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Biochemistry

Sp-1 binds promoter elements regulated by the RB protein and Sp-1-mediated transcription is stimulated by RB coexpression

(anti-oncogene/tumor-suppressor gene)

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ABSTRACT **The retinoblastoma (RB) protein is implicated in transcriptional regulation of at least five cellular genes, including c-*fos*, c-*myc*, and transforming growth factor β1. Cotransfection of RB and truncated promoter constructs has defined a discrete element (retinoblastoma control element; RCE) within the promoters of each of these genes as being necessary for RB-mediated transcription control. Previously, we have shown that RCEs form protein–DNA complexes *in vitro* with three heretofore unidentified nuclear proteins and mutation of their DNA-binding site within the c-*fos* RCE results in an abrogation of RCE-dependent transcription *in vivo*. Here, we demonstrate that one of the nuclear proteins that binds the c-*fos*, c-*myc*, and transforming growth factor β1 RCEs *in vitro* is Sp-1 and that Sp-1 stimulates RCE-dependent transcription *in vivo*. Moreover, we show that Sp-1-mediated transcription is stimulated by the transient coexpression of RB protein. We conclude from these observations that RB may regulate transcription in part by virtue of its ability to functionally interact with Sp-1.**

The retinoblastoma susceptibility gene (*RB1*) is a member of a class of cellular genes variously termed tumor-suppressor genes, antioncogenes, or recessive oncogenes (1). The frequent loss or mutational inactivation of the *RB* gene has been implicated in the etiology of a subset of human cancers, including retinoblastoma, small cell lung cancer, osteosarcoma, and carcinomas of the bladder and breast (1). The RB protein has also been shown to form complexes *in vivo* with the products of several viral oncogenes, such as simian virus 40 large tumor antigen and adenovirus E1A (1).

The product of the retinoblastoma (*RB*) gene is a ubiquitously expressed, nuclear phosphoprotein with nonspecific affinity for DNA. Given these attributes, a role for the RB protein in regulating gene expression was suggested. Indeed, five cellular genes have been identified as targets of transcription regulation by RB. Robbins *et al.* (2) demonstrated that the c-*fos* promoter was negatively regulated by RB in transient transfection assays. A 30-bp promoter segment, termed the retinoblastoma control element (RCE), was defined by deletion analysis as being necessary for RB-mediated transcription repression. This same promoter segment was shown to be sufficient to confer sensitivity to RB if linked to a heterologous promoter, such as herpes simplex virus (HSV) thymidine kinase (*TK*). The transient coexpression of wild-type, but not functionally impaired, *RB* cDNAs was shown to be required for RB-mediated control of the c-*fos* RCE. Pietenpol *et al.* (3) subsequently showed that the c-*myc* promoter is negatively regulated by treatment with transforming growth factor β1 (TGF-β1) or by cotransfection with wild-type RB. Promoter deletions defined a 23-bp element necessary for transcription regulation of c-*myc* by RB or TGF-β1. Similarly, Kim *et al.* (4) used transient transfection to identify a 32-bp element within the TGF-β1 promoter (TGFβ1) that is necessary and sufficient for transcription regulation mediated by the RB protein. Interestingly, the latter workers also showed that the response of a given RB-regulated promoter to RB cotransfection is dependent on the recipient cell: cotransfections of NIH 3T3 cells or AKR-2B cells led to repression of *TGFβ1* transcription, whereas a stimulation of transcription was noted in cells derived from lung epithelium. Most recently, Kim *et al.* (5) and Yu *et al.* (6) have shown the promoters of the *IGF2* gene and the *neu* gene to be RB-responsive *in vivo*.

RB has been shown to form cell cycle-regulated complexes with E2F, a transcription factor that regulates viral and cellular gene expression, and such complexes result in a marked reduction in E2F-dependent transcription (7–10). RB has also been shown to bind another transcription factor, ATF-2, *in vitro* and to stimulate ATF-2 activity *in vivo* (11). Given that RB has been reported to bind at least 10 additional cellular proteins *in vitro*, it is possible that a diverse collection of transcription factors may be targets of RB function (12–14). As one means of identifying targets of RB function, we have pursued the identification and characterization of nuclear proteins that bind the RCEs *in vitro* and *in vivo*. Our analyses have defined three ubiquitously expressed nuclear proteins of 80, 95, and 115 kDa (retinoblastoma control proteins; RCPs) that specifically bind the RCEs from the c-*fos*, c-*myc*, and *TGFβ1* promoters (15). A 13-bp sequence within the c-*fos* RCE is involved in binding the RCPs, including a 6-bp core sequence, 5′-GCCACC-3′, whose mutation blocks or strongly interferes with protein–DNA interactions *in vitro* (15). Mutations that blocked the binding of the RCPs to the c-*fos* RCE *in vitro* were shown to eliminate transcription activity *in vivo*, suggesting that the interaction of one or more of the RCPs with the c-*fos* RCE is functionally important (15). Pietenpol *et al.* (3) have also shown that the c-*myc* RCE is capable of forming three protein–DNA complexes *in vitro* with extracts prepared from whole cells. It is not yet clear whether the proteins that give rise to these latter complexes are similar to those we have identified.

In this report, we provide evidence that one of the three RCPs is Sp-1, a well-characterized transcription factor, and that Sp-1 stimulates RCE-dependent transcription *in vivo*.

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Abbreviations: RCE, retinoblastoma control element; TGF-β1, transforming growth factor β1; RCP, retinoblastoma control protein; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; HSV, herpes simplex virus.

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Furthermore, we show that the stimulation of RCE transcription by Sp-1 is enhanced by coexpression of RB protein.

MATERIALS AND METHODS

Cell Culture. NIH 3T3, EJ, C-33A, and A549 cells were acquired from the American Type Culture Collection; PC12 cells were a gift from Luis Parada (National Cancer Institute, Frederick, MD); Schneider SL2 cells were a gift of Cheaptip Benyajati (University of Rochester); and COS cells were a gift of Bryan Cullen (Duke University Medical Center). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, except PC12 cells were grown in DMEM supplemented with 10% horse serum/5% calf serum and Schneider SL2 cells were grown in Schneider's medium supplemented with 10% heat-inactivated fetal calf serum. Mammalian cells were grown in humidified incubators under 5% CO_2/95% air and Schneider SL2 cells were grown at room temperature on laboratory bench tops.

Oligonucleotides and Protein–DNA Binding Assays. Oligonucleotides were synthesized on an Applied Biosystems automated DNA synthesizer, deprotected, and then partially purified through Sephadex G-25. To ensure sequence fidelity, each oligonucleotide pair was cloned into an appropriate vector and sequenced by dideoxynucleotide chain termination (16). The following oligonucleotide sequences were used in these studies:

Fos,	5′-CCCGCGCGCCACCCCTCTGGCGCCACCGTG-3′ (15);
5′Fos-4,	5′-CCCTTGCGCCACCCCTCT-3′ (15);
5′Fos-5,	5′-CCCGCGCGCCATTCCTCT-3′ (15);
RCP$^-$,	5′-CCCGCGCGAAATTCCTCTGGCGCCACCGTG-3′ (15);
dbl RCP$^-$,	5′-CCCGCAAAAAACCCCTCTGAAAAAACCGTG-3′;
AP-1,	5′-TAAAATGAGTCAAGTGG-3′ (17);
Myc,	5′-GCAGAGGGCGTGGGGGAAAAGAA-3′ (3, 15);
TGF-β1,	5′-GGAGCCCGCCCACGCGAGATGAGGACGGTGGC-3′ (4, 15);
Sp-1,	5′-GATGGGCGGAGTTAGGGGCGGGACTATC-3′ (18).

Oligonucleotides were labeled with [^{32}P]dNTPs and purified from unincorporated radioactivity as described (15). Nuclear extracts were prepared and DNA-binding assays were performed as described (15). For DNA-binding assays in which antibodies were included, antibodies were added to binding assay mixtures before addition of radiolabeled oligonucleotides. Monoclonal antibodies 5M3 and M73 are an anti-synthetic peptide antibody prepared against human RB protein and an anti-E1A antibody (19), respectively. Sp-1 protein purified from HeLa cells was obtained from Promega and used in DNA-binding assays as suggested by the supplier.

Anti-Sp-1 Antibody Preparation, Immunoprecipitations, and Western Blotting. To generate polyclonal antisera against Sp-1, a full-length human Sp-1 cDNA (kindly provided by Robert Tijan, University of California, Berkeley) supplied in plasmid pBSK$^+$ was cleaved with *Bam*HI and a 1.8-kbp fragment was inserted in-frame into pGEX-1, a bacterial fusion protein expression vector (20). This portion of the Sp-1 cDNA encodes the N-terminal 603 aa of Sp-1 protein (21). After bacterial transformation, fusion proteins were induced with isopropyl β-D-thiogalactopyranoside and a 105-kDa GST–Sp-1 fusion protein was purified as described (20). For immunizations, a single New Zealand White rabbit was sequentially immunized with 150 μg of affinity-purified fusion protein in Freund's complete and incomplete adjuvants. Anti-Sp-1 immunoreactivity, as judged by immunoprecipitation and Western blotting, was detected in serum harvested after the first booster injection with immunogen.

For anti-Sp-1 immunoprecipitations, cells were metabolically labeled, and extracts were prepared and immunoprecipitated as described (22). For Western blotting, denatured protein extracts were resolved on SDS/polyacrylamide gels, transferred to nitrocellulose using a semidry transfer apparatus, and incubated with a 1:5000 dilution of rabbit anti-Sp-1 antibody. After incubation with a 1:20,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit secondary antibody, Sp-1 was detected by using an enhanced chemiluminescent system (ECL; Amersham) and exposure at ambient temperature to Hyperfilm.

Expression Constructs, Transfections, and Chloramphenicol Acetyltransferase (CAT) Assays. Plasmid pP_{ac}Sp-1, a Sp-1 expression construct, was obtained from Robert Tjian (23). PCR was used to generate an epitope-tagged Sp-1 cDNA by DNA amplification with primers immediately flanking the Sp-1 cDNA open reading frame. Each primer resulted in addition of an *Eco*RI site to the Sp-1 cDNA and the 3′ PCR primer incorporated a 10-aa influenza hemagglutinin epitope (N-YPYDVPDYAS-C), recognized by monoclonal antibody 12CA5 (gift of Rene Bernards, Massachusetts General Hospital Cancer Center, Charlestown), at the C terminus of Sp-1. After PCR and cloning of amplified DNA into pUC12, dideoxynucleotide sequencing was performed to ensure that the fusion of Sp-1 and hemagglutinin sequences had occurred in-frame (16). The epitope-tagged Sp-1 cDNA was then recloned into the *Hin*dIII site of pCMV-4, a cytomegalovirus (CMV) immediate-early promoter expression vector (gift of Stefan Doerrer, Duke University Medical Center) after addition of *Eco*RI/*Hin*dIII adapters. Reporter constructs were prepared by cloning dimers of RCE-containing oligonucleotides upstream of the HSV *TK* promoter and the bacterial CAT gene as described (15). Wild-type and mutated *DHFR*–CAT constructs (24) were kindly provided by J. Azizkhan (University of North Carolina, Chapel Hill). A human RB expression construct, pCMV-HRb, driven by the CMV immediate-early promoter was prepared by cloning a wild-type human *RB* cDNA into plasmid pIENH (gift of Jeffrey Marks, Duke University Medical Center). Transient transfections of COS cells were performed by a protocol incorporating DEAE-dextran followed by treatment with chloroquine and a dimethyl sulfoxide shock as described (25). COS whole cell extracts were prepared 48 hr posttransfection for immunoprecipitation and DNA-binding assays following solubilization in ELB$^+$ and removal of cell debris (22). Transfection of Schneider SL2 cells was performed as described (26). CAT assays were performed as described and results were normalized against the abundance of total cell protein in a portion of each extract.

RESULTS

Nuclear Factors That Bind RCEs Also Bind to Oligonucleotides Containing Sp-1 Binding Sites. Given that the c-*fos*, c-*myc*, and *TGFβ1* RCEs are G+C-rich promoter elements resembling Sp-1-binding sites and that two of three RCPs we have identified (95 and 115 kDa) approximate Sp-1 in molecular mass, we performed a series of experiments to test whether Sp-1 encodes one or more RCE-binding proteins. In the first experiment, an oligonucleotide containing two Sp-1-binding sites from the simian virus 40 early promoter was used as an unlabeled competitor for the formation of RCP–RCE complexes in a DNA-binding assay. We have previously shown that a full-length c-*fos* RCE probe forms three distinct protein–DNA complexes *in vitro* (15). These three complexes are also formed by a radiolabeled probe encompassing the 5′ half of the c-*fos* RCE (labeled 1A, 1B, and 2 in Fig. 1*A*; ref. 15). Also, as reported (15), these protein–DNA complexes are abolished by inclusion of a 200-fold molar excess of unlabeled oligonucleotides containing RCEs from the c-*fos*, c-*myc*, and *TGFβ1* promoters but not with a heterologous, unlabeled oligonucleotide (AP-1 in Fig. 1*A*; ref. 15). As shown in Fig. 1*A* (*Left*), an oligonucleotide containing two Sp-1-binding sites also abolished the appear-

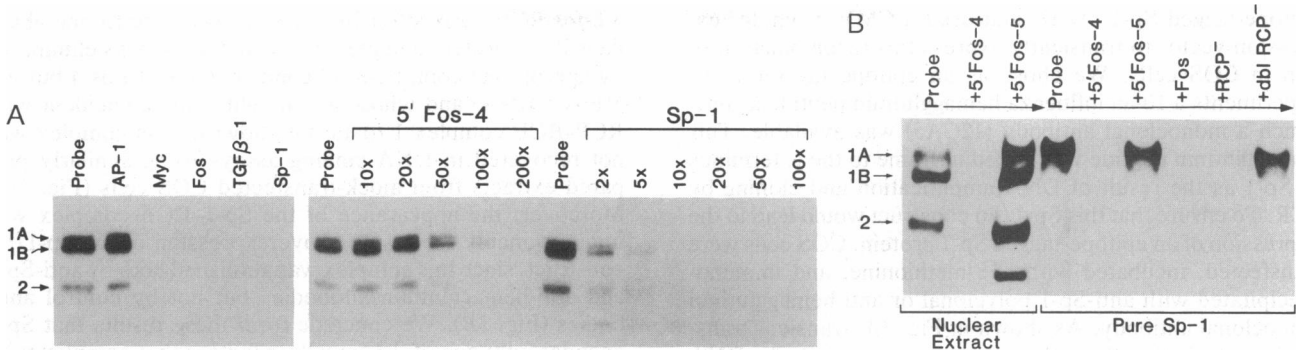

FIG. 1. c-*fos* RCE–RCP complexes are abolished by RCE and Sp-1 oligonucleotides and are formed by purified Sp-1. (*A*) (*Left*) DNA-binding assays were performed with NIH 3T3 nuclear extracts and a 5'Fos-4 probe. A 200-fold molar excess of unlabeled RCE-containing (Myc, Fos, and TGF-β1), heterologous (AP-1), or Sp-1 oligonucleotides was included as competitor DNA. RCE-specific complexes 1A, 1B, and 2 are indicated by arrows. (*Right*) Increasing concentrations of unlabeled 5'Fos-4 oligonucleotides (10- to 200-fold molar excess) or Sp-1 oligonucleotides (2- to 100-fold molar excess) were included in DNA-binding assays with a 5'Fos-4 probe (Probe). DNA-binding assays were exposed to Hyperfilm for 18 hr at −80°C. (*B*) A 5'Fos-4 probe was included in DNA-binding assays with nuclear extracts prepared from C-33A cells (18 μg; Nuclear Extract) or purified Sp-1 protein (30 ng; Pure Sp-1). Unlabeled oligonucleotides that form complexes with the RCPs (5'Fos-4, Fos) or do not form complexes with the RCPs (5'Fos-5, RCP⁻, dbl RCP⁻) were included as competitor DNAs. DNA-binding assays were exposed to Hyperfilm for 18 hr at −80°C.

ance of complexes 1A, 1B, and 2. Not apparent from this experiment is the result, shown in Fig. 1*A* (*Right*), that complete competition for RCE–RCP binding is achieved by incorporation of only a 10-fold molar excess of unlabeled Sp-1 oligonucleotides, suggesting that the simian virus 40-derived oligonucleotide is a more efficient competitor DNA than is the c-*fos*-derived RCE.

We next investigated whether purified Sp-1 would bind to the c-*fos* RCE and result in a protein–DNA complex that comigrates with one or more of the RCP–RCE complexes recovered from nuclear extracts. As shown in Fig. 1*B*, protein–DNA complexes formed in DNA-binding assays with purified Sp-1 resulted in the appearance of a single RCE–Sp-1 complex that comigrated with RCE complex 1A from nuclear extracts. These results are consistent with previous UV cross-linking experiments showing that complex 1A is composed of a single photoaffinity-labeled protein of 95 kDa (15). As shown in Fig. 1*B*, the Sp-1-induced protein–DNA complex was sensitive to competition with wild-type but not with mutated partial RCE oligonucleotides (5'Fos-5) that do not form complexes with the RCPs (15). Surprisingly, the complex formed by Sp-1 was abolished by full-length c-*fos* RCE oligonucleotides with a mutated RCP-binding site (RCP⁻). We have previously shown that this mutated oligonucleotide does not form RCE–RCP complexes in DNA-binding assays using nuclear extracts and is functionally inactive as measured in transient transfections (15). This unexpected result suggested that purified Sp-1 can bind to an alternative binding site within the c-*fos* RCE *in vitro*, perhaps at a closely related 3' site we have previously identified (15). To test this proposition, Sp-1 DNA-binding assays were performed with another RCE oligonucleotide that is mutated within each of two directly repeated 5'-GCGCCACC-3' sequences (dbl RCP⁻). As predicted, dbl RCP⁻ is not able to compete for Sp-1 binding *in vitro* (Fig. 1*B*). Taken together, we conclude that (*i*) the nuclear factors that interact with the c-*fos* RCE *in vitro* also bind to a heterologous oligonucleotide containing bona fide Sp-1-binding sites, (*ii*) purified Sp-1 forms a protein–DNA complex with the c-*fos* RCE that comigrates with RCP–RCE complex 1A, (*iii*) purified Sp-1 and the RCPs share a c-*fos* RCE-binding site, and (*iv*) purified Sp-1 can bind to a specific site within the c-*fos* RCE that is not bound *in vitro* by proteins in nuclear extracts.

One of Three RCPs Is Bound by Antibodies Reactive with Sp-1 Protein. The aforementioned results suggest that Sp-1 forms a single RCE gel shift complex similar to that of one of the RCPs. To determine whether one or more RCPs are antigenically related to Sp-1, we obtained an anti-Sp-1 monoclonal antibody against the Sp-1 DNA-binding domain and prepared polyclonal antibodies against the Sp-1 transactivation domain and included these reagents in DNA-binding assays. As shown in Fig. 2, inclusion of anti-Sp-1 monoclonal or polyclonal antibodies in DNA-binding assays with a c-*fos* RCE-derived probe resulted in the depletion of complex 1A but not complex 1B or 2. Neither a control monoclonal antibody nor rabbit preimmune serum diminished the recovery of RCP–RCE complexes, suggesting that anti-Sp-1 antibody–RCP interactions are specific (Fig. 2). We conclude from these results that the diminution of RCE–RCP complex 1A by anti-Sp-1 antibodies is the result of the binding of each antibody to Sp-1 or an antigenically related protein required for the formation of complex 1A.

Sp-1 Encodes One of Three Nuclear Proteins That Bind RB-Regulated Promoter Elements. Although the results presented in Figs. 1 and 2 suggest that the 95-kDa RCP may be Sp-1, we wished to establish whether Sp-1 indeed encoded this RCP. Moreover, it remained formally possible that the 80- and 115-kDa RCPs that form RCE complexes 1B and 2 are also Sp-1 related, perhaps modified or partially degraded Sp-1 derivatives. To address these possibilities, we prepared an

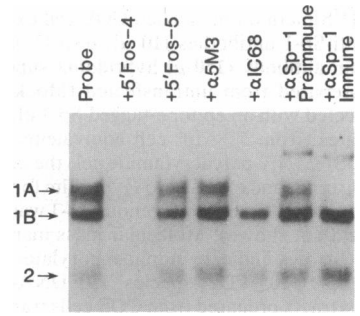

FIG. 2. RCP complex 1A is formed by a nuclear protein that is antigenically related to Sp-1. DNA-binding assays were performed with C-33A nuclear extracts and a 5'Fos-4 probe. As in Fig. 1, unlabeled RCE-derived oligonucleotides that bind (5'Fos-4) or do not bind RCPs (5'Fos-5) were included as competitor DNAs. Also included in these assays are monoclonal (4 μl; IC68) and polyclonal (3 μl; Immune) anti-Sp-1 antibodies or similar volumes of control antibodies (5M3, Preimmune). Monoclonal antibodies were added as ascites fluid. DNA-binding assays were exposed to Hyperfilm for 24 hr at −80°C.

epitope-tagged Sp-1 cDNA and used a CMV promoter expression vector to transiently express this recombinant protein in COS cells. We chose as an epitope tag for these experiments a 10-aa influenza hemagglutinin peptide against which a monoclonal antibody (12CA5) was available. This hemagglutinin peptide was linked in-frame to the C terminus of Sp-1 as the result of DNA amplification and cloning by PCR. To ensure that this Sp-1/flu construct would lead to the expression of an epitope-tagged Sp-1 protein, COS cells were transfected, incubated with [^{35}S]methionine, and immunoprecipitated with anti-Sp-1 polyclonal or anti-hemagglutinin monoclonal antibody. As shown in Fig. 3*A*, transient transfection of COS cells with the epitope-tagged Sp-1 cDNA resulted in overexpression of 95- to 105-kDa proteins that are immunoprecipitable by rabbit anti-Sp-1 antiserum as well as monoclonal antibody 12CA5. Neither antibody detected a similarly abundant protein in mock-transfected COS cells, suggesting that transient transfection of the Sp-1/flu construct results in overexpression of bona fide fusion protein (Fig. 3*A*).

To determine whether Sp-1 encodes one or more RCPs, DNA-binding assays were performed with a radiolabeled RCE probe. As shown in Fig. 3*B*, DNA-binding assays using a c-*fos* RCE-derived oligonucleotide gave rise to one abundant RCE–protein complex whose detection was eliminated by appropriate competitor oligonucleotides (5'Fos-4 but not 5'Fos-5 DNA) and whose gel mobility was coincident with RCP–RCE complex 1 (data not shown). This complex was not recovered in DNA-binding assays using similarly prepared extracts from mock-transfected COS cells (Fig. 3*B*). Moreover, the appearance of the Sp-1–RCE complex was unquestionably the result of overexpression of the Sp-1/flu construct, since this complex was abolished both by anti-Sp-1 and anti-hemagglutinin antibodies but not by control antibodies (Fig. 3*B*). We conclude from these results that Sp-1 encodes only one of three nuclear factors that complex with RCEs *in vitro*. The identity of the 80- and 115-kDa RCPs remains to be established.

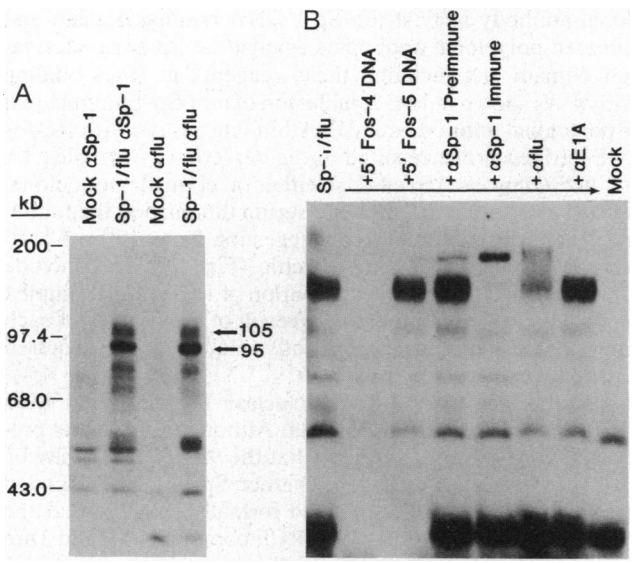

FIG. 3. Immunoprecipitation and DNA-binding assays of COS cells transfected with an epitope-tagged Sp-1 cDNA. (*A*) Immunoprecipitation of [^{35}S]methionine-labeled COS cell extracts with rabbit polyclonal anti-Sp-1 antibodies (10 μl; αSp-1) or a monoclonal anti-hemagglutinin antibody (150-μl hybridoma supernatant; αflu). Extracts were prepared from untransfected (Mock) COS cells or COS cells transfected with an epitope-tagged Sp-1 cDNA (Sp-1/flu). Immunoprecipitates (from 5 × 10^6 cell equivalents per lane) were resolved on a SDS/8%/4% polyacrylamide gel; the gel was prepared for fluorography, dried, and exposed to Hyperfilm for 20 hr at −80°C. Upon extended exposure times, endogenous Sp-1 was detected in the leftmost lane (data not shown). Molecular mass markers are shown on the left and arrows indicate unphosphorylated (95 kDa) and phosphorylated Sp-1 (105 kDa) species. (*B*) DNA-binding assays performed with extracts prepared from COS cells transfected with an epitope-tagged Sp-1 cDNA. Whole cell extracts (8 μg) were prepared from mock-transfected COS cells (Mock), or after transfection with a Sp-1/flu recombinant cDNA, and incubated with a 5'Fos-4 probe (Sp-1/flu). A 200-fold molar excess of unlabeled wild-type (5'Fos-4) or mutated c-*fos* RCE oligonucleotides (5'Fos-5) was included as competitor DNA. Rabbit preimmune (1 μl) and immune (1 μl) anti-Sp-1 antiserum or monoclonal antibodies (10-μl hybridoma supernatant) against influenza hemagglutinin (αflu) or adenovirus E1A (αE1A, M73; ref. 19) were included to identify transiently expressed Sp-1/flu fusion protein. Binding assays were exposed to Hyperfilm for 15 hr at −80°C.

Sp-1 Stimulates Transcription of RCEs *in Vivo* and Sp-1-Mediated Transcription Is Stimulated by the RB Protein. Given that Sp-1 interacts with RCEs *in vitro*, we wished to determine whether Sp-1 influences RCE transcription *in vivo*. Since the expression of Sp-1 and other RCE-binding proteins in most cells would obscure transcription mediated by exogenous Sp-1, we chose to transfect *Drosophila* Schneider SL2 cells, a Sp-1-deficient cell type that does not express detectable *in vitro* RCE-binding activity (ref. 23; A.J.U. and J.M.H., unpublished observations). For Schneider SL2 transfections, we used a Sp-1 expression construct driven by the *Drosophila* actin promoter (pP$_{ac}$Sp-1; ref. 23). To ensure that exogenous expression of Sp-1 would transactivate a bona fide Sp-1-dependent promoter, we performed cotransfections with a wild-type reporter construct prepared from the *DHFR* promoter and a mutated derivative lacking E2F-binding sites. Cotransfection with the Sp-1 expression vector significantly stimulated (30- to 40-fold) *DHFR*–CAT and *DHFR*–E2F$^-$–CAT expression in SL2 cells (Table 1). In contrast, differential levels of transcription resulted from Sp-1 cotransfec-

Table 1. *Drosophila* Schneider SL2 transfection results

Reporter construct	Addition	% acetylation, mean (SD)
DHFR	None	0.5 (0.4)
	RB	0.7 (0.6)
	Sp-1	18.1 (2.3)
	RB + Sp-1	79.0 (12.6)
DHFR–E2F$^-$	None	0.4
	RB	0.6
	Sp-1	13.2
	RB + Sp-1	60.0
TK	None	5.8 (3.8)
	Sp-1	15.5 (1.3)
	RB + Sp-1	60.2 (25.0)
FOS	None	5.2 (3.8)
	Sp-1	23.5 (16.0)
	RB + Sp-1	120.0 (63.0)
MYC	None	6.7 (1.0)
	Sp-1	44.7 (7.3)
	RB + Sp-1	247.9 (96.0)
TGF-β1	None	2.0 (0.42)
	Sp-1	27.9 (4.6)
	RB + Sp-1	218.1 (72.0)

Schneider SL2 cells were transfected with 5 μg of TK–CAT, FOS–CAT, MYC–CAT, TGFβ–CAT, or 2 μg of DHFR–CAT or DHFR–E2F$^-$–CAT reporter constructs alone or with 0.1 μg of pP$_{ac}$Sp-1, 20 μg of pCMV-HRB, or both. Control DNA (pUC12) was included in each transfection to bring the final total DNA concentration to 30 μg. Expression values are expressed as mean percentage acetylation (SD) of [^{14}C]chloramphenicol per A_{600} of 1 μl of total cell extract. Control DNAs consisting of equivalent amounts of Sp-1 and RB expression vectors lacking their respective cDNAs were included in transfections to determine basal transcription activities.

tion with RCE–CAT constructs (Table 1). Sp-1 marginally stimulated expression from the c-*fos* RCE–CAT construct above that of the HSV *TK* promoter alone. Consistent with our previous observations that the c-*myc* and *TGFβ1* RCEs bind to Sp-1 *in vitro* with greater affinity than does the c-*fos* RCE (ref. 15; A.J.U. and J.M.H., unpublished observations), cotransfection of Sp-1 with these reporter constructs resulted in greater stimulation, 7-fold and 14-fold, respectively, of RCE transcription *in vivo*. This stimulation of the c-*myc* and *TGFβ1* reporters was clearly due to the presence of linked RCE sequences as Sp-1 mediated transcription was observed to be 4- to 5-fold greater than for the HSV *TK* promoter alone (Table 1). We conclude from these results that Sp-1 interacts with RCEs *in vivo* as well as *in vitro* and that this protein–DNA interaction is functional.

To determine whether RB coexpression affects Sp-1-mediated transcription, SL2 cells were cotransfected with $pP_{ac}Sp\text{-}1$ and a wild-type human RB expression construct (pCMV-HRb). Cotransfection of RB with Sp-1 resulted in a marked increase in *DHFR* transcription independent of the presence of E2F sites within the *DHFR* promoter (Table 1). To determine whether the effect of RB cotransfection is Sp-1 dependent, we performed cotransfections of the *DHFR* promoter and the RB expression vector alone. As shown in Table 1, stimulation of *DHFR* transcription by RB was completely dependent on the coexpression of Sp-1 as cotransfection of RB alone did not affect *DHFR* transcription. Similarly, cotransfection of RB with Sp-1 resulted in an additional stimulation of the c-*fos*, c-*myc*, and *TGFβ1* reporter constructs of 5- to 8-fold. We conclude from these analyses that RB directly or indirectly regulates Sp-1 activity *in vivo*.

DISCUSSION

To clarify the mechanism by which RB regulates transcription we have identified and partially characterized nuclear proteins that interact with RCEs *in vitro*. In this report, we demonstrate that one of three RCE-binding proteins is encoded by Sp-1. Moreover, we show that Sp-1 stimulates RCE transcription *in vivo* and that transactivation by Sp-1 may be enhanced by the coexpression of RB.

Recently, the transfection experiments of Kim *et al.* (5) have also suggested that Sp-1 is regulated by RB. Yet, taken together with our data the mechanism by which RB stimulates Sp-1-mediated transcription remains to be established. It is clear from our cotransfection experiments with the *DHFR* promoter that RB stimulates *DHFR* transcription in a Sp-1-dependent manner. At least two mechanistic possibilities may account for the stimulation of Sp-1-mediated transcription by RB: (*i*) RB may augment Sp-1 transcription by liberating Sp-1 from a negative regulator, or (*ii*) RB may facilitate the interaction of Sp-1 with components of the basal transcription complex, a function previously referred to as "coactivation." Each of these possibilities appears to be supported by our cotransfection results with RCEs linked to the HSV *TK* promoter. As for *DHFR*, in cotransfections with Sp-1 and RB, a significant synergistic activation of transcription was noted for each RCE reporter construct. Moreover, the relative levels of synergism are directly correlated with the affinity of Sp-1 for each RCE *in vitro* and the degree of Sp-1 transactivation of each RCE *in vivo*. Given that we have not as yet detected RB–Sp-1 complexes *in vivo* or *in vitro*, we speculate that RB indirectly stimulates Sp-1 transactivation via the physical interaction of RB with targets or regulators of Sp-1 function. Further analyses *in vitro* and *in vivo* will be necessary to define the mechanism(s) by which RB augments Sp-1-mediated transcription.

It is important to note that the functional significance of the 80- and 115-kDa non-Sp-1-encoded RCPs for RCE-dependent transcription or RB regulation has not been addressed by our experiments. It is possible that, together with Sp-1, the 80- and 115-kDa RCPs function to regulate RCE transcription. Indeed, the c-*fos* RCE was only weakly stimulated by Sp-1 in SL2 cells, whereas its transcriptional activity in mammalian cells is significantly greater (15). Given the diversity of cellular promoters that may be regulated by Sp-1, it is unlikely that each promoter is a target of RB function. Instead, the RCEs may represent a functional subset of Sp-1-regulated promoter elements, perhaps those that interact with all three RCPS, whose activity is subject to RB regulation (27). In this regard, it will be of interest to determine whether the *DHFR* promoter is a target for binding by all three RCPs.

Since each RCP shares a c-*fos* RCE-binding site and each RCP binds to bona fide Sp-1 sites *in vitro*, then either (*i*) the RCPs share a common DNA-binding domain with Sp-1, or (*ii*) the RCPs possess structurally distinct DNA-binding domains that share with Sp-1 a common cognate DNA-binding sequence. It is worth noting that two recently cloned Sp-1-related cDNAs encode zinc finger proteins that approximate in size the 115- and 80-kDa RCPs we have described here and elsewhere (15). It will be of interest to determine whether these two RCPs are encoded by Sp-1-related genes and whether they are also targets of RB function.

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