Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 3953–3957, April 1995
Biochemistry

Functional interactions between the retinoblastoma (Rb) protein and Sp-family members: Superactivation by Rb requires amino acids necessary for growth suppression

(anti-oncogene/tumor-suppressor gene)

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Communicated by Gordon G. Hammes, Duke University Medical Center, Durham, NC, January 3, 1995

ABSTRACT **The transient expression of the retinoblastoma protein (Rb) regulates the transcription of a variety of growth-control genes, including c-*fos*, c-*myc*, and the gene for transforming growth factor $\beta 1$ via discrete promoter sequences termed retinoblastoma control elements (RCE). Previous analyses have shown that Sp1 is one of three RCE-binding proteins identified in nuclear extracts and that Rb functionally interacts with Sp1 *in vivo*, resulting in the "superactivation" of Sp1-mediated transcription. By immunochemical and biochemical criteria, we report that an Sp1-related transcription factor, Sp3, is a second RCE-binding protein. Furthermore, in transient cotransfection assays, we report that Rb "superactivates" Sp3-mediated RCE-dependent transcription *in vivo* and that levels of superactivation are dependent on the trans-activator (Sp1 or Sp3) studied. Using expression vectors carrying mutated Rb cDNAs, we have identified two portions of Rb required for superactivation: (*i*) a portion of the Rb "pocket" (amino acids 614–839) previously determined to be required for physical interactions between Rb and transcription factors such as E2F-1 and (*ii*) a novel amino-terminal region (amino acids 140–202). Since both of these regions of Rb are targets of mutation in human tumors, our data suggest that superactivation of Sp1/Sp3 may play a role in Rb-mediated growth suppression and/or the induction of differentiation.**

The human retinoblastoma susceptibility gene *RB1* is believed to participate in the orchestration of orderly cell growth and/or differentiation (1). Deletion or mutational inactivation of *RB1* is correlated with the genesis of a variety of human cancers including retinoblastoma, osteosarcoma, and carcinomas of the breast, bladder, and lung (2–6). The retinoblastoma protein (Rb) may also be sequestered by viral oncoproteins, such as simian virus 40 large tumor antigen, via conserved regions of the oncoproteins that are essential for viral-induced transformation (7–11).

The Rb gene encodes a set of ubiquitously expressed nuclear phosphoproteins that are distinguished by their extent of posttranslational modification (3, 12–14). Given its nuclear localization and an associated nonspecific affinity for DNA, a role for Rb in the regulation of gene expression has been suggested (3, 12). This supposition was supported by experiments demonstrating that a 30-bp sequence within the c-*fos* promoter is a target of Rb function (15). This sequence, termed "retinoblastoma control element" (RCE), was shown to be necessary and sufficient for Rb-mediated transcription control. Subsequently, similar elements have been described within the promoters of a variety of genes that themselves encode growth regulatory molecules (16–22). Interestingly, Kim *et al.* (18) showed that the transcriptional response of a given RCE to Rb coexpression is cell-type dependent and may be stimulatory or inhibitory. While a mechanism for Rb-mediated transcriptional regulation was not indicated by these experiments, recent evidence strongly suggests that Rb functions to regulate transcription via its physical interaction with sequence-specific DNA-binding proteins (23–29).

The binding of viral oncoproteins to Rb occurs within a discrete portion of the Rb carboxyl terminus that is a "hotspot" for mutation in human cancers (2, 3, 30–36). This region, often referred to as the Rb "pocket," is also the site of interaction of Rb with a variety of proteins that are believed to be targets of Rb function, including transcription factors E2F-1, ATF-2, and myoD (23, 25, 27, 28). The functional consequence of the formation of such complexes can be quite distinct. Rb forms cell-cycle-regulated complexes with E2F-1 and down-regulates E2F-dependent transcription *in vivo* (for a review, see ref. 23). In contrast, the formation of complexes between Rb and ATF-2 or myoD leads to an increase in ATF-2 or myoD-mediated transcription (25, 27, 28). The precise role of these functional interactions in Rb-mediated growth control has yet to be determined. However, it is believed that the interaction of Rb with transcription factors such as ATF-2 and myoD leads to an increase in the transcription of growth-inhibitory genes or differentiation-inducing genes and that interactions between Rb and factors such as E2F-1 inhibit the synthesis of gene products required for cell-cycle progression. A more complete understanding of Rb-mediated growth control is complicated by two additional findings. First, two Rb-related proteins, p107 and p130, have recently been cloned and shown to share a high degree of sequence similarity with Rb, especially within their respective pocket regions. As a consequence, members of the Rb family have been shown to complex with a common set of viral and cellular proteins (28, 37–41). Second, affinity chromatography experiments have identified cellular proteins that interact with the 380 amino acids of p105/Rb that are amino-terminal to the Rb pocket (57). The association of at least one of these proteins, a serine/threonine-protein kinase, with the Rb amino terminus has been shown to be dependent upon Rb sequences that are required for cell-growth regulation. It is not as yet clear what role, if any, these amino-terminal Rb-associated proteins play in Rb-mediated transcriptional regulation.

Previously, we reported that three nuclear proteins of 80, 95, and 115 kDa, termed "retinoblastoma control proteins"

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Abbreviations: Rb, retinoblastoma protein; RCE, retinoblastoma control elements; RCP, retinoblastoma control protein (REC-binding protein); HA, hemagglutinin; DHFR, dihydrofolate reductase; CAT, chloramphenicol acetyltransferase; GST, glutathione *S*-transferase; CMV, cytomegalovirus; TGF-$\beta 1$, transforming growth factor type $\beta 1$.

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(RCPs), specifically bind to RCEs within the c-*fos*, c-*myc*, and transforming growth factor type $\beta1$ (TGF-$\beta1$) gene promoters and that their binding is correlated with RCE-dependent transcriptional activity *in vivo* (42). We subsequently demonstrated that the 95-kDa RCP is Sp1 and that Sp1 activates transcription of RCEs *in vivo* (43). Moreover, the coexpression of Rb and Sp1 proteins results in the "superactivation" of Sp1-mediated transcription (20, 22, 43). Recently, three Sp1-related genes have been cloned based on their homology with the Sp1 DNA-binding domain (44, 45). In this report we demonstrate that a previously identified 115-kDa RCE-binding protein is Sp3, that Sp3 protein functionally interacts with RCEs *in vivo*, and that Sp3 protein is "superactivated" by coexpression of Rb protein. In addition, we report that superactivation requires portions of Rb that are subject to mutation in tumor cells, including amino acids previously determined to be necessary for E2F-1-binding.

MATERIALS AND METHODS

Oligonucleotide-Probe Preparation and Protein–DNA Binding Assay. Complementary oligonucleotide pairs were synthesized and purified as described (42). The sequence of each oligonucleotides used in these studies has been described (14, 17, 18, 42, 43). To ensure sequence fidelity, each oligonucleotide pair was cloned into an appropriate vector and sequenced by dideoxynucleotide chain termination (46). Oligonucleotides were end-labeled with [γ-^{32}P]ATP (ICN; 4500 Ci/mmol; 1 Ci = 37 GBq) and T4 polynucleotide kinase (New England Biolabs) and were purified from unincorporated radionuclide as described (42). Nuclear extracts were prepared and utilized in protein–DNA binding assays as described (42).

Construction of Expression Plasmids and Transient Transfections. An epitope-tagged Sp3 expression construct was generated by PCR amplification of a plasmid carrying an Sp3 cDNA, pSP72Sp3 (ref. 44; provided by Astar Winoto, University of California, Berkeley), utilizing oligonucleotide primers flanking the Sp3 cDNA, Vent polymerase (New England Biolabs), and a thermal cycler. This amplified Sp3 DNA was cloned into pCMV-4, a cytomegalovirus (CMV) immediate-early promoter expression vector (43), to create pCMV-Sp3/flu and was subcloned in pP_{ac}, a *Drosophila* β-actin promoter expression vector (a gift of Robert Tjian, University of California, Berkeley), creating pP_{ac}Sp3/flu; flu refers to an incorporated 10-amino acid influenza hemagglutinin (HA) epitope (43). pP_{ac}Sp1, a Sp1 expression construct, has been described (43, 47, 48). Expression plasmids carrying an epitope-tagged Sp1 cDNA, pCMV-Sp1/flu, and a wild-type human Rb cDNA, pCMV-HRb, have been described (43). For mapping studies, wild-type and mutated Rb cDNAs cloned in a simian virus 40 promoter-dependent vector, pSVE, were utilized in transient transfections (49). Reporter-gene constructs were prepared and utilized as described (43). African Green monkey kidney COS-1 cells, human C-33A cervical carcinoma cells, and *Drosophila* Schneider SL-2 cells were cultured and transiently transfected as described (42, 43).

Antibodies, Immunoprecipitations, and Immunoblotting (Western Blotting). A bacterial expression plasmid that encodes the amino-terminal 300 amino acids of Sp3 fused in-frame with glutathione *S*-transferase (GST) was constructed by cloning a 900-bp Sp3 *Bam*HI–*Bgl* II fragment from pCMV-Sp3/flu into the *Bam*HI site of pGEX2T (Pharmacia), creating pGEXSp3-N. A second GST-Sp3 fusion construct, pGEXSp3-C, was constructed by fusing a 1245-bp *Bgl* II–*Bam*HI Sp3 cDNA fragment encoding the carboxyl-terminal 415 amino acids of Sp3 with pGEX2T. GST-Sp3 fusion proteins were induced, harvested, and partially purified as described (50). To generate polyclonal Sp3 antisera, New Zealand White rabbits were sequentially immunized with affinity-purified GST-Sp3 fusion proteins in Freund's complete and incomplete adjuvants. Anti-Sp3 antisera (anti-Sp3-1, an anti-amino-terminal Sp3 antiserum, and anti-Sp3-2, an anti-carboxyl-terminal Sp3 antiserum provided by Astar Winoto) were used in immunoprecipitations and protein–DNA binding assays as described (43). IC68 (a gift of Steve Jackson and Robert Tjian, University of California, Berkeley) is a previously described Sp1 monoclonal antibody (51). IC68 was incorporated in protein–DNA binding assays as ascites fluid. M73 and 12CA5 are monoclonal antibodies prepared against adenovirus E1A (52) and the influenza HA peptide described above. M73 and 12CA5 were incorporated in protein–DNA binding assays as hybridoma supernatants. For immunoprecipitations, cells were metabolically labeled with Tran^{35}S-label (ICN), and extracts were prepared and immunoprecipitated as described (3).

RESULTS

cDNA for Sp1-Related Transcription Factor Sp3 Encodes One of Three Nuclear Proteins That Bind to Rb-Regulated Promoter Elements. We have previously established that Sp1 cDNA encodes one of three RCE-binding proteins, RCPs, that are detected in nuclear extracts with DNA–protein binding assays (42, 43). We have also established that each RCP requires identical RCE nucleotides to form protein–DNA complexes *in vitro* and that mutation of these nucleotides abrogates RCE-dependent transcriptional activity *in vivo* (42). Given their common nucleotide-binding specificity, we reasoned that the DNA-binding domains of the remaining two unidentified RCPs might be structurally similar to the DNA-binding domain of Sp1. This supposition was bolstered by the recent identification of a family of Sp1-related transcription factors (44, 45, 53). Given the molecular mass of RCP 1B (115 kDa), we considered the possibility that RCP 1B is formed by Sp3, an Sp1-related transcription factor. To test this hypothesis, we constructed an expression plasmid (pCMV-Sp3/flu) containing an Sp3 cDNA driven by the CMV immediate-early promoter. Since Sp3 cDNAs cloned to date lack nucleotides encoding the extreme amino terminus of full-length Sp3 protein, a consensus translational start sequence was added to the Sp3 cDNA by the PCR (44, 45).

To confirm that the recombinant Sp3 cDNA we prepared expressed a stable protein of the predicted size (110 kDa), C-33A human cervical carcinoma cells were transiently transfected with pCMV-Sp3/flu, and nascent proteins were radiolabeled with [^{35}S]methionine. A protein of the predicted size was immunoprecipitated from Sp3-transfected cells with a monoclonal antibody directed against the HA tag (Fig. 1, lane 3) and with two distinct anti-Sp3 polyclonal antisera (Fig. 1, lanes 5 and 7). Immunoprecipitates prepared from untransfected cells with anti-HA (Fig. 1, lane 1) or anti-Sp3 antisera (Fig. 1, lanes 4 and 6) and immunoprecipitates from Sp3-transfected cells with homologous rabbit preimmune sera (Fig. 1, lanes 8 and 9) did not detect a protein of similar size. Immunoprecipitates of C-33A cells transfected in parallel with a HA-tagged Sp1 cDNA showed somewhat greater amounts of exogenous protein expression (Fig. 1, lane 2).

Nuclear extracts prepared from COS-1 cells transiently transfected with pCMV-Sp3/flu were then examined for RCE-binding activity by using a protein–DNA binding assay (Fig. 2). Compared with extracts prepared from untransfected cells (Fig. 2, lane 15), an additional protein–DNA complex that migrated slightly ahead of RCP 1B was detected with a c-*fos* RCE probe in Sp3-transfected cell extracts (Fig. 2, Sp3/flu band). As for previously identified RCPs, the DNA-binding activity associated with this additional complex is specific, since it was eliminated by the inclusion of excess unlabeled RCE oligonucleotides from the c-*fos*, TGF-$\beta1$ gene, and c-*myc* promoters (Fig. 2, lanes 11–13) but not by heterologous oligonucleotides (Fig. 2, lane 16; refs. 42 and 43). All RCE–RCP complexes are also eliminated by the addition of excess unlabeled oligonucleotides containing Sp1-binding sites (Fig. 2, lane 14; ref. 43). To determine whether this protein–DNA

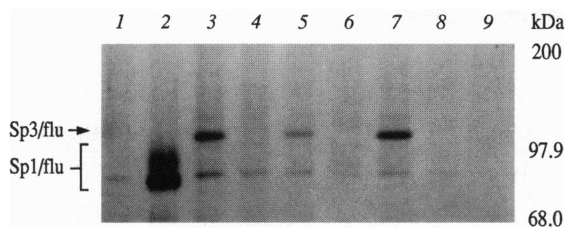

FIG. 1. Immunoprecipitation of recombinant Sp1 and Sp3 proteins. Equal amounts of radiolabeled cell extracts from mock-transfected C-33A cells (lanes 1, 4, and 6) and C-33A cells transiently transfected with pCMV-Sp1/flu (lane 2) or pCMV-Sp3/flu (lanes 3, 5, and 7–9) were immunoprecipitated with an anti-HA monoclonal antibody (12CA5, lanes 1–3), two anti-Sp3 polyclonal rabbit antisera (anti-Sp3-2, lanes 4 and 5; anti-Sp3-1, lanes 6 and 7), or homologous preimmune sera (lanes 8 and 9). Recombinant proteins are indicated on the left and molecular weight markers on the right.

complex detected in transiently transfected cells and the RCPs require similar RCE nucleotides for binding, we included a series of unlabeled and mutated c-*fos* RCE oligonucleotides as competitors in protein–DNA binding assays (Fig. 2). Mutated c-*fos* RCE oligonucleotides 4 and 8 (Fig. 2, lanes 6 and 10) competed for Sp3/flu- and RCP-binding as efficiently as did wild-type RCE oligonucleotides (Fig. 2, lane 2), whereas mutants 3, 6, and 7 (Fig. 2, lanes 5, 8, and 9) were less effective, and mutants 1, 2, and 5 (Fig. 2, lanes 3, 4, and 7) did not detectably compete for RCP binding. Thus, each mutated-RCE oligonucleotide equally affects the abundance of exogenous and endogenous protein–DNA complexes.

Importantly, the protein–DNA complex detected in transiently transfected cells is clearly formed by the binding of epitope-tagged Sp3 protein, since it is eliminated by the inclusion of an antibody directed to the epitope tag (Fig. 2, lane 19) but not a control monoclonal antibody (Fig. 2, lane 20) or a monoclonal antibody (IC68; ref. 51) prepared against Sp1 (Fig. 2, lane 21). As we have shown (43), inclusion of IC68 leads to a faint supershift of the RCE–RCP complex (RCP 1A) formed by Sp1 (Fig. 2, asterisk). To determine if RCE–RCP complex 1B is formed by a protein that is antigenically related to Sp3, we included a polyclonal anti-Sp3 antiserum (anti-Sp3-1) in parallel protein–DNA binding assays. The protein–DNA complexes RCP 1B and Sp3/flu were greatly diminished by anti-Sp3-1 (Fig. 2, lane 18) but not by homologous preimmune serum (Fig. 2, lane 17). We conclude from these experiments that (*i*) recombinant Sp3 protein binds to RCEs via RCE nucleotides that are identical to those bound by RCPs and (*ii*) Sp3 in all likelihood forms RCP 1B.

Sp3 Stimulates c-*fos*-, c-*myc*-, and TGF-β1 Gene RCE-Dependent Transcription, and Sp3-Mediated Transcription Is Superactivated by Rb *in Vivo*. To determine the functional consequence of Sp3–RCE binding *in vivo*, we performed transient cotransfections using a *Drosophila* embryo cell line (SL-2). *Drosophila* cells were chosen as recipients for these transfections because they are Rb- and Sp1/Sp3-deficient yet are responsive to these transcriptional regulators (43, 48, 50). At the outset, we performed a series of titration experiments in which increasing quantities of Sp1 or Sp3 cDNAs driven by the *Drosophila* β-actin gene promoter (pP$_{ac}$Sp1/flu and pP$_{ac}$Sp3/flu, respectively) were used with a dihydrofolate reductase (DHFR; ref. 54) reporter gene construct to compare the relative transcriptional activities of Sp1 and Sp3. As previously reported for Sp1 expression in *Drosophila* cells, DHFR gene transcription increased linearly with increasing quantities of transfected Sp3 DNA (ref. 48 and data not shown). We note, however, that Sp3 appeared to be less potent than Sp1 at stimulating DHFR gene transcription at low input levels of Sp3 DNA (i.e., 1–40 ng). For all subsequent experiments, we chose to transfect 100 ng of pP$_{ac}$Sp3/flu, since this quantity of DNA stimulated reporter gene activity to levels that were similar to that of pP$_{ac}$Sp1/flu and well within the linear range of our enzymatic assays.

We next examined the efficiency with which Sp3 trans-activates RCEs derived from the c-*fos*, c-*myc*, and TGF-β1 gene promoters utilizing reporter constructs previously analyzed for their transcriptional response to Sp1 (43). As we have previously shown for Sp1, differential levels of RCE transcription result after cotransfection of *Drosophila* SL-2 cells with Sp3 cDNA, reporter gene constructs, and pCMV-4 vector (Table 1 and ref. 43). Sp3 did not appreciably stimulate the activity of the c-*fos* RCE, whereas the activity of the c-*myc* and TGF-β1 gene RCEs was increased up to 4-fold by Sp3 cDNA transfection. When compared with our previous results using Sp1 cDNA (43), cotransfection of Sp3 cDNA with each reporter plasmid resulted in comparable levels of relative CAT activity. Additionally, Sp3-mediated transcription of each RCE was qualitatively similar to that of Sp1 (43)—i.e., transcription mediated by Sp1 and Sp3 followed the same hierarchy of RCE sequence preference: TGF-β1 gene, c-*myc* > c-*fos* ≥ thymidine kinase gene. These data are entirely consistent with our previous observation that the extent of RCE trans-activation *in vivo* is directly correlated with the relative binding activity of RCPs for each RCE *in vitro* (42, 43). We conclude that, as for Sp1, Sp3 can interact both physically and functionally with RCEs.

Given the previously demonstrated functional interaction between Rb and Sp1, we next addressed whether Rb can functionally interact with Sp3 (20, 22, 43). As we have previously reported for Sp1, Rb coexpression led to the stimulation of Sp3 trans-activation (superactivation; Table 1). However, we note that the pattern of Rb-mediated Sp3 superactivation is distinctly different from that of Sp1. For example, Rb reproducibly superactivates Sp3-mediated RCE transcription 3- to 4-fold, whereas our previous experiments have indicated that Rb superactivates Sp1-mediated transcription of the c-*fos*

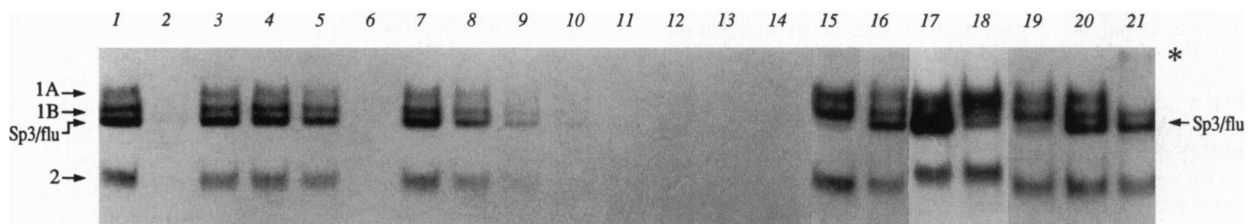

FIG. 2. Protein–DNA binding assays using a radiolabeled c-*fos* RCE probe, unlabeled oligonucleotide competitors, and antisera. A radiolabeled c-*fos* probe (5′Fos-4; refs. 42 and 43) was incubated with whole-cell extracts prepared from untransfected COS cells (lane 15) or cells transiently transfected with pCMV-Sp3/flu (lanes 1–14) (16–21). Excess unlabeled competitor oligonucleotide constructs were added as follows: 5′Fos-WT (wild-type) (lane 2), 5′Fos-1 (lane 3), 5′Fos-2 (lane 4), 5′Fos-3 (lane 5), 5′Fos-4 (lane 6), 5′Fos-5 (lane 7), 5′Fos-6 (lane 8), 5′Fos-7 (lane 9), 5′Fos-8 (lane 10), Fos RCE (lane 11), TGF-β1 gene RCE (lane 12), Myc RCE (lane 13), Sp1 (lane 14), no competitor (lane 15), and AP-1 (lane 16). Antisera added to protein–DNA complexes were as follows: anti-Sp3-1 preimmune (lane 17), anti-Sp3-1 immune (lane 18), anti-HA (lane 19), anti-E1A (lane 20) (M73; ref. 52), and anti-Sp1 (lane 21) (IC68; ref. 51). Oligonucleotides and binding assays have been described (42, 43), and protein–DNA complexes are indicated by arrows. A supershifted Sp1–DNA complex is indicated by an asterisk.

Table 1. Stimulation of RCE-dependent transcription by Sp3 and superactivation of Sp3 by Rb coexpression in transiently transfected *Drosophila* SL-2 cells

Reporter construct	Vector addition: CMV	Vector addition: Sp3	Mean relative CAT activity (± SE)
HSV TK-CAT	pCMV-HRb	None	0.2
	pCMV-4	+	1.0 (0.01)
	pCMV-HRb	+	2.6 (0.26)
Fos RCE-CAT	pCMV-4	+	0.8 (0.33)
	pCMV-HRb	+	3.3 (0.32)
Myc RCE-CAT	pCMV-4	+	3.7 (0.46)
	pCMV-HRb	+	9.2 (1.81)
TGFβ-1 RCE-CAT	pCMV-4	+	4.2 (0.28)
	pCMV-HRb	+	11.7 (2.83)

Drosophila SL-2 cells were cotransfected with an Sp3-expression construct ($pP_{ac}Sp3/flu$), the indicated RCE-CAT reporter construct, and CMV vector either without (pCMV-4) or with wild-type Rb (pCMV-HRb). Resulting levels of chloramphenicol acetyltransferase (CAT) activity were quantified as described (43). Shown are mean values for percentage acetylation (± SE) of [^{14}C]chloramphenicol per A_{600} of 1 μl of total cell extract normalized to the amount of activity recorded for the herpes simplex virus (HSV) thymidine kinase (TK)-CAT reporter construct. Mean values from five or six independent plates of transfected cells are presented.

and c-*myc* RCEs >5-fold and the TGF-β1 gene RCE nearly 8-fold. Thus, Rb can superactivate Sp3-mediated transcription *in vivo*, and levels of RCE superactivation are dependent on the coexpressed Sp-family member.

Regions of Rb Necessary for Maximal Superactivation Coincide with Those Involved in Cell-Growth Regulation. To better understand the mechanism of Rb-mediated superactivation, we utilized a series of mutated-Rb cDNAs to identify regions of Rb protein necessary for maximal superactivation. The mutated Rb cDNAs we chose for these experiments have previously been shown to express stable proteins *in vivo* and have been characterized for their ability to suppress cell growth and interact with E2F (49). Results of these experiments delineate at least two distinct regions of Rb that if deleted significantly decrease Rb-mediated Sp1 superactivation (Table 2). Both of these regions have previously been shown to be subject to inactivating mutations in human tumors and to be required for Rb-mediated growth suppression of tumor cells in culture. Deletions in the Rb gene that caused the greatest reduction in Sp1 superactivation map to exons 19–24, corresponding to amino acids 614–839 encompassing the carboxyl-terminal end or B domain of the Rb pocket. A second Rb region required for wild-type levels of Sp1 superactivation is located more than 400 amino acids upstream of the B domain (amino acids 140–202, corresponding to a portion of exon 4, all of exon 5, and the 5′ end of exon 6). The identification of this Rb region as being important for Sp1 regulation is unique in that all previously studied Rb-regulated transcription factors have been shown to require only the Rb pocket region for physical or functional interaction. We note that this amino-terminal Rb region is also subject to inactivating mutations in retinoblastoma and that *in vitro* mutagenesis of this region results in Rb proteins that are defective in growth-suppression activity yet maintain their E2F-1-binding activity *in vivo* (49, 55, 56). Thus, while our transfection results suggest that Sp1 and E2F-1 functionally interact with Rb via regions that are required for cell-growth regulation, they each exploit distinct but partially overlapping portions of Rb. Finally, we note that deletion of a portion of exon 1 and exon 2 (corresponding to amino acids 37–89) results in an apparent gain-of-function mutation. This deletion produces a mutated Rb protein that superactivates Sp1 more than twice as effectively as wild-type Rb (Table 2).

DISCUSSION

The results presented in this report support and extend previous observations that Rb can collaborate with sequence-specific DNA-binding proteins to stimulate transcription. Rb has previously been shown to stimulate the expression of the TGF-β2 gene via its physical interaction with ATF-2 (25). Similarly, the physical interaction of Rb with myoD and myogenin has been suggested to stimulate the transcription of myogenic genes (27, 28). However, in contrast to functional interactions with transcription factors such as these, physical complexes between Rb and Sp family members have not as yet been detected *in vitro* or *in vivo* (20, 22, 24, 42, 43, 50). Instead, recent evidence indicates that the Sp1/Sp3 trans-activation domains are bound by cellular proteins that may negatively regulate their activity *in vivo* (20, 50). Thus, Rb coexpression may superactivate Sp1/Sp3 by liberating these factors from negative regulation. However, functional interactions between Rb and Sp1 or Sp3 do not appear to result in equivalent levels of superactivation. Results from the present study suggest that the degree to which Rb superactivates a given RCE is dependent on the trans-activator analyzed (Sp1 or Sp3). Although "basal" Sp3-mediated RCE-dependent transcription is quantitatively and qualitatively similar to that of Sp1, the degree to which Sp1 and Rb superactivate RCEs is substantially greater than that for Sp3 and Rb. Additionally, whereas levels of Sp1 superactivation appear to be directly correlated with the affinity of Sp1 for its cognate RCE-binding site, the degree to which Rb superactivates Sp3 is independent of the particular RCE examined—i.e., each RCE is superactivated to similar levels. Given that the present and previous studies indicate that Sp1 and Sp3 appear to bind similar, if not identical, nucleotide sequences with similar affinity, we hypothesize that Sp1 and Sp3 compete for their cognate DNA-binding sites and Rb in mammalian cells. We propose that this competition at least partly determines the degree to which superactivation governs the activity of a particular RCE-regulated promoter. We further speculate that the abundance of active Sp family members and additional RCE-binding proteins may account

Table 2. Superactivation of Sp1 by wild-type (wt) and mutated Rb proteins in transiently transfected *Drosophila* SL-2 Cells

Transfected Rb DNA	n*	Mean Sp1 superactivation† (± SE)	Significance‡
Control	8	1.2 (0.1)	$P = 0.001$
Wild-type Rb	8	8.6 (1.9)	NC
Mutant Rb			
Δ37–89	3	18.3 (4.0)	$P < 0.05$
Δ89–140	3	12.4 (1.3)	NS
Δ140–202	7	4.4 (0.6)	$0.1 > P > 0.05$
Δ202–249	6	4.7 (1.0)	NS
Δ249–309	2	10.3 (1.3)	NS
Δ309–343	2	12.0 (2.3)	NS
Δ343–389	2	6.3 (0.6)	NS
Δ389–580	6	5.0 (1.4)	NS
Δ580–614	4	10.1 (3.5)	NS
Δ614–662	3	2.6 (1.0)	$0.1 > P > 0.05$
Δ662–775	7	2.1 (0.4)	$P < 0.01$
Δ775–817	10	3.0 (0.6)	$P < 0.01$
Δ817–839	7	2.6 (0.5)	$P < 0.05$
Δ839–892	3	5.4 (1.0)	NS
Δ892–926	3	5.0 (3.5)	NS

Drosophila SL-2 cells were cotransfected with an Sp1 expression construct ($pP_{ac}Sp1$), a DHFR-CAT reporter construct, and vector pSVE without (control) or with wild-type Rb cDNA (pSVE-HRb; ref. 49) or mutated Rb cDNA encoding Rb lacking the indicated amino acids (49).
*n, number of independent plates of transfected SL-2 cells analyzed.
†Mean Sp1 superactivation is expressed as levels of CAT activity quantified as described (43).
‡Significance of the difference in Sp1 superactivation by mutated Rb cDNAs or control relative to wild-type Rb was calculated by using Student's t test. NC, not calculated; NS, not significant.

for the differential cell specificity of Rb-regulated RCE-dependent transcription reported by Kim *et al.* (18).

In cotransfection assays utilizing a battery of internally deleted Rb cDNAs, we have located amino acids required for superactivation in two noncontiguous regions of Rb that are often mutated in tumor cells and have been shown to be necessary for growth suppression. The first is a portion of the Rb pocket (amino acids 614–839) previously shown to be required for the binding of Rb to transcription factors such as E2F-1 and viral oncoproteins. However, in contrast to the requirements for the formation of Rb/E2F-1 complexes, deletion of the amino-terminal portion (A domain) of the Rb pocket, or amino acids 839–928, did not significantly alter levels of superactivation. A second Rb region required for superactivation corresponds to amino acids 140–202. To our knowledge this is the first indication that portions of Rb outside of the Rb pocket region are required for functional interactions between Rb and a transcription factor. Since, as mentioned above, the mechanism of Rb-mediated superactivation may involve the formation of a multimeric protein complex of Rb, Sp1/Sp3, and negative regulators of Sp1/Sp3, it is conceivable that this amino-terminal portion of Rb may play a role in bridging protein–protein interactions. Alternatively, this upstream portion of Rb might serve to tether cellular proteins that can modify Sp1/Sp3 or Sp1/Sp3-binding proteins leading to the stimulation of transcription. Consistent with this latter hypothesis, at least one additional cellular protein, a cell cycle-regulated serine/threonine kinase, has been shown to associate with Rb, depending on the presence of amino acids that overlap with this upstream portion of Rb (57). Whether this Rb-associated kinase plays a role in superactivation remains to be explored.

This work was supported in part by National Cancer Institute Grant CA53248 and by American Cancer Society Faculty Research Award A-73970. J.M.H. is supported by the Pew Scholars Program in the Biomedical Sciences.

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