-acid peptide that appears to specifically stimulate and/or stabilize Sp1 binding was used in lanes 1 to 9. (C) Effect of Sp1 antibody. Nuclear extracts from F9, CCL-64, and 3T3 cells were incubated with either preimmune control serum (−) or Sp1 immune serum (+) prior to addition of the RCE probe.

to the c-*fos* RCE was identical to migration of RCF-1 in the gel shift analysis. Moreover, the Sp1–β-Gal fusion proteins also bound to the RCE and have the same binding specificity as does RCF-1. Increasing amounts of a 14-amino-acid peptide derived from the human Rb protein were used in the gel shift experiment. We have demonstrated that this peptide is able to stimulate Sp1 binding but have not determined whether the effect of this specific Rb-derived peptide reflects a function contained within the full-length Rb protein. A complete characterization of this peptide and its effect on Sp1 binding will be published elsewhere (28a).

To provide further evidence that RCF-1 is indeed Sp1, we used a rabbit polyclonal antibody (2873) raised against Sp1 in the gel shift analysis. Preincubation of the nuclear extract with the Sp1 immune serum resulted in a reduction of RCF-1 binding in contrast to the preimmune serum (Fig. 3C). A similar effect of the Sp1 antibody on RCF-1 binding was observed in nuclear extracts from F9 (lanes 1 and 2), CCL-64 (lanes 3 and 4), and 3T3 (lanes 5 and 6) cells. Interestingly, preincubation of the extracts with the Sp1 antibody did not affect binding of RCF-2. This finding suggests that RCF-2 may be distinct from Sp1 but can bind to a similar site. Alternatively, RCF-2 may represent a form of Sp1 that is not recognized as efficiently by the Sp1 antibody as is RCF-1.

Regulation of the P4 promoter of the IGF-II gene by Sp1. As shown above, we have demonstrated that Sp1 can bind directly to the RCE. To demonstrate that Sp1 can also bind to the IGF-II RCEs, bacterially purified LacZ-Sp1 protein (168C) was used in a gel shift analysis using the I7, I5, and I8 promoter fragments as probes. As shown in Fig. 4a, Sp1 binds with high affinity to the I7 and the I5 probes but only with a greatly reduced efficiency to the I8 probe. To determine directly whether the RCEs in the IGF-II P4 promoter were involved in mediating Sp1-directed transcription, the effect of Sp1 on transcription was examined in *Drosophila* Schneider cells. The IGF-II–CAT chimeric constructs were cotransfected with a *Drosophila* actin promoter-Sp1 expression vector (7), and the level of CAT activity was determined (Fig. 4b). The activities of constructs I5 and I7 were induced 94- and 108-fold, respectively, by Sp1, whereas the activity of construct I8 was induced only 6-fold. This finding suggests that one or both of the RCEs located between −58 and −36 in the IGF-II P4 promoter can bind Sp1 and that the bound Sp1 is transcriptionally active.

Rb regulates expression of the IGF-II gene through Sp1. Although our results suggest that the Sp1 binding sites in the TGF-β1, c-*fos*, c-*myc*, and IGF-II promoters are involved in Rb-mediated transcriptional regulation, we have not directly implicated the RCE sequence in Rb regulation. The c-*fos*, c-*myc*, and TGF-β1 promoters contain multiple Sp1 binding sites as well as binding sites for many other transcription factors, including E2F. Thus, the use of these promoters for the analysis of regulation of Sp1 by Rb is limited. In contrast, the IGF-II promoter construct I7 appears to contain only the identified Sp1 binding sites and a TATA box, allowing for the analysis of Sp1 and its role in mediating Rb transcriptional regulation. To examine the role of the RCEs in mediating Rb-mediated transcriptional regulation, both RCEs in plasmid I7 were inactivated by changing the internal three base pairs of each RCE (I7M3; Fig. 5a). Since the RCEs shown to be regulated by Rb all contain the Sp1 variant binding site CCACCC, we wanted to contrast the ability of the CCACCC and the CCGCCC Sp1 binding sequences to confer Rb-mediated transcriptional regulation. Thus, to determine whether the consensus Sp1 binding site can also be regulated by Rb, single T-to-C base changes in each of the RCEs (I7M1

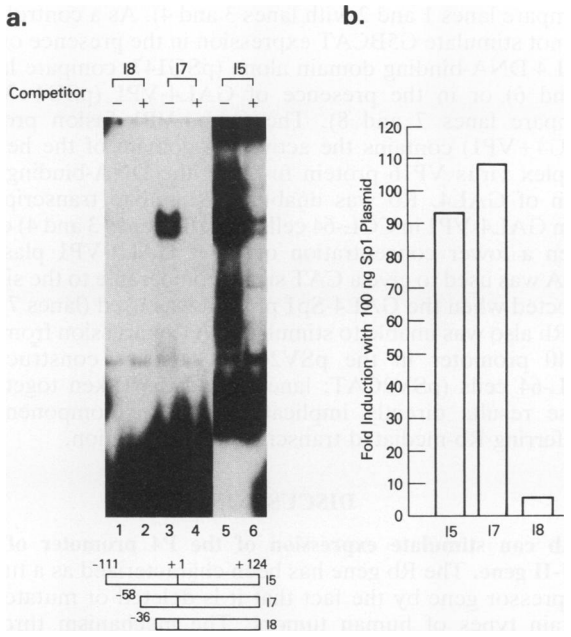

FIG. 4. Stimulation by Sp1 of the P4 promoter of the IGF-II gene. (A) Binding of Sp1 to the IGF-II promoter. Oligonucleotides from the I5, I7, and I8 deletions were labeled to the same specific activity and used as probes in a gel shift analysis. The Sp1–β-Gal fusion protein 168C was used for the analysis, and the TGF-β1 RCE was used as a competitor in lanes 2, 4, and 6. (B) Stimulation of IGF-II transcription by Sp1 in *Drosophila* Schneider cells. Constructs I5, I7, and I8 were cotransfected with either 100 ng of a control vector or 100 ng of a *Drosophila* actin promoter-Sp1 expression vector. The fold induction with 100 ng of Sp1 plasmid is shown. The results are representative of three independent experiments.

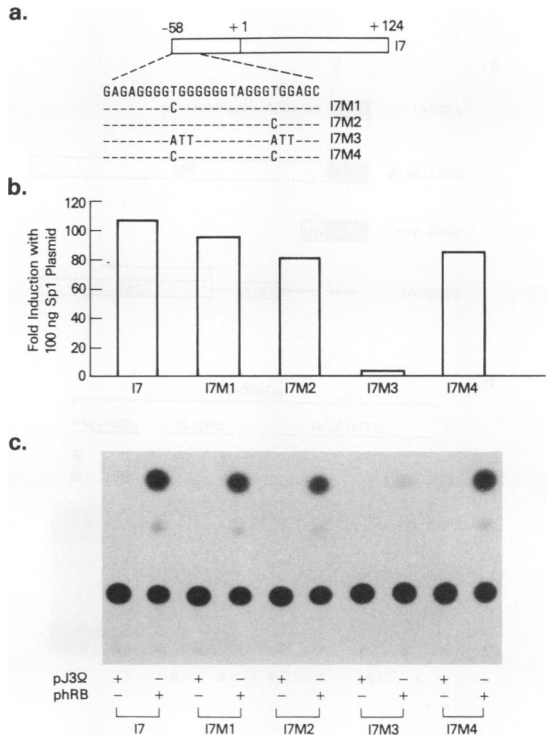

FIG. 5. Evidence that Rb regulates IGF-II expression through the two Sp1 binding sites. (A) Diagram of the mutations introduced into the two RCE elements contained within construct I7. The T in either the 5′, 3′, or both RCEs was changed to a G in M1, M2, and M4, respectively. Both RCEs were mutated by inserting three base changes in each RCE in mutant M3. (B) Effect of Sp1 on transcription of the RCE mutants in *Drosophila* Schneider cells. The parental plasmid I7 and the four mutants were cotransfected with either a control vector or a *Drosophila* actin promoter-Sp1 expression vector into Schneider cells. The level of Sp1-mediated activation is shown. (C) Effect of Rb on transcription of the RCE mutants plasmids. The parental plasmid I7 and the four mutants plasmids were cotransfected with either a control plasmid (pJ3Ω, labeled −) or an Rb expression plasmid (phRb, labeled +) into CCL-64 mink lung epithelial cells. The level of CAT activity is shown.

and I7M2) and in both RCEs (I7M4) were introduced into plasmid I7.

To first assess the ability of Sp1 to activate the RCE promoter mutations, the different constructs were transfected into Schneider cells with or without an Sp1 expression vector (Fig. 5b). Mutant constructs I7M1, I7M2, and I7M4 were induced 80- to 95-fold by Sp1. However, I7M3 was induced only twofold. Thus, as expected, the change of the CCACCC RCE to the consensus Sp1 binding site CCGCCC had no effect on the ability of Sp1 to activate transcription, whereas inactivation of both Sp1 sites abolished Sp1 activation.

The effect of Rb on the RCE mutants was examined by cotransfection of the plasmids into CCL-64 cells with or without an Rb expression vector (Fig. 5c). The parental construct I7 was induced 22-fold by Rb, whereas the mutant constructs that had either a single or double base change from T to C were induced 20-fold by Rb. In contrast, the mutant that can no longer bind Sp1 (I7M3) was induced only 1.5-fold. These results strongly suggest that Rb can stimulate transcription of the IGF-II P4 promoter through the identified Sp1 binding sites. Moreover, in the context of the IGF-II P4 promoter, the CCACCC and CCGCCC Sp1 binding sites can confer equal responsiveness to Rb.

Rb can stimulate GAL4-Sp1-mediated transcription. The results presented above strongly suggest that Rb can regulate transcription through an Sp1 binding site. However, it is still possible that another factor that binds to a sequence similar or identical to Sp1 could be mediating the effect of Rb on transcription. To directly implicate Sp1 in conferring Rb-mediated transcriptional regulation, we used a plasmid construct (pSG4+Sp1N) that expresses a GAL4-Sp1 fusion protein under the regulation of the simian virus 40 (SV40) early promoter (Fig. 6a). This GAL4-Sp1 fusion protein is dependent upon the GAL4 DNA-binding domain for DNA binding, since the zinc finger DNA-binding domain of Sp1 has been deleted (Sp1N). The GAL4-Sp1N fusion protein has been demonstrated to stimulate transcription in mammalian cells dependent upon a GAL4 binding site (12a, 25).

To determine the effect of Rb on GAL4-Sp1-mediated transcription, a CAT reporter construct (G5BCAT) containing five GAL4 binding sites upstream from the E1B TATA box (26), the GAL4-Sp1 expression construct (pSG4+Sp1N), and either the human Rb expression plasmid (phRB) or the control expression vector (pJ3Ω) were cotransfected into NIH 3T3 (Fig. 6b) and CCL-64 (Fig. 6c) cells. The SV40-growth hormone plasmid was included as an internal control for transfection efficiency. As shown in Fig. 6b, the coexpression of Rb significantly stimulated Sp1-mediated transcription in duplicate transfections in 3T3 cells

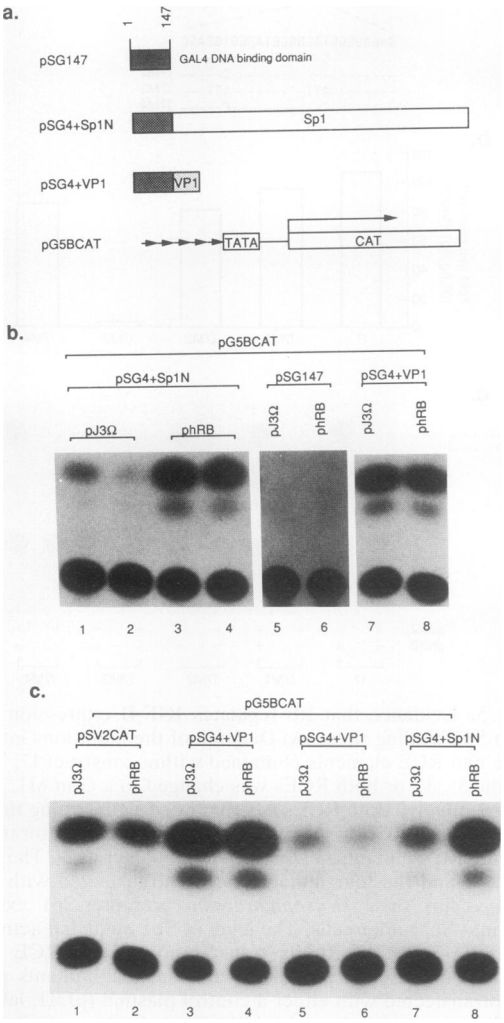

FIG. 6. Stimulation by Rb of Sp1-mediated transcription. (A) Diagram of the transactivator and reporter plasmids. pSG4+Sp1N contains the Sp1 coding domain fused to the GAL4 DNA-binding domain. The GAL4-Sp1N fusion protein contains a C-terminal truncation that deletes the zinc finger DNA-binding domain of Sp1. pSG4+VP1 contains VP16 activator sequences between amino acids 411 and 454 fused to the GA14 DNA-binding domain. The G5BCAT reporter construct contains five GAL4 binding sites upstream of the E1B TATA box. Downstream of the initiation site is the CAT reporter gene. (B) Stimulation by Rb of GAL4-Sp1-dependent transcription in 3T3 cells. Lanes: 1 to 4, 2 μg of G5BCAT, 0.4 μg of pSG4+Sp1N, and 2 μg of either pJ3Ω or phRB were transfected into 3T3 cells; 5 and 6, 0.4 μg of pSG147 was used instead of pSG4+Sp1N; 7 and 8, 0.4 μg of pSG4+VP1 was used instead of pSG147. pSVGH (1 μg) was used as an internal control for transfection efficiency. The results shown are from the same transfection experiment, but the amount of extract in lanes 5 and 6 was increased threefold, whereas the amount of extract used in lanes 7 and 8 was reduced fivefold, to obtain CAT activity within the linear range. (C) Stimulation by Rb of GAL4-Sp1-dependent transcription in CCL-64 cells. Two micrograms of G5BCAT, 0.4 μg of pSV2CAT (lanes 1 and 2), 0.4 μg of pSG4+VP1 (lanes 3 and 4), 0.05 μg of pSG4+VP1 (lanes 5 and 6), 0.4 μg of pSG4+Sp1N (lanes 7 and 8), and either pJ3Ω (lanes 1, 3, 5, and 7) or phRb (lanes 2, 4, 6, and 8) were cotransfected into CCL-64 cells; 0.5 μg of pSVGH was included as an internal control.

(compare lanes 1 and 2 with lanes 3 and 4). As a control, Rb did not stimulate G5BCAT expression in the presence of the GAL4 DNA-binding domain alone (pSG147; compare lanes 5 and 6) or in the presence of GAL4-VP1 (pSG4+VP1; compare lanes 7 and 8). The GAL4-VP1 fusion protein (pSG4+VP1) contains the activation domain of the herpes simplex virus VP16 protein fused to the DNA-binding domain of GAL4. Rb was unable to stimulate transcription from GAL4-VP1 in CCL-64 cells (Fig. 6c, lanes 3 and 4) even when a lower concentration of input GAL4-VP1 plasmid DNA was used to give a CAT signal comparable to the signal detected when the GAL4-Sp1 protein was used (lanes 7 and 8). Rb also was unable to stimulate CAT expression from the SV40 promoter in the pSV2CAT reporter construct in CCL-64 cells (pSV2CAT; lanes 1 and 2). Taken together, these results directly implicate Sp1 as a component in conferring Rb-mediated transcriptional regulation.

DISCUSSION

Rb can stimulate expression of the P4 promoter of the IGF-II gene. The Rb gene has been characterized as a tumor suppressor gene by the fact that it is deleted or mutated in certain types of human tumors. The mechanism through which Rb functions to constrain cell proliferation is unclear, but recent observations have suggested that Rb can function as a regulator of transcription. In this regard, we have previously identified regions of the human c-*fos* and TGF-β1 promoters that are able to confer regulation by Rb to a heterologous promoter (21, 31). Contained within these regions are several shared motifs, one of which we have termed the RCE. This RCE motif (CCACCC) is also present in other promoters that are regulated by Rb, including the c-*myc* promoter (28, 29). In this report, we demonstrate that Rb can stimulate transcription from the P4 promoter of the IGF-II gene. Moreover, the region of the P4 promoter responsible for conferring Rb-mediated transcriptional regulation contains the two identified copies of an RCE-like sequence (CCACCC).

Sp1 can bind to the RCE. We have identified one predominant factor (RCF-1) that binds to the RCE sequence. By several binding criteria, our results suggest that RCF-1 is identical to the Sp1 transcription factor. First, Sp1 purified from HeLa cells and Sp1–β-Gal fusion proteins purified from *E. coli* bind to the RCE with the same affinity as does RCF-1. Second, the same single and double base changes in the RCE that reduce RCF-1 binding also reduce Sp1 binding, whereas both RCF-1 and Sp1 can bind to a consensus Sp1 site. Third, the binding of both RCF-1 and Sp1 can be inhibited by treatment with 1,10-phenanthroline, and binding can be restored by the introduction of zinc to the binding reaction mixture. Fourth, we have demonstrated that an Sp1-specific antibody is able to bind to and alter the mobility of RCF-1 specifically. We also have demonstrated that Sp1 functionally binds to and activates transcription from the RCE in vivo, using a *Drosophila* actin promoter-Sp1 vector to coexpress Sp1 in *Drosophila* cells. Using both wild-type and mutant RCEs, we have established a correlation between the ability of Sp1 to bind to the IGF-II RCEs with Sp1-mediated transcription. We also have demonstrated that Sp1 can activate transcription from the human c-*fos* promoter through the RCE sequence(s) in cotransfection experiments in Schneider cells (20a). In a similar analysis, it recently has been shown that Sp1 binds efficiently to an RCE-like sequence in the P5 promoter of bovine papilloma virus (25). Taken together, these results demonstrate that Sp1 can bind

to and activate transcription through the RCE. However, we have not ruled out the possibility that additional regulatory factors may interact with the RCE. Indeed, the inability of the Sp1 antibody to alter RCF-2 binding suggests that a factor distinct from Sp1 can interact with the RCE.

Sp1 is a target for Rb-regulated transcription. We also have demonstrated that a functional RCE is required for Rb-mediated stimulation of transcription. Mutations in the RCE that prevent Sp1 transcriptional activation in Schneider cells also prevent induction by Rb in CCL-64 cells. In contrast, a base change in the RCE that still allows binding of Sp1 and transcriptional activation by Sp1 in Schneider cells is also stimulated by Rb in CCL-64 cells. These results strongly suggest that a Sp1 binding site is involved in Rb-mediated transcriptional regulation. To directly implicate Sp1 in the stimulation of transcription by Rb, we have used a GAL4-Sp1 expression construct containing a deletion of the zinc finger DNA-binding domain (pSG44+Sp1N). In these experiments, the transfected cells do not have an endogenous factor that can bind to and activate transcription from the GAL4 reporter plasmid. Thus, the observed expression of CAT from the GAL4-CAT reporter construct is dependent solely upon the level and activity of the cotransfected fusion transactivator protein. In cotransfection experiments, we have demonstrated that Rb is able to significantly stimulate transcription mediated by GAL4-Sp1 from the GAL4 binding sites in the G5BCAT reporter construct. In contrast, Rb is not able to induce transcription when a plasmid expressing either the GAL4 binding domain alone (pSG147) or the GAL4-VP16 fusion protein (pSG4+VP1) was used in the cotransfection assay. We have also demonstrated that both the A and B activation domains of Sp1 together and the B activation domain alone are sufficient for conferring positive transcriptional regulation by Rb (25a). Moreover, we have examined the ability of point mutations and deletions found in the Rb protein in naturally occurring tumors to regulate Sp1. Our results suggest that certain mutations found in certain bladder and breast cancers are not inactivating mutations but instead are altered in their ability to regulate transcription mediated by Sp1 and E2F (32a).

Interestingly, we have observed that Rb can stimulate transcription conferred by a GAL4–ATF-2 fusion protein, whereas transcription conferred by GAL4–ATF-1 is slightly repressed by Rb. The differential regulation of ATF-1– and ATF-2–GAL4 fusion proteins is consistent with our observations concerning the regulation of transcription by Rb conferred through ATF binding sites. We have implicated an ATF-1 binding site in the negative regulation of the adenovirus E2 promoter by Rb (22), whereas we have demonstrated that the TGF-β2 promoter is stimulated by Rb through an ATF-2 binding site (22a). Although the results reported in this communication demonstrate that the transcription factor Sp1 is a target for Rb-mediated induction of transcription, our results with ATF-2 demonstrate that Sp1 is not the sole target for mediating positive transcriptional regulation by Rb. Thus, Rb may act as a cell cycle-specific regulator of transcriptional initiation by regulating the activity of certain transcription factors in either a positive or negative manner.

Mechanism of Rb action. Recently, Rb has been found to be present in a complex that also contains the E2F transcription factor (1, 2, 5, 6). Additionally, Rb has been shown to form a complex in vitro with both c-*myc* and N-*myc* proteins (32) as well as with several unidentified factors (8, 17, 20). The interaction of Rb with the E2F complex results in a dramatic inhibition of E2F transcriptional activity, whereas the effect of Rb on Myc-mediated transcription is unknown. However, in contrast to the ability of Rb to repress E2F transcriptional activity, we have demonstrated that Sp1-mediated transcription can be induced by Rb. Indeed, we have observed significant inhibition of E2F-mediated transcription by Rb in CCL-64 cells, whereas in the same experiment, IGF-II transcription is stimulated by Rb 20-fold (22). These observations suggest that Rb is not functioning merely by binding to and inactivating a specific transcription factor but instead is actively involved in regulating transcriptional initiation in both a positive and negative manner. It is possible that Rb is interacting directly with Sp1 or with a complex that interacts with Sp1. In this model, Rb could be functioning as a specific coactivator to bridge Sp1 with the initiation complex. Alternatively, Rb could be regulating Sp1 activity through a more indirect mechanism. For example, the transient overexpression of Rb at certain stages of the cell cycle may cause negative, Rb-containing complexes to disassociate, resulting in the freeing of certain positive factors such as Sp1 for transcription. This may occur if the Rb-containing complexes in the cell require a strict stoichiometry. In this model, the effect of Rb on transcription would be similar to the squelching phenomenon observed by Gill and Ptashne (12b). However, this potential negative squelching by Rb would have a positive effect on transcription, whereas classical squelching results in a reduction in transcription.

It is of interest to note that Chittenden and coworkers (6) have identified several DNA motifs which represent consensus DNA sequences bound by transcription factors that are able to interact with Rb. In their experiments, a nuclear extract was allowed to interact with immobilized Rb and subsequently exposed to random oligonucleotides. The bound oligonucleotides were eluted and amplified by polymerase chain reaction, and the process was then repeated five or six times. The predominant motif observed following cloning and sequencing of the selected oligonucleotides was the binding site for E2F. However, several oligonucleotides containing Sp1-like binding sites were also detected. This result is provocative in that it suggests that Sp1 can be retained on an Rb affinity column. It is important to note, however, that we have been unable to demonstrate a direct interaction between Sp1 and Rb in coimmunoprecipitation experiments and unable to observe an effect of Rb on Sp1 binding in gel shift and DNase I footprinting experiments. Clearly, the elucidation of the mechanism of Rb function in stimulating and inhibiting transcription requires further in vivo and in vitro analysis.

Selectivity of Sp1 sites for Rb regulation. The common motif identified in several Rb-regulated promoter elements is a variant of the consensus Sp1 binding site (21, 28, 31). This observation led us to examine whether Rb can regulate Sp1 transcription specifically from the variant Sp1 binding site or whether the consensus Sp1 binding site can also confer Rb regulation. Interestingly, in the context of the IGF-II promoter, changing the CCACCC motif to the consensus sequence CCGCCC had no effect on the ability of Rb to stimulate transcription. This result is paradoxical since certain Sp1-dependent promoters are not significantly stimulated by Rb. For example, the SV40 promoter that contains six Sp1 sites and was used as an internal control in these experiments is not dramatically affected by Rb (less than twofold). Thus, the position or the context of the Sp1 site within a given promoter must be important for conferring Rb regulation. In this regard, we have observed that Rb can inhibit transcription from the adenovirus E2 promoter

through a E2F binding site (22). However, only transcription mediated by the proximal E2F site is inhibited by Rb. The effect of the position of the Sp1 site relative both to the binding sites of other transcription factors and to the initiation site of transcription on Rb stimulation is currently being analyzed. It is important to note that E2F binding sites are situated immediately 3′ to the RCEs in the c-*fos* and TGF-β1 promoters. Regulation of these promoters by Rb may involve a complex interaction between Sp1, E2F, and Rb. Indeed, mutation of the Sp1 binding site in the region of the TGF-β1 promoter responsible for conferring Rb regulation leads to negative regulation of TGF-β1 expression in CCL-64 cells, apparently mediated through ATF-1 and E2F binding sites (20b).

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