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The Retinoblastoma Gene Product RB Stimulates Sp1-Mediated Transcription by Liberating Sp1 from a Negative Regulator

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Studies have demonstrated that the retinoblastoma susceptibility gene product, RB, can either positively or negatively regulate expression of several genes through *cis*-acting elements in a cell-type-dependent manner. The nucleotide sequence of the retinoblastoma control element (RCE) motif, GCCACC or CCACCC, and the Sp1 consensus binding sequence, CCGCCC, can confer equal responsiveness to RB. Here, we report that RB activates transcription of the c-*jun* gene through the Sp1-binding site within the c-*jun* promoter. Preincubation of crude nuclear extracts with monoclonal antibodies to RB results in reduction of Sp1 complexes in a mobility shift assay, while addition of recombinant RB in mobility shift assay mixtures with CCL64 cell extracts leads to an enhancement of DNA-binding activity of Sp1. These results suggest that RB is directly or indirectly involved in Sp1-DNA binding activity. A mechanism by which RB regulates transactivation is indicated by our detection of a heat-labile and protease-sensitive Sp1 negative regulator(s) (Sp1-I) that specifically inhibits Sp1 binding to a c-*jun* Sp1 site. This inhibition is reversed by addition of recombinant RB protein, suggesting that RB stimulates Sp1-mediated transactivation by liberating Sp1 from Sp1-I. Additional evidence for Sp1-I involvement in Sp1-mediated transactivation was demonstrated by cotransfection of RB, GAL4-Sp1, and a GAL4-responsive template into CV-1 cells. Finally, we have identified Sp1-I, a ~20-kDa protein(s) that inhibits the Sp1 complexes from binding to DNA and that is also an RB-associated protein. These findings provide evidence for a functional link between two distinct classes of oncoproteins, RB and c-Jun, that are involved in the control of cell growth, and also define a novel mechanism for the regulation of c-*jun* expression.

The retinoblastoma susceptibility gene product $p105^{Rb}$ (RB) is generally believed to be an important regulator in the control of cellular proliferation and regulation of the cell cycle (7, 10, 13, 28, 29). The biochemical mechanisms for RB action remain unclear, although one possibility is transcriptional control of cell growth-related genes. Indeed, six cellular genes have been identified as targets of transcriptional regulation by RB (20–22, 31, 33, 50). Robbins et al. (33) demonstrated that a 30-bp segment of the c-*fos* promoter, termed the retinoblastoma control element (RCE), was necessary for RB-mediated transcriptional repression of c-*fos* expression and reduction of AP-1 stimulatory activity. Subsequently, Pietenpol et al. (31) identified a 23-bp element in the c-*myc* promoter that was necessary for negative regulation by transforming growth factor β1 (TGF-β1) or by cotransfection with human *Rb* expression plasmid. In contrast to these findings, RB has also been shown to stimulate transcription, particularly from the TGF-β1 and insulin-like growth factor II gene promoters (20, 21). Moreover, Kim et al. (20) demonstrated that the TGF-β1, c-*fos*, and c-*myc* promoters can be both positively and negatively regulated by RB through the same *cis*-acting element, dependent upon the cell type. With all of these studies, a common sequence, CCACCC, termed the RCE motif, has been identified as being important for conferring RB-mediated regulation of gene expression (21). Furthermore, changing CCACCC to a consensus Sp1-binding sequence, CCGCCC, has no effect on the ability of RB to activate transcription (21).

Recent reports have suggested that RB directly interacts with three important transcription factors, E2F, ATF-2 and Elf-1 (3, 8, 21, 47). E2F regulates both viral and cellular gene expression, and RB-E2F interactions are thought to be important events in cell cycle control (3, 8). RB–ATF-2 interactions are important for conferring *Rb* regulation of the TGF-β2 promoter (22). Elf-1 is a lymphoid-specific Ets transcription factor that regulates inducible gene expression during T-cell activation (47). The RB–Elf-1 interaction may be important for coordinating lineage-specific effector functions, such as cytokine production with cell cycle progression in activated T cells (47). Taken together, these findings show that RB binds a number of cellular proteins, which may be directly or indirectly involved in the transcriptional regulation of a set of genes required for controlling cell growth. These results reveal a new mechanism by which RB constrains cellular proliferation.

Given that the RB protein has a nuclear localization and represses the expression of the c-*fos* gene, whose gene product Fos is one component of the heterodimeric transcription factor AP-1 (11, 32), it is possible that RB can be functionally linked to c-*jun* and play a role in transcriptional control. Constitutive activation of the nuclear-acting proto-oncogene c-*jun* leads to increased transcription of a set of genes which generate malignant phenotypes in some cells (41, 42), and inactivation of the *Rb* gene is also associated with the etiology of many human cancers (5, 15, 17, 18, 43, 44, 48, 49). These two classes of genes, c-*jun* acting positively on cell growth and the other acting negatively, may directly influence each other through an unknown mechanism.

The rat c-*jun* promoter contains G+C-rich sequences between −125 and −100 that include a consensus Sp1 binding site (−115 to −100) and a sequence, CCACCC (−118 to −123) (24, 25), which is identical to the previously identified

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RCE. Since the sequence CCACCC is found in the rat c-*jun* promoter, we examined whether RB might also regulate c-*jun* promoter activity through this element. Our results demonstrate that transient expression of human RB significantly stimulated the activity of the rat c-*jun* promoter in murine fibroblast, 3T6, and NIH 3T3 cells. Deletion and mutagenesis analysis reveal that the RB responsiveness in the c-*jun* promoter is conferred via the Sp1-binding sites between nucleotides −125 and −100. In addition, we demonstrate that three predominant complexes in mobility shift assays, including the transcription factor Sp1 (23), can be inhibited by addition of RB monoclonal antibodies. This finding suggests that the RB protein is directly or indirectly involved in Sp1 binding. Furthermore, we demonstrate that CV-1 cell nuclear extracts contain an Sp1 negative regulator(s) (Sp1-I), which inhibits formation of the mobility-shifted complexes. The inhibition of the DNA-binding activity of Sp1 can be reversed by addition of baculovirus-expressed RB. Finally, we have identified Sp1-I as a ~20-kDa protein(s) that is also an RB-binding protein(s). These findings reveal a novel mechanism involved in transcriptional activation by an RB protein.

MATERIALS AND METHODS

Cell culture. NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium plus 10% calf serum. 3T6 mouse fibroblasts, CV-1 cells (a green monkey kidney cell line), COS-1 cells (simian virus 40-transformed CV-1 cells), CCL64 mink lung epithelial cells, HeLa human epithelioid carcinoma cells, 3Y1 mouse embryonal fibroblasts, and Saos2 human osteogenic sarcoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Molt4 human acute lymphoblastic leukemia cells were grown in RPMI 1640 plus 10% fetal bovine serum. Cells were grown at 37°C in a humidified incubator under 6% CO_2.

Plasmid constructs, transfections, and CAT assays. The construction of a series of deletion mutants of the rat c-*jun* gene promoter linked to the bacterial chloramphenicol acetyltransferase (CAT) gene has been described elsewhere (24). −126/+874c-*jun*CAT and its Sp1 mutant (mSp1−126/+874c-*jun*CAT) were generated by PCR amplification of a DNA fragment between nucleotides −126 and −20. The amplified fragment was digested with restriction enzymes *Xba*I and *Sac*I and was used to replace the −730 to −20 fragment within the −730/+874c-*jun*CAT construct. Primers used for PCR amplification were 5′-TCCATCTAGACGGGTGGGCCCGCCCC CTT-3′ (contains an *Xba*I site [underlined] and wild-type Sp1 sites), 5′-TCCATCTAGACGGGTGaattCGCCCCCTT-3′ (contains an *Xba*I site [underlined] and a mutated Sp1 site [in lowercase]), and 5′-GTCCTTATCCAGCCAGCCTGAGCT C-3′ (contains a *Sac*I site at −20 relative to the transcription start site). The mutant sequences were confirmed by dideoxy-chain termination DNA sequencing (40). A baculovirus-expressed *Rb* plasmid was constructed by insertion of an *Ava*II-to-*Sca*I fragment isolated from a pSVE-hRb vector (4) into the *Sma*I site of the baculovirus transfer plasmid pVL1393 (PharMingen). The GAL4-Sp1 fusion plasmid has been described by Kim et al. (21) and was provided by Jay Gralla. The human *Rb* expression plasmid phRb and control vector pJ3Ω were provided by Robert Weinberg and have been previously described (4). G_5E4T.CAT and GAL4-VP1 (14, 36, 37) were provided by Michael Carey.

Transfections were performed by the calcium phosphate precipitation method (16). Unless otherwise specified, each 100-mm-diameter culture dish was incubated for 14 h with calcium phosphate-DNA coprecipitates containing 2.5 μg of a reporter plasmid and 7.5 μg of a control vector (pJ3Ω) or human *Rb* expression plasmid (phRb). Forty-eight hours after transfection, protein extracts were prepared and CAT activity was determined. All transfections were repeated at least three times. For normalization of transfection efficiencies in 3T6 and CV-1 cells, a luciferase reporter gene driven by Rous sarcoma virus promoter (RSV-Luc) was included in the cotransfection. The level of luciferase expression was determined by using a luminometer (Monolight 1500; Analytical Luminescence Laboratory).

Purification of recombinant human RB protein. The cleared insect lysate containing recombinant human RB protein was applied to an anti-human RB G3-245–Sepharose matrix (50 mg of anti-human RB monoclonal antibody G3-245 [PharMingen] to 5 ml of cyanogen bromide-activated Sepharose [Pharmacia]). The flowthrough fraction was reapplied a second time to achieve higher binding efficiencies. The chromatography matrix was washed extensively with Triton X-100-based lysis buffer containing 1 M NaCl. The RB protein was eluted with 50 mM diethylamine (pH 11.3), and the eluant was neutralized immediately by adding 1 M Tris-HCl (pH 7.0). The peak fractions were pooled and dialyzed against 250 mM NaCl–10% glycerol–10 mM Tris-HCl (pH 7.0).

Oligonucleotides and electrophoretic mobility shift assays (EMSAs). Oligonucleotides were synthesized on an Applied Biosystems automated DNA synthesizer, deprotected, and then partially purified through Sephadex G-25 spin columns. The sequence of the wild-type human c-*jun* Sp1 site (hcj-Sp1) oligonucleotide used in these experiments was 5′-TGACGG GCGGGCCCGCCCCCCT-3′. Complementary oligonucleotides were annealed and labeled at their 5′ ends, using [γ-^{32}P] ATP (4,500 Ci/mmol; ICN) and T4 polynucleotide kinase (New England Biolabs). Radiolabeled double-stranded oligonucleotides were purified through a Sephadex G-25 spin column. Typically, the specific activities of oligonucleotide probes were 10^5 cpm/ng of DNA. Probes were stored at −20°C.

EMSAs were performed by incubating for 30 min at 4°C in binding buffer (10 mM Tris-HCl [pH 7.5], 40 mM NaCl, 1 mM MgCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 4% glycerol) 4 to 10 μg of nuclear extracts, 5,000 cpm of oligonucleotide probe, 1 μg of acetylated bovine serum albumin, and 2 μg of poly(dI-dC) (Pharmacia LKB Biotechnology) in a total reaction volume of 20 μl. For the competition study, a 100-fold molar excess of unlabeled oligonucleotides was added to the reaction mixture prior to the addition of radiolabeled probe, and the mixture was then incubated at room temperature for 15 min. For supershift assays, the antiserum was preincubated with nuclear extracts for 1 h at 4°C prior to initiation of the binding reaction. Mobility shift reactions were resolved on 4% nondenaturing polyacrylamide gels that were electrophoresed at 200 V at 4°C for 3 h. Gels were then dried and exposed to X-ray film with an intensifying screen at −80°C.

Western blot (immunoblot) analysis. Nuclear extracts (20 μg) were electrophoresed on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels and transferred to nitrocellulose filters. Filters were probed with an anti-Sp1 polyclonal antibody (a generous gift from Stephen Jackson), and an alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G antibody was used as a secondary reagent. The Sp1 antibodies were raised against a synthetic peptide (PEP2) corresponding to amino acid residues 520 to 538 of the Sp1 protein (19).

Cell labeling, immunoprecipitation, and gel electrophoresis. Cell proteins were biosynthetically labeled for 2 h by addition of [^{35}S]methionine (500 μCi; Amersham) to 4×10^6 *Spodoptera frugiperda* Sf9 cells infected with 10^7 viral particles for 2

days. The labeled cells were washed three times in phosphate-buffered saline and lysed in Triton X-100-based lysis buffer. The lysate was incubated with 2 μg of anti-RB monoclonal antibody G3-245 (PharMingen) per ml at 4°C for 16 h. Protein G-Sepharose was added, and the immunoprecipitates were washed five times in Triton X-100-based lysis buffer. Finally, the Sepharose beads were boiled in a reducing SDS sample buffer, and the supernatant was separated on an SDS–5 to 15% polyacrylamide gradient gel. The radiolabeled proteins were visualized by autoradiography of the dried gel.

Partial purification of an RB-associated Sp1-I from CV-1 nuclear extracts. Two milligrams of CV-1 nuclear extracts was applied to a 200-μl bed volume of a heparin-agarose column which was preequilibrated with buffer A containing 20 mM HEPES (pH 7.9), 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 20% (vol/vol) glycerol. After the column was washed with a 10× bed volume of buffer A, Sp1-I was eluted with buffer A containing 1 M KCl. The fractions containing Sp1-I activity were pooled and subjected to a preequilibrated Sephadex G-150 column (1.0 by 27 cm) and eluted with buffer A containing 0.1 M KCl. Aliquots of each fraction of 0.5 ml were used to monitor the inhibition of DNA-binding activity of Sp1. The approximate molecular weight of Sp1-I was determined by using alcohol dehydrogenase, bovine serum albumin, carbonic anhydrase, and cytochrome *c* as markers.

RB-Sepharose affinity columns were prepared by immobilizing the baculovirus-expressed RB proteins to cyanogen bromide-activated Sepharose 4B. Sepharose used as a negative control was prepared by inactivating cyanogen bromide-activated Sepharose 4B by using 1 M glycine at pH 8.0. CV-1 nuclear extracts were applied to a preequilibrated RB-Sepharose affinity column. After the column was washed extensively with buffer A, the bound proteins were eluted with buffer A containing 1 M KCl and dialyzed against mobility shift assay binding buffer for the determination of activity inhibitory to Sp1 complex formation.

RESULTS

RB-stimulated c-*jun* gene expression is mediated through Sp1-binding sites. Six cellular genes, c-*fos*, c-*myc*, the TGF-β1 and TGF-β2 genes, *neu*, and the insulin-like growth factor II gene, have been identified as targets of transcriptional regulation by RB (20–22, 31, 33, 50). One identified RB-responsive element has a 6-bp core sequence, CCACCC, which has been termed the RCE. The rat c-*jun* promoter contains G+C-rich sequences between −125 and −100 that include a consensus Sp1-binding site (−115 to −100) and a CCACCC sequence (−118 to −123) which is identical to the previously identified RCE.

To determine whether RB regulates the c-*jun* promoter in a manner similar to that observed in the TGF-β1 and insulin-like growth factor II promoters, a construct containing the rat c-*jun* promoter, −730/+874, fused to a bacterial CAT gene (24) was used in cotransfection assays into murine 3T6 cells with either a control plasmid (pJ3Ω) or a human Rb expression vector (phRb). As shown in Fig. 1, lane 2, the CAT activity was increased about sixfold by cotransfection with phRb as compared with the control (Fig. 1, lane 1). To map the RB-responsive element in the c-*jun* promoter, a series of 5′ deletion constructs were used in the cotransfection experiments. The CAT activities of the constructs −328/+874 and −126/+874 were induced four- to sixfold by RB (Fig. 1). However, the induction of CAT activity by RB decreased to basal levels similar to control levels when the deletion series reached −79. These results suggest that sequences between −126 and −79, including the RCE and Sp1-binding sites, are important for RB-mediated transactivation. To directly examine the role of these RCE and Sp1-binding sites in response to RB, mutant mSp1−126/+874CAT, containing nonfunctional RCE and Sp1 sites (5′-GGGTGAATTCGCCCCCC-3′), was used for cotransfection experiments. As expected, the construct mSp1−126/+874CAT, with mutated RCE and Sp1 sites, eliminated RB responsiveness (Fig. 1). These results are consistent with previous reports that Sp1-mediated transcription is stimulated by the transient coexpression of the RB protein (20, 21).

RB antibodies diminish Sp1 multigene product binding activities. To investigate whether the c-*jun* Sp1 site would bind a similar or identical set of binding factors which resemble RCE-retinoblastoma control binding proteins, we performed EMSAs using the hcj-Sp1 probe or a rat c-*jun* Sp1 probe. We observed three predominant mobility shift complexes with COS-1 nuclear extracts (Fig. 2A, lane 1; complexes 1a, 1b, and 2). Crude nuclear extracts from CCL64, HeLa, Molt4, 3Y1, and Saos2 (an *Rb*-negative osteosarcoma cell line) cells also give similar mobility shift patterns. It has been reported that Sp1 encodes one of three nuclear proteins that bind RB-regulated promoter elements (21).

To provide further evidence that the predominant complex 1a is Sp1 protein, we used either rabbit polyclonal antibody 2892 or monoclonal antibody IC6 (a generous gift from Stephen Jackson) raised against Sp1 in an EMSA. Preincubation of the nuclear extracts with the Sp1 antibodies resulted in a supershift and concomitant reduction of complex 1a but did not affect the binding of complexes 1b and 2 (Fig. 2A, lanes 3 and 4). Interestingly, preincubation of the extracts with RB monoclonal antibodies RB-Ab1 (Rb-IF8; Santa Cruz Biotechnology) and RB-Ab2 (XZ133; PharMingen) resulted in diminishing the binding of all three Sp1 complexes, 1a, 1b, and 2 (Fig. 2A, lanes 5 and 6; Fig. 2B, lane 2). In contrast, the Sp1 complexes were not affected by preincubation with either an anti-c-Jun (AB-1; Oncogene Science) or anti-Fos (c-*fos* 4; Santa Cruz Biotechnology) antibody (Fig. 2B, lanes 3 and 4). These findings suggest that RB or an RB-associated factor(s) is involved in the binding of these predominant complexes. To distinguish between these two possibilities, Saos2 nuclear extracts lacking RB were used for EMSAs. Preincubation of Saos2 nuclear extracts with the Sp1 antibodies resulted in a supershift (Fig. 2C, lane 2) as observed with other extracts, but preincubation with RB antibodies did not affect the binding complexes (Fig. 2C, lane 3) as observed in Fig. 2A, lanes 5 and 6. These results suggest that RB is most likely indirectly involved in the DNA-binding complexes of Sp1.

Sp1-binding activity is enhanced by recombinant RB. To investigate more directly whether RB associates directly with Sp1, thus affecting DNA-binding complexes of Sp1, we performed EMSAs in the presence of affinity column-purified baculovirus-expressed RB protein. The recombinant RB protein was identified by immunoprecipitation of [^{35}S]methionine-labeled insect cell lysates containing recombinant RB (Fig. 3A). As shown in Fig. 3B, lanes 4 and 5, RB protein quantitatively enhanced Sp1-binding activity instead of altering the pattern of the Sp1 complexes. This enhanced binding activity can be blocked by preincubation of recombinant RB with an RB antibody (Santa Cruz Biotechnology) (Fig. 3B, lane 6), suggesting that RB-mediated Sp1 binding is specific and that increased Sp1-binding activity mediated by RB is possibly due to the RB protein titrating away a negative regulator(s) (Sp1-I), which can affect Sp1 binding.

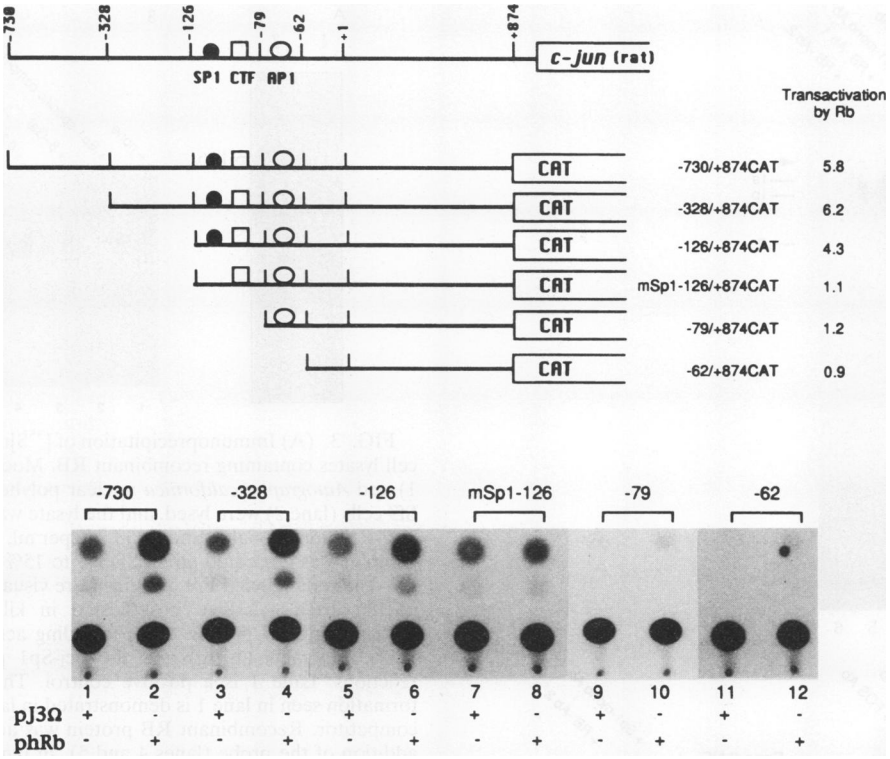

FIG. 1. Mapping of the RB-responsive element in the rat c-*jun* promoter. The schematic diagram shows the rat c-*jun* gene (−730 to +874) and a series 5′ deletion mutants (nucleotides −328, −126, −79, and −62) fused to the CAT reporter gene. The Sp1, CTF, and AP-1 binding sites are indicated with filled circles, squares, and open circles, respectively. The mSp1−126/+874CAT construct contains a mutated Sp1-binding site (underlined), as indicated by the following sequence: 5′-GGGTGAATTCGCCCCCC-3′. The rat c-*jun*CAT plasmids were cotransfected into 3T6 fibroblasts with either a control plasmid (pJ3Ω) or a human *Rb* expression vector (phRb). The CAT activities stimulated by RB represent averages of three or more experiments of the relative fold increase versus the control lacking RB. An autoradiogram from a representative CAT assay is shown.

A negative regulator(s) inhibits Sp1 multigene product binding activities. During the course of study of DNA-binding complexes with various sources of nuclear extracts, we demonstrate that CV-1 cell nuclear extracts have undetectable levels of Sp1, in both DNA mobility shift (Fig. 4A, lane 5) and Western blot (Fig. 4B, lane 5) analysis, compared with extracts prepared from COS-1 cells. COS-1 cells are derived from CV-1 cells with the incorporation of simian virus 40 large T antigen. The mechanism for the lack of Sp1 protein in CV-1 cells is not clear. One possibility is a negative regulator preventing expression of the Sp1 gene product.

To examine whether CV-1 cells contain such an Sp1 negative regulator, we mixed CV-1 and COS-1 cell nuclear extracts in various ratios for EMSAs. Mixing of CV-1 and COS-1 extracts in a 1:1 ratio resulted in depletion of Sp1 binding compared with COS-1 nuclear extracts alone (Fig. 4A, lanes 1 and 4). The inhibition of the formation of Sp1 complexes was also observed when we mixed CV-1 with CCL64, HeLa, Molt4, or 3Y1 cell nuclear extracts (data not shown). The quality of CV-1 nuclear extracts was ensured by its retaining tumor promoter-responsive element (TRE)-binding activity (Fig. 4C, lane 1). In contrast to the inhibition of Sp1 binding by nuclear extracts prepared from CV-1 cells, TRE-binding activity with COS-1 or CCL64 cell nuclear extracts was increased in an additive manner rather than being inhibited by addition of CV-1 nuclear extracts (Fig. 4C, lane 5). Taken collectively, these results indicate that CV-1 nuclear extracts contain an Sp1 negative regulator(s) that specifically inhibits the formation of Sp1 complexes. We will henceforth refer to this Sp1 negative regulator as Sp1-I.

Inhibition of Sp1-binding activity can be reversed by recombinant RB. The results presented above suggest that RB is indirectly involved in the DNA-binding activity of Sp1. To test the possibility that Sp1-I from CV-1 nuclear extracts associates with RB protein, we performed EMSAs by addition of recombinant RB in experiments as described above. As shown in Fig. 4D, addition of recombinant RB protein reversed the inhibited Sp1-binding activity in mixed CV-1 and CCL64 cell extracts without changing mobility shift patterns (Fig. 4D, lanes 1, 4, and 5). A similar result was obtained, although with less effectiveness to restore the Sp1-binding activity, when COS-1 instead of CCL64 nuclear extracts were used (data not shown). These results provide strong evidence that there is no direct association between RB and Sp1 proteins; rather, RB liberates Sp1 from Sp1-I, resulting in increased Sp1-binding activity.

Functional evidence for the presence of Sp1-I in CV-1 cells. Our in vitro data presented above strongly suggest that RB is involved in Sp1 binding via interaction with Sp1-I. However, we have not observed DNA-binding activity by Sp1-I (Fig. 4A, lane 5), suggesting that Sp1-I inhibition of the DNA-binding activity Sp1 is not due to competition for the same Sp1-binding sequence but instead forms a non-DNA-binding Sp1–Sp1-I complex.

To provide functional evidence for an Sp1-I, we used a GAL4-Sp1 fusion construct lacking an Sp1 DNA-binding domain to determine the effect of RB on GAL4-Sp1-mediated

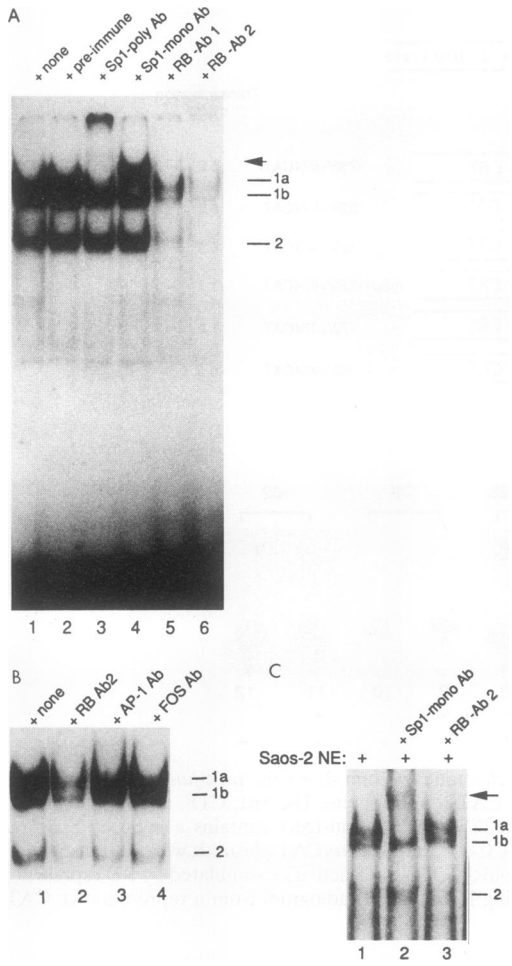

FIG. 2. Characterization of the factors binding to hcj-Sp1 in EMSAs. (A) COS-1 nuclear extracts (4 μg) were preincubated with preimmune serum, polyclonal (poly) or monoclonal (mono) Sp1 antibodies (Ab), or monoclonal RB antibodies (Rb-Ab1 and Rb-Ab2) prior to initiation of binding reactions (lanes 2 to 6). The supershifted band is indicated with an arrow. The mobility shift assay shown in lane 1 was carried out without pretreatment. (B) COS-1 nuclear extracts (4 μg) were preincubated with or without monoclonal RB antibody 2, c-Jun/AP-1 antibody, or FOS antibody; 0.5 μg of each antibody was used in the indicated reactions in panel A and B. (C) Saos2 nuclear extracts (NE; 4 μg) were preincubated with monoclonal Sp1 or RB antibodies prior to initiation of binding reactions (lanes 2 and 3). The EMSA in lane 1 was carried out without pretreatment.

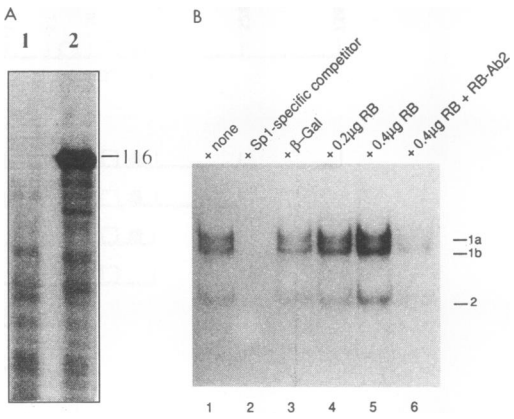

FIG. 3. (A) Immunoprecipitation of [^{35}S]methionine-labeled insect cell lysates containing recombinant RB. Mock-infected Sf9 cells (lane 1) and *Autographa californica* nuclear polyhedrosis virus-RB-infected Sf9 cells (lane 2) were lysed, and the lysate was incubated with 2 mg of anti-RB monoclonal antibody G3-245 per ml. The immunoprecipitated complex was separated on an SDS–5 to 15% polyacrylamide gradient gel. The radiolabeled RB proteins were visualized by autoradiography of the dried gel. Size is indicated in kilodaltons. (B) Effect of recombinant RB protein on Sp1-binding activity in EMSAs. CCL64 nuclear extracts (1 μg) and the hcj-Sp1 probe were used in all reactions. Lane 1 is a positive control. The specificity of complex formation seen in lane 1 is demonstrated in lane 2 with an Sp1-specific competitor. Recombinant RB protein was added to EMSAs prior to addition of the probe (lanes 4 and 5). In lane 3, 0.4 μg of β-galactosidase (β-Gal; Sigma) was used as a negative control. In lane 6, 0.4 μg of recombinant RB protein was preincubated with monoclonal RB antibody on ice for 1 h prior to EMSAs.

transcriptional regulation in CV-1 cells. The GAL4-Sp1 fusion construct was cotransfected into CV-1 cells in the presence of either a control expression vector (pJ3Ω) or a human *Rb* expression plasmid (phRb) with a CAT reporter, G_5E4T.CAT (14). GAL4-VP1 (14) was used as a positive control (Fig. 5, lanes 3 and 4). Transfections with either GAL4(1–147) or pJ3Ω did not stimulate CAT activities of the G_5E4T.CAT reporter (Fig. 5, lanes 1, 2, and 9). Surprisingly, increased amounts of GAL4-Sp1, even up to 1 μg, did not result in increased CAT activity (Fig. 5, lanes 5 to 8). However, transcriptional stimulation was observed by cotransfecting GAL4-Sp1 and phRb, while RB on its own did not stimulate the GAL4-responsive promoter (data not shown). This stimulation increased in a linear fashion dependent upon increasing amounts of RB (Fig. 5, lanes 10 to 12). This result is similar to that in which E2 markedly superactivates the GAL4-Sp1 fusion protein over a range of GAL4-Sp1 levels in CV-1 cells (27). The ability of GAL4-Sp1 to induce transcriptional activity in vivo in the presence of RB also correlates with our in vitro results (Fig. 3B and Fig. 4D). These results provide additional evidence for Sp1-I, a negative regulator(s), being involved in the Sp1-mediated transactivation and also substantiate the notion that Sp1 itself is able to mediate RB activation (21, 45).

Sp1-I is a heat-labile and a proteinase K-sensitive protein. To characterize the nature of Sp1-I, we performed a heat inactivation study. CV-1 nuclear extracts were preincubated at 50, 75, or 100°C for 5 min prior to performing EMSAs. As shown in Fig. 6A, heat-treated CV-1 extracts lose inhibition of Sp1-binding activities, indicating the heat-labile nature of Sp1-I. Furthermore, to examine whether Sp1-I is sensitive to protease digestion, we pretreated CV-1 nuclear extracts with proteinase K (Sigma) prior to mixing with COS-1 extracts for EMSAs. Preincubation of 8 μg of CV-1 extracts with 0.001 U of proteinase K at 25 or 37°C results in loss of inhibitory activities to different degrees (Fig. 6B, lanes 4 and 5). Collectively, these findings suggest that Sp1-I is a heat-labile and proteinase K-sensitive protein.

Identification of Sp1-I, a ~20-kDa protein(s) that inhibits the Sp1 complexes from binding to DNA. The data presented above clearly suggest that Sp1-I is a component of proteins in CV-1 nuclear extracts. Identification and purification of this negative regulator, Sp1-I, are important for examining its inhibition of the DNA-binding activity of Sp1. The extracts were fractionated successively through heparin-agarose and Sephadex G-150 column chromatography (Fig. 7A). The column fractions were assayed in EMSA reaction mixtures containing COS-1 nuclear extracts with Sp1 DNA-binding activi-

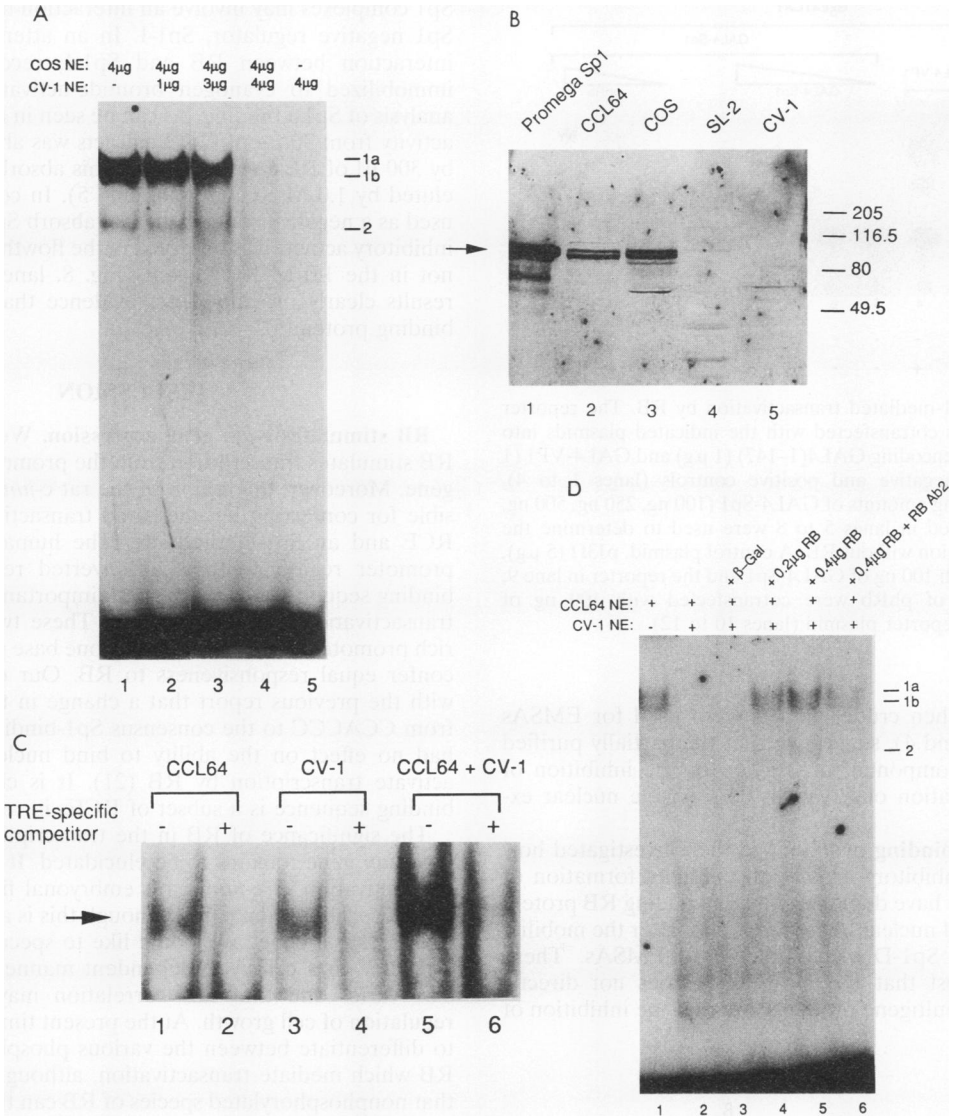

FIG. 4. Inhibition of Sp1 binding activity by CV-1 cell nuclear extracts. The hcj-Sp1 probe was used in all EMSAs. (A) Experiments in which CV-1 and COS-1 nuclear extracts (NE) were mixed in various ratios for EMSAs. COS-1 nuclear extracts only (lane 1), a constant amount of COS-1 nuclear extracts with increased amounts of CV-1 nuclear extracts as indicated (lanes 2 to 4), and CV-1 nuclear extracts alone (lane 5) were used for EMSAs. The amounts of nuclear extracts used in each reaction are indicated. (B) Western blot analysis of Sp1 proteins. Five footprint units of purified Sp1 (Promega) and 20 μg of nuclear extracts prepared from CCL64, COS-1, SL2, and CV-1 cells were separated on an SDS–10% polyacrylamide gel and transferred to nitrocellulose filters. Filters were probed with polyclonal Sp1 antibody 2892. The position of Sp1 protein in the autoradiography is indicated with an arrow. The sizes of molecular weight markers in kilodaltons are presented. (C) CV-1 nuclear extract contains TRE-binding factors. A TRE oligonucleotide that contains the AP-1-binding sequence 5′-CGCTTGATGAGTCAGCC-GGAA-3′ was obtained from Promega. Ten micrograms of CCL64 nuclear extracts (lanes 1 and 2), 10 μg of CV-1 nuclear extracts (lanes 3 and 4), and a combination of 10 μg each of CCL64 and CV-1 nuclear extracts were used for EMSAs. Prior to the addition of the TRE probe, lanes 2, 4, and 6 were subjected to competition with a 100-fold molar excess of unlabeled TRE to show the specificity of TRE-binding activity. (D) Recombinant RB protein reverses the inhibitory effect of CV-1 nuclear extracts. The hcj-Sp1 probe with a combination of 2 μg each of CCL64 and CV-1 nuclear extracts was used for EMSAs (lanes 2 to 6). Lane 1 contains CCL64 nuclear extracts only; 0.2 and 0.4 μg of recombinant RB were added in lanes 4 and 5, respectively; 0.4 μg of β-galactosidase protein (Sigma) was added as a negative control (lane 3). In lane 6, 0.4 μg of recombinant RB was preincubated with RB-Ab2 on ice for 1 h prior to EMSAs.

ties and a specific c-*jun* Sp1 DNA probe (Fig. 7B). In the course of these fractionations, fractions 36 to 40 clearly contained an activity inhibitory to Sp1 binding (Fig. 7B). The major protein in the active fractions of the Sp1-I partially purified through Sephadex G-150 chromatography is a polypeptide of approximately 20 kDa. Moreover, the partially purified Sp1-I has no DNA-binding activity to the Sp1 site (Fig. 7C, lane 2) and inhibits the DNA-binding activity of Sp1 from CCL64 nuclear extracts (Fig. 7C, lane 3). To determine whether the inhibitory function of this partially purified Sp1-I is regulated by RB protein as demonstrated previously for CV-1 extracts, we added recombinant RB in the mixtures of CCL64 nuclear extracts and partially purified Sp1-I for EMSA. We observed that the inhibition of Sp1 binding by a partially purified Sp1-I is also reversed by addition of recombinant RB (Fig. 7C, lanes 4 and 5). These results are similar to those

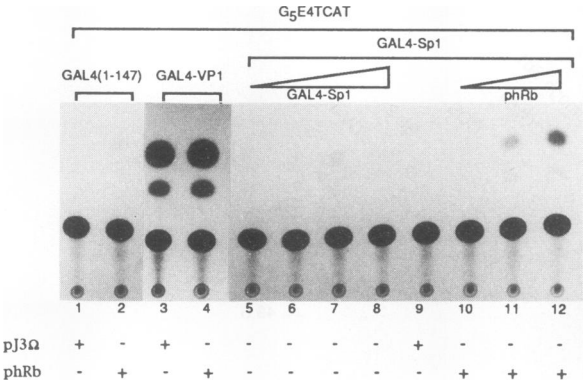

FIG. 5. GAL4-Sp1-mediated transactivation by RB. The reporter plasmid (2.5 μg) was cotransfected with the indicated plasmids into CV-1 cells. Plasmids encoding GAL4(1–147) (1 μg) and GAL4-VP1 (1 μg) were used as negative and positive controls (lanes 1 to 4), respectively. Increasing amounts of GAL4-Sp1 (100 ng, 250 ng, 500 ng, and 1 μg) as indicated in lanes 5 to 8 were used to determine the degree of transactivation without RB. A control plasmid, pJ3Ω (5 μg), was cotransfected with 100 ng of GAL4-Sp1 and the reporter in lane 9; 2.5, 5, and 7.5 μg of phRb were cotransfected with 100 ng of GAL4-Sp1 and the reporter plasmid (lanes 10 to 12).

described above when crude extracts were used for EMSAs (Fig. 4D, lanes 3 and 4), suggesting that the partially purified Sp1-I is a major component of activity for the inhibition of Sp1 complex formation observed in CV-1 crude nuclear extracts.

Sp1-I is an RB-binding protein. We then investigated how RB reverses the inhibitory activity and induces formation of Sp1 complexes. We have demonstrated that adding RB protein to COS-1 or CCL64 nuclear extracts does not alter the mobility but enhances the Sp1-DNA complexes in EMSAs. These observations suggest that RB most likely does not directly interact with Sp1 multigene products and that the inhibition of

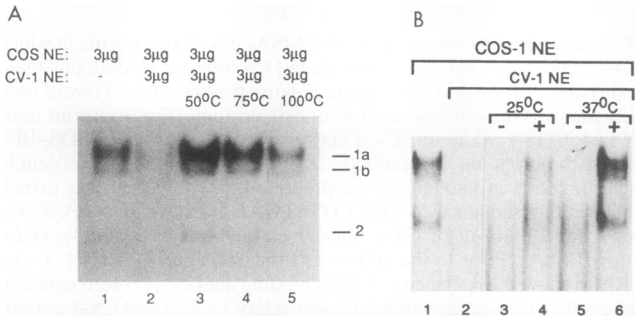

FIG. 6. Sp1-I is a heat-labile and proteinase K-sensitive protein. (A) CV-1 nuclear extracts (NE) were heated at 50, 75, or 100°C for 5 min (lanes 3 to 5) and then mixed with COS-1 nuclear extracts for EMSAs. A positive and a negative control are shown in lanes 1 and 2, respectively. (B) Experiments in which COS-1 and CV-1 nuclear extracts were mixed in a 1:1 ratio with the hcj-Sp1 probe for EMSAs. Eight micrograms of COS-1 nuclear extracts alone (lane 1), COS-1 and CV-1 extracts mixed in a 1:1 ratio (lane 2), COS-1 and heat-pretreated CV-1 extracts at 25°C for 10 min or at 37°C for 3 min (lanes 3 and 5), and COS-1 extracts with proteinase K (0.001 U)-pretreated CV-1 extracts at 25°C for 10 min or at 37°C for 3 min (lanes 4 and 6) are shown. Proteinase K enzymatic activity was stopped by adding 25 μM phenylmethylsulfonyl fluoride prior to mixing with COS-1 extracts for EMSA.

Sp1 complexes may involve an interaction between RB and an Sp1 negative regulator, Sp1-I. In an attempt to see specific interaction between RB and Sp1-I, recombinant RB was immobilized to cyanogen bromide-activated Sepharose for analysis of Sp1-I binding. As can be seen in Fig. 8, lane 3, Sp1-I activity from 30 μg of CV-1 extracts was absorbed completely by 300 μl of RB-Sepharose, and this absorbed Sp1-I could be eluted by 1.0 M KCl (Fig. 8, lane 5). In contrast, Sepharose, used as a negative control, did not absorb Sp1-I. Therefore, an inhibitory activity was observed in the flowthrough fraction and not in the 1.0 M KCl eluant (Fig. 8, lanes 6 and 8). These results clearly provide direct evidence that Sp1-I is an RB-binding protein(s).

DISCUSSION

RB stimulates c-*jun* gene expression. We demonstrate that RB stimulates transcription from the promoter of the rat c-*jun* gene. Moreover, the region of the rat c-*jun* promoter responsible for conferring RB-mediated transactivation contains an RCE and an Sp1-binding site. The human c-*jun* G+C-rich promoter region contains an inverted repeat of two Sp1-binding sequences, which are also important for RB-mediated transactivation (data not shown). These two different G+C-rich promoter elements, with only one base pair difference, can confer equal responsiveness to RB. Our observations agree with the previous report that a change in the RCE sequence from CCACCC to the consensus Sp1-binding site, CCGCCC, had no effect on the ability to bind nuclear factors and to activate transcription by RB (21). It is clear that the Sp1-binding sequence is a subset of RCEs in response to RB.

The significance of RB in the transcriptional regulation of the c-*jun* gene remains to be elucidated. It has been reported that activation of c-*jun* in rat embryonal fibroblast promotes cellular proliferation (41). Although this is a correlation rather than a causal effect, we would like to speculate that as RB is regulated in a cell cycle-dependent manner, c-*jun* expression also varies and that this correlation may be essential for regulation of cell growth. At the present time, we have no data to differentiate between the various phosphorylated forms of RB which mediate transactivation, although our data suggest that nonphosphorylated species of RB can titrate away the Sp1 negative regulator. Taken altogether, our results suggest that Sp1-mediated transactivation of c-*jun* expression by RB is one of the mechanisms whereby RB exerts control on cell growth. Furthermore, RB-mediated transactivation of c-*jun* can be distinguished from the induction of c-*jun* by other exogenous stimuli such as phorbol esters, growth factors, and lymphokines (1, 2, 6, 26, 30, 34, 35).

RB also regulates one of the TGF-β isoforms, TGF-β2. However, the TGF-β2 promoter is stimulated by RB through an ATF-2 binding site, a different RB-responsive element. Therefore, it is reasonable to speculate that RB can mediate the transcriptional regulation of many other genes, which have yet to be discovered. Indeed, we have mapped a novel DNA sequence in the human *junB* promoter which can confer RB responsiveness (12a). Whether the physical interaction between RB and the *cis*-acting element-binding factor accounts for the stimulation of *junB* by RB protein remains to be determined.

Sp1 multigene products are targets for RB-mediated transactivation. We have shown that a G+C-rich Sp1-binding sequence forms three predominant protein-DNA complexes in vitro with nuclear extracts (Fig. 2A, lane 1). These are similar to RCE complexes previously shown to be regulated by RB (45, 46). Moreover, it has been demonstrated that an oligonu-

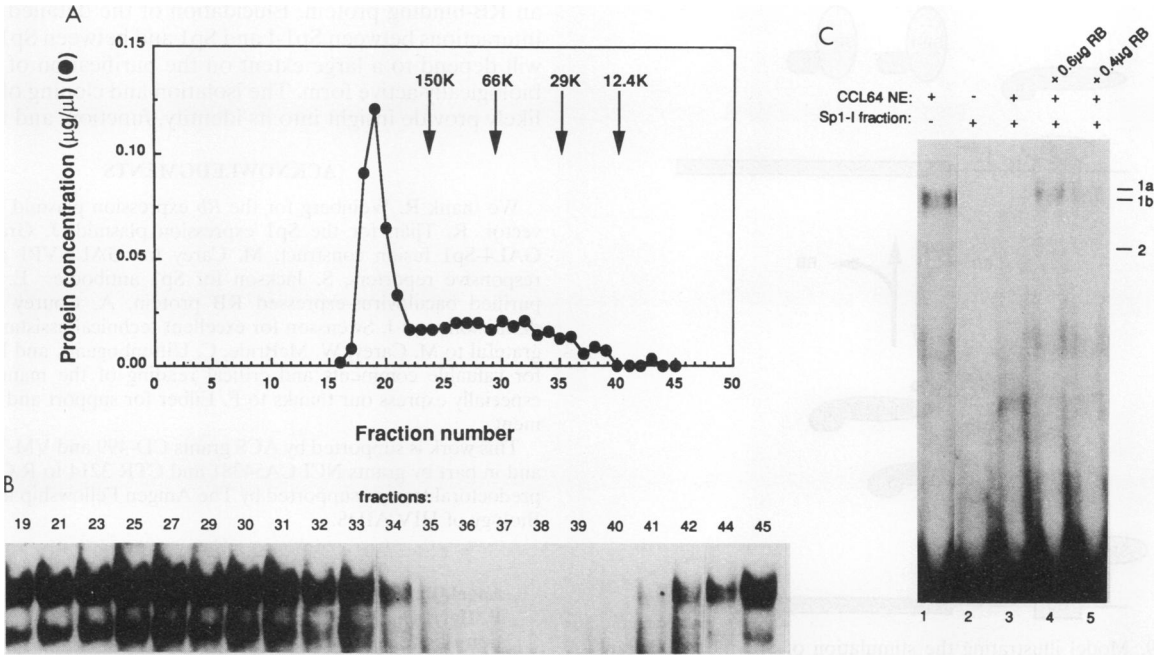

FIG. 7. Purification and characterization of Sp1-I. (A) Sephadex G-150 gel filtration of CV-1 nuclear extracts. Gel filtration was performed as described in Materials and Methods. The molecular size markers (alcohol dehydrogenase [150 kDa], bovine serum albumin [66 kDa], carbonic anhydrase [29 kDa], and cytochrome *c*, 12.4 kDa) are indicated with arrows. (B) The hcj-Sp1 probe was used for EMSAs. Aliquots of each fraction were added to 4 μg of COS-1 nuclear extracts for EMSA to detect the activity inhibitory to the DNA-binding activity of Sp1. Fractions 36 to 40, corresponding to the molecular size range of 24 to 13 kDa, showed significant activity inhibitory to Sp1 binding. (C) Partially purified Sp1-I (pooled fractions 36 to 40) has no DNA-binding activity for the Sp1 sequence (lane 2) as compared with 2 μg of CCL64 nuclear extracts (NE) (lane 1) for EMSA with the hcj-Sp1 probe. Partially purified Sp1-I inhibits the DNA-binding activity of Sp1 from CCL64 extracts (lane 3), and this inhibition was reversed by addition of 0.6 or 0.4 μg of recombinant RB (lane 4 or 5, respectively).

cleotide containing two Sp1-binding sites can abolish the appearance of RCE complexes (45). These results suggest that a subset of RB-regulated promoter elements, the Sp1-binding sequence, can also interact with a common set of nuclear factors (retinoblastoma control binding protein) in vitro. Mutation of one of the inverted repeats, either the upstream or downstream Sp1-binding site, did not affect protein-DNA complex formation (data not shown). However, mutations that prevented Sp1 binding to the G+C-rich promoter element in vitro also abrogate Sp1-mediated transactivation by RB protein in vivo (Fig. 1, lane 8). These data clearly indicate that a single Sp1-binding site is sufficient for conferring RB-mediated transactivation.

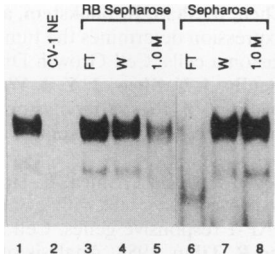

FIG. 8. Sp1-I is an RB-binding protein. RB-Sepharose was used for analysis of Sp1-I binding, while Sepharose was used as a negative control. The Sp1 DNA-binding complexes from 2 μg of COS-1 nuclear extracts (NE) with the hcj-Sp1 probe for EMSA (lane 1), with addition of 2 μg of CV-1 nuclear extracts (lane 2), with the flowthrough (FT) fraction (lanes 3 and 6), with the washed (W) fraction (lanes 4 and 7), and with the 1.0 M KCl-eluted fraction (lanes 5 and 8) are shown.

The predominant complex 1a, identified as RCF-1 (21), comigrates with the only DNA-Sp1 complex formed with purified Sp1 (Promega). Anti-RB antibodies reduce or diminish all three predominant complexes, suggesting that these Sp1-binding proteins are required for Sp1-mediated transcription and RB-mediated transactivation of gene expression. The inability of an Sp1 antibody to alter either complex 1b or complex 2 suggests that factors distinct from Sp1 can also interact with the Sp1-binding sequence. GT box-binding proteins have been identified as members of a novel Sp1 multigene family (23). Two of them, Sp2 and Sp3, contain zinc fingers and transactivation domains similar to those of Sp1. Sp3, in particular, also binds to the Sp1 consensus GC box and has binding activity similar to that of Sp1. After comparison of our results and those of Kingsley and Winoto (23) for mobility shift analysis of GC and GT box-binding proteins, we speculate that 1b could be the Sp3 transcription factor.

Mechanism of RB-stimulated transcription mediated through the Sp1 site. Recently, the transfection experiments of Kim et al. (21) and Udvadia et al. (45) suggested that Sp1 is regulated by RB. The mechanism by which RB stimulates Sp1-mediated transactivation remains to be elucidated. We speculate that transactivation is in part due to the ability of RB to functionally interact with Sp1, as it has recently been reported that RB regulates transcription by virtue of its physical associations with transcription factors (3, 8, 21, 47). However, we have not detected RB-Sp1 complexes in vitro; therefore, we speculate that RB indirectly stimulates Sp1

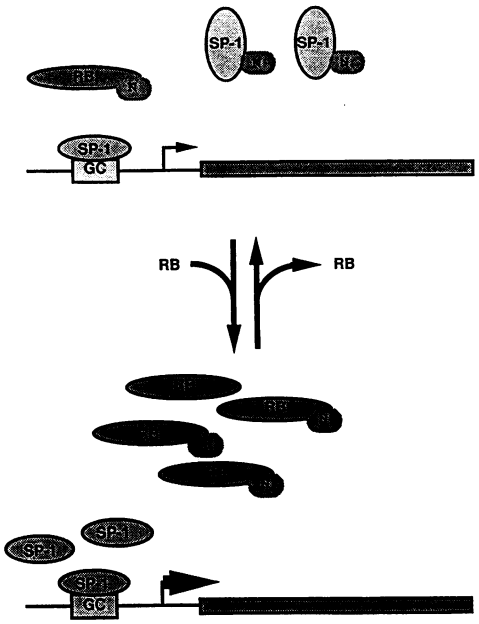

FIG. 9. Model illustrating the stimulation of Sp1-mediated transactivation by RB protein. Sp1 is illustrated as horizontal and vertical ellipsoid shapes. The horizontal ellipsoid-shaped Sp1 binds to an Sp1 DNA-binding site, and the vertical ellipsoid-shaped Sp1 associates with a negative regulator(s) (Sp1-I). After stimulation with RB protein, RB depletes an Sp1-I and leads to enhanced Sp1-mediated transactivation by free Sp1 proteins. Sp1-I, RB protein, and Sp1 protein are denoted as N, RB, and SP-1, respectively.

transactivation via the physical interaction of RB with regulators of Sp1 function. To clarify the mechanism by which RB regulates Sp1-mediated transcription, we have observed an inhibitor(s), Sp1-I, that inhibits the DNA-binding activity of Sp1 and other Sp1-predominant complexes to the Sp1-binding sites and that is also an RB-associated protein. We conclude that RB modulates the DNA-binding activity of Sp1 via direct interaction with Sp1-I, an Sp1 negative regulator(s).

Taking these findings collectively, we presented in Fig. 9 one model which accounts for the stimulation of Sp1-mediated transcription by RB. Evidence for RB regulating Sp1-binding activity through Sp1-I is as follows. (i) An anti-RB antibody interferes with binding of nuclear proteins to the Sp1 site, suggesting that the RB antibody dissociates RB from an RB-associated factor(s), likely a negative regulator for Sp1 binding. (ii) Sp1-binding activity recovered from CCL64 or COS-1 nuclear extracts is enhanced by adding baculovirus-expressed RB protein. (iii) Mixing of COS-1 and CV-1 nuclear extracts resulted in a loss of Sp1-binding activity in EMSAs, suggesting that an Sp1-binding negative regulator, Sp1-I, exists in CV-1 cells. (iv) RB recovers the inhibited Sp1-binding activity from the mixtures of CV-1 and COS-1 cell extracts, suggesting that Sp1-I is an RB-associated protein. (v) In CV-1 cells, increased amounts of GAL4-Sp1 in a transient transfection assay do not increase CAT activity, while increasing amounts of RB when phRb is cotransfected with GAL4-Sp1 resulted in increased CAT activity. These results suggest that RB depletes an Sp1-associated negative regulator(s) and leads to enhanced stimulation by free GAL4-Sp1. (vi) The partially purified ~20-kDa Sp1-I has an activity inhibitory to Sp1 binding similar to that observed in crude extracts. (vii) Using RB-Sepharose binding analysis, we demonstrated that Sp1-I is an RB-binding protein. Elucidation of the detailed molecular interactions between Sp1-I and Sp1 and between Sp1-I and RB will depend to a large extent on the purification of Sp1-I in a biologically active form. The isolation and cloning of Sp1-I will likely provide insight into its identity, function, and regulation.

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