Sp-1 Binds Promoter Elements That Are Regulated by Retinoblastoma and Regulate CTP:Phosphocholine Cytidylyltransferase-α Transcription*

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The retinoblastoma (Rb) protein is implicated in transcriptional regulation of at least five cellular genes. Co-transfection 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 transcriptional control. In the present report we demonstrate that two RCEs identified within the CTP:phosphocholine cytidylyltransferase-α (CTα) proximal promoter are essential to promote transcription. Mutations that abolished each RCE markedly decreased CTα transcription. Co-transfection of Rb and truncated promoter constructs demonstrated that Rb regulates CTα expression by different mechanisms depending on the phase of the cell cycle. The regulation of CTα expression by Rb required both the Sp1 and the RCEs. Maximal expression occurred when both Rb and Sp1 were overexpressed. Moreover, RCEs were required for Rb association with the DNA. This regulatory mechanism alters CTα activity and thereafter changes PC availability and cell physiology. This is the first report demonstrating not only that surrounding Sp1 binding sites alter regulation mediated by Rb, but also that the expression of a gene involved in PC biosynthesis shares a common regulatory pathway with genes responsible for cell growth and differentiation.

Cytidylyltransferase (CT) pathway in which CTP:phosphocholine cytidylyltransferase (CT) catalyzes the regulated and rate-limiting step (1–3). Two genes (Pcyta1 and Pcytb1, for CTα and CTβ, respectively) encode CT activity (4–8). CTα is ubiquitously expressed in nucleated cells (9), and its expression is tightly regulated. CTα is also regulated post-translationally by reversible association with membrane lipids, which are required for its activity (10–14). At the level of gene expression, CTα mRNA has been shown to increase after growth factor stimulation (15), during liver development (16), in proliferating liver tissue following partial hepatectomy (17), and during the S phase of the cell cycle (18). We recently reported that the expression of CTα is activated in late G1-S phase by the action of Sp1 (19) and repressed in quiescent cells by the action of histone deacetylase (20).

The retinoblastoma gene product (Rb) was the first tumor suppressor to be identified (21). Rb regulates the expression of several genes through cis-acting elements in a cell type-dependent manner through the retinoblastoma control element (RCE), which is present in some genes responsible for cell growth and differentiation (22). This element can be positively or negatively regulated by Rb and by three nuclear proteins of 115, 95, and 80 kDa (retinoblastoma control proteins (RCPs)) that bind RCE in vitro (23–26). The RCPs bind to the RCEs within the c-fos, c-myc, and transforming growth factor-β1 promoters (26). RCPs were shown to be members of the Sp1 transcription factor family. The 115- and 95-kDa RCP proteins correspond to Sp1 and Sp3 transcription factors, respectively, and activate RCE-mediated transcription. In contrast, the 80-kDa RCP, which is produced by internal initiation of transcription from Sp1 mRNA, acts as a potent inhibitor of Sp1/Sp3-mediated transcription (26–29). RCP-mediated transcription is controlled by Rb protein (23). It seems that Rb regulates RCP-dependent transcription by direct interaction with RCPs rather than by binding to the RCE DNA sequences.

Through sequence analysis, we discovered two putative RCEs in the promoter of the mouse Pcyta 1 gene. We explored the nature of these putative RCEs and their possible role in CTα regulation in C3H10T1/2 mouse embryonic fibroblasts.

EXPERIMENTAL PROCEDURES

Materials—The luciferase vector, pGL3-basic, which contains the cDNA for Photinus pyraris luciferase, and the dual-

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The abbreviations used are: PC, phosphatidylcholine; CT; CTP:phosphocholine cytidylyltransferase; FBS, fetal bovine serum; LUC, luciferase; Rb, retinoblastoma protein; RCE, retinoblastoma control element; RCP, retinoblastoma control protein; qPCR, quantitative PCR; CMV, cytomegalovirus; ChIP, chromatin immunoprecipitation.

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3 The abbreviations used are: PC, phosphatidylcholine; CT; CTP:phosphocholine cytidylyltransferase; FBS, fetal bovine serum; LUC, luciferase; Rb, retinoblastoma protein; RCE, retinoblastoma control element; RCP, retinoblastoma control protein; qPCR, quantitative PCR; CMV, cytomegalovirus; ChIP, chromatin immunoprecipitation.
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Luciferase Reporter Assay System were obtained from Promega (Madison, WI). Lipofectamine and PLUS reagent, Dulbecco’s modified Eagle’s medium, Schneider’s medium, fetal bovine serum, and fetal calf serum were from Invitrogen. Anti-Rb and anti-Sp1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). ECL® immunoblotting reagents were purchased from Amersham Biosciences.

Cell Culture—C3H10T1/2 mouse embryo fibroblasts and human osteosarcoma (SaOs-2) cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with penicillin G (100 units/ml), streptomycin (100 μg/ml), and 10–15% fetal bovine serum in a 5% CO₂ humidified incubator at 37 °C. Schneider SL2 cells were cultured in Schneider’s medium supplemented with 10% heat-inactivated fetal calf serum at room temperature. C3H10T1/2 fibroblasts were arrested in G₀ by incubation in culture medium with low serum (0.5%) for 72 h and released by addition of fresh medium containing 10% fetal bovine serum.

Transient transfections with CTα promoter-luciferase reporter plasmids containing mutations that alter transcription factor binding, LUC.C7 (−1268/+38), LUC.C7delRCE1, LUC.C7delRCE2, LUC.C7delSp1B, and LUC.C7delSp1C (1 μg), were performed using a cationic liposome method (30). LUC.C7 (−1268/+38) inserted into the promoter-less luciferase vector pGL3-basic (Promega) was prepared as described previously (31). Mutations in each of the indicated sites were performed using QuickChange (Stratagene). All dishes received 1 μg of pSV-β-galactosidase (Promega) as a control for transfection efficiency. Luciferase and β-galactosidase assays were performed using the Promega dual assay system, as recommended by the manufacturer, and luminometric measurements were made using Fluskan Ascent FL Type 374 (ThermoLabsystems). Luciferase activity was normalized to β-galactosidase activity. A vector enabling expression of recombinant human Rb (CMV-Rb) was obtained from Dr. L. Schang (University of Alberta), and vectors enabling expression of recombinant human Sp1 protein were obtained from Dr. R. Tjian (pPacSp1 and pPac0) (32, 33).

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assays—Total nuclear extracts of C3H10T1/2 cells grown to different stages of the cell cycle were prepared as described by Andrews and Faller (34). Oligonucleotides carrying the RCE and Sp1 elements: RCE sense (5’-GCCACCGGCCCCCGTTCTTGG-3’) and RCE antisense (5’-TCGAGCGCCCCGTTCTTGG-3’); RCEB sense (5’-GAGGTTGCAGCATGACAGGAGGCGGGATCGGAGC-3’) and RCEB antisense (5’-CCCCGGCCTCCCCGCCCCCTTCTCATT-3’); RCEB-C and DRCEB-C elements: DRCEC sense (5’-AAATAAAGCCCGGCCCCCGTTTCTTGGACG-3’), DRCEB antisense (5’-CGACAGGGCCGCCTTGATTCTGATTG-3’), and DRCEB sense (5’-GGAACAACATTTAGCAGAGGGCGGGAGGACG-3’) and DRCEB antisense (5’-CCCCGGCCTCCCCGCCCCCTTCTTGGACG-3’) sequences were synthesized by the University of Alberta DNA Core Facility. Complementary oligonucleotides (100 μM of each) were heated at 90 °C for 5 min and then slowly cooled to room temperature. Five picomoles of double-stranded oligonucleotide was 5’-labeled using T4 kinase (Invitrogen) and [γ-32P]ATP (PerkinElmer Life Sciences). For each binding reaction (40 μl), 1 μg of poly(dl-dC)-poly(dl-dC), 20 μl of 2× binding buffer (100 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 250 mM NaCl, 5 mM EDTA, 50% glycerol, 0.5% Nonidet P-40, 5 mM dithiothreitol), 1 μg of nuclear extract, and labeled probe (20,000 cpm) were incubated for 30 min at room temperature.

For supershift analysis, after incubation of the probe with nuclear protein, 5 μl of antibody specific for Rb (c-15) and Sp1 (sc-59) (Santa Cruz Biotechnology) was added for 15 min. Binding reactions were terminated by the addition of 4 μl of gel loading buffer (30% (v/v) glycerol, 0.1% (w/v) bromphenol blue, 0.1% (w/v) xylene cyanol). The complex was separated on a non-denaturing 6% (w/v) polyacrylamide gel and visualized by autoradiography of the dried gel.

Chromatin Immunoprecipitation Assay—C3H10T1/2 fibroblasts were grown for 24 h in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. After serum deprivation for 72 h, cells were incubated with 1% formaldehyde for 5 min at 37 °C. Cells were collected, lysed, and sonicated three times for 10 s each time at 2.5% with an ultrasonic processor XL from Heat Systems and treated as recommended by the manufacturer (Upstate). Anti-Rb (c-15) and anti-Sp1 (sc-59) antibodies were from Santa Cruz Biotechnology. For PCR analysis, we used the following set of primers: ChIPCT1 (5′-TTGCGCT CCTTCTACCTGTGCTC/CT) and ChIPCT2 (5′-CTCCTTCCCCCGT CTCTTGTC/CT) and CB001 (5′-CCACACATCCgAAATTCC/ CB002 (5-CACgACTAgCgAAgTC). PCR was performed using 2.5 μl of template DNA, 1.5 mM MgCl₂, and 20 pmol of each primer for 30 cycles at 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min. For the assays with LUC.C7delRCE plasmids we used a set of primers, ChIPCT1 and GLP2, chosen specifically to detect the episomal CTα promoter but not the chromosomal promoter.

CT Activity—Cells were collected in 1 ml of homogenization buffer A. The cells were counted then sonicated for 20 s at 4 °C. Cell lysates were centrifuged at 7,000 × g for 5 min to pellet nuclei and unbroken cells. Aliquots of supernatant were used for CT activity assays. All protein concentrations were measured using the Bio-Rad protein assay with bovine serum albumin as a standard. CT activity in the homogenate was determined in the presence of PC:oleate vesicles by monitoring the conversion of phospho[3H]choline to CDP-[3H]choline, as previously described (35).

Quantitative Reverse Transcription-PCR—For each transcript of interest, PCR primers (mouse CTα-F, GCT AAA GTC AAT TCG AGG AA; mouse CTα-R, CAT AGG GCT TAC TAA AGT CAA CT; mouse B2 MG-F, GCT ATC CAG AAA ACC CCT CAA; mouse B2 MG-R, CAT GTC TCG ATC CCA GTA GAC GGT; human Rb1-F, ATT CTG CAT TGG TGC TAA AAG; human Rb1-R, TCT CTG TTC TTA CCA CCT CGC; human Sp1-F, GCC GTG GGG AAA GTG TAT G; and human Sp1-R, TTC TGG GGC TTT TTA ATA TGT) suitable for SYBR green-based qPCR (Simpson et al. (45)) were designed using Oligo6 Software by Molecular Biology Insights. PCR reactions (25 μl) included Platinum Quantitative PCR Supermix-UDG (Invitrogen, 12.5 μl), 250 nM of each PCR primer, and 5 μl of cDNA template per reaction. Triplicate reactions and a “no
growth factor genes (26). CTRCE1 and CTRCE2 are located, respectively, in positions −145 and −80 relative to the transcriptional start point. CTRCE1 and CTRCE2 are shown in Fig. 1, and these RCEs are identical to those identified in the c-myc (for CTRCE1, see Fig. 1) and c-fos (for CTRCE2, see Fig. 1) promoters.

**Mutations That Alter the RCEs Abolish CTα Expression**—To evaluate the role of these putative RCEs in CTα expression we generated two reporter plasmids in which the 1268-bp CTα proximal promoter (Luc.C7 (31)) harbors mutations that alter each of the RCE elements (the RCE2 ggtgg was changed to aattg and the RCE1 cctcg was changed to cctcg). We named these plasmids Luc.C7delRCE1 and Luc.C7delRCE2, respectively.

C3H10T1/2 embryonic mouse fibroblasts were transfected with each of these plasmids plus pSV-β-galactosidase as a transfection control. After transfection, cells were synchronized by serum deprivation, and samples were taken at both G0 and S (20 h after cell cycle induction) phases of the cell cycle. Luciferase and β-galactosidase activities were measured. The results shown in Fig. 2 indicate that each of the RCE binding sites is essential to maintain basal CTα transcription. A pronounced decrease in luciferase activity was evident with both of the mutated plasmids at both the G0 and S phases of the cell cycle.

**Rb Overexpression Alters CTα Promoter Activity but Does Not Restore CTα Expression from Promoters with Deletions in either RCE1 or RCE2**—To investigate the role of Rb in the regulation of CTα expression, we co-transfected each of the reporter constructs with a plasmid designed to overexpress human Rb (CMV-Rb) or an empty plasmid (CMV-0), and pSV-β-galactosidase as a transfection control. Rb overexpression was assessed using qPCR; the relative Rb mRNA copy number was 0 in untransfected cells and 20 in transfected cells. We measured luciferase and β-galactosidase activities in extracts obtained from cells that had been arrested in G0 or in cells synchronized to S phase. The results obtained by reporter assays (Fig. 3A) indicate that Rb is involved in regulating CTα expression in different ways depending on the phase of the cell cycle. In quiescent cells Rb induced CTα expression, but during the S phase Rb repressed CTα expression. When either of the RCE elements was mutated, these two effects were abolished such that the activity of the promoter reached a very low level, as previously demonstrated (Figs. 2 and 3). These results indicate that RCE elements are involved in maintaining the basal level of CTα transcription and that the regulation by Rb requires intact RCE elements.

To understand the physiological consequences of the effect of Rb on CTα transcription, we measured CT activity in the template” control reaction were performed for each primer pair. Thermocycling was performed using the Rotor-Gene RG-3000 Thermocycler (Corbett Research), and the thermocycling conditions were 50 °C for 2 min, 95 °C for 4 min, followed by 45 cycles of 95 °C for 20 s, 55–58 °C for 20 s, and 72 °C for 20 s. Upon completion of a thermocycling run, melt curve analysis was performed on the double-stranded amplicons to identify nonspecific amplification products. Normalized CT data were used to calculate expression ratios between the sample and control groups.

**RESULTS**

**CTα Proximal Promoter Contains Two RCEs**—Rb regulates transcription of the c-fos, c-myc, and transforming growth factor-β promoters through the RCE in transient assays in either a positive or negative manner depending upon cell type (26). Sequence examination of the CTα proximal promoter has revealed that it contains target sequences for Rb termed RCEs, which are present in the promoter regions of a number of
same extracts. When Rb was overexpressed during G₀ phase, CT activity was increased by 21%; this increase was not masked by the activity of the endogenous enzyme. However, we did not observe any change in CT activity after Rb overexpression during S phase (Fig. 3B). The latter result might be explained by the low (10%) transient transfection efficiency of these cells. In this case, the decrease observed in CT mRNA (measured as reporter assay) was not reflected in enzyme activity due to the endogenous CT activity in non-transfected cells. We did not attempt to overexpress Rb in stable cell lines, because this would be expected to alter cell cycle progression (36).

Rb Regulation of CȚα Expression Involves Sp1 Binding Elements—Udvadia et al. (26, 27) have demonstrated that one of the factors (RCP) that binds to the RCE elements is a member of the Sp1 family. In previous experiments we identified Sp1 as an important regulator of CȚα expression during the cell cycle. We found that phosphorylated Sp1 binds to the Sp1 binding site B thereby activating CȚα expression when the cells reach S phase (19, 37). Otherwise, in quiescent cells the complex between Sp1/E2F-Rb-histone deacetylase represses CȚα expression (20). In the latter case, Sp1 binds to Sp1 binding site C that was identified as a negative modulator of CȚα expression (19). Because both RCE sites are located close to Sp1 binding sites (Fig. 1), we investigated the relative effect of Rb overexpression on CȚα expression when Sp1 binding site B or C was mutated. We co-transfected cells with the reporter constructs

![FIGURE 3. Rb overexpression alters CȚα transcription and activity but does not restore CȚα expression when either RCE element is altered.](image)

![FIGURE 4. Sp1 binding sites adjacent to the RCEs alter the Rb-mediated effect on CȚα expression.](image)
reporter was induced by overexpression of Sp1 and Rb. As shown in Fig. 5A, stimulation of CTα transcription by Rb was much higher upon co-expression of Sp1 compared with transfection of Rb alone. Maximum activation was achieved when both Sp1 and Rb proteins were co-expressed.

We also evaluated whether the stimulation of luciferase expression that we observed in the reporter assay had any consequence for CT activity and cell physiology. CT activity in SL2 cells overexpressing Rb and Sp1 was 32% higher than in control cells (Fig. 5B). It is important to note that Rb and Sp1 expression in those cells was confirmed by qPCR (see Fig. 5C). These observations indicate that Rb directly or indirectly regulates the ability of Sp1 to modulate CTα expression in intact cells.

Sp1 and Rb Bind in Vivo to the CTα Promoter—The association between Rb and Sp1 and the CTα promoter was monitored by ChIP assay using chromatin prepared from C3H10T1/2 fibroblasts. Target sequences were detected by PCR using the sets of primers described under "Experimental Procedures." A portion of the CTα promoter was found in association with anti-Sp1 and anti-Rb immunoprecipitates in chromatin extracted from these cells (Fig. 6). As a control chromatin was immunoprecipitated with anti-IgG. In these samples, no CTα promoter was detected (Fig. 6, lane 2).

Sp1 and Rb Bind in Vitro to Probes Containing RCE and Sp1 Elements—The previous experiments demonstrate that Sp1 and Rb cooperatively regulate CTα expression. To determine if Sp1 and Rb form a DNA-protein complex with the Sp1 and/or RCE-DNA motif, we performed gel-shift assays and supershift experiments using anti-Rb and anti-Sp1 antibodies. We probed two different DNA motifs, one containing the RCE1 and Sp1C elements (named RCEC) and the other having the RCE2 and Sp1B elements (named RCEB). Nuclear extracts were isolated from C3H10T1/2 cells synchronized to G0 and S phases. With nuclear extract from G0 and RCEB as a probe, two complexes (Fig. 7, lane 2, arrows a and b) were detected. After incubation with anti-Sp1 antibody (Fig. 7, lane 3) the intensity of the lower band b decreased. Upon incubation with anti-Rb antibody the upper band a disappeared (Fig. 7, lane 4). With the RCEC probe, we observed two bands whose mobilities were so similar that they could not be properly defined (Fig. 7, lane 6). The intensities of the two bands decreased when the extract was incubated with anti-Sp1 antibody (Fig. 7, lane 7). However, the intensity of only the upper band decreased when the extract was incubated with anti-Rb antibody (Fig. 7, lane 8). A similar experiment was performed using nuclear extracts from cells in S phase (Fig. 8). When the extract was incubated with the RCEB probe, one complex (Fig. 8, lane 2 arrow a) was detected whose intensity and mobility did not change when the assay was performed in the presence of anti-Sp1 antibody (Fig. 8, lane 3). However, incubation with anti-Rb antibodies decreased the intensity of the band (Fig. 8, lane 4). When the same extract was incubated with RCEC probe one band was observed (Fig. 8, lane 5).
6), whose intensity decreased after incubation with anti-Sp1 antibodies (Fig. 8, lane 7). Furthermore, with anti-Rb we observed a supershifted band (Fig. 8, lane 8, arrow b). Together these results demonstrate that Sp1 and Rb directly or indirectly bind and form complexes with probes containing RCE and Sp1 consensus binding elements in vitro.

To determine if mutations in either of the RCE motifs altered the binding of Sp1 and/or Rb, we performed supershift assays with the DRCEB probe (mutated to alter RCE2 and wild-type Sp1B) and DRCEC probe (mutated to alter RCE1 and wild-type Sp1C). As shown in Fig. 9A, when extracts from G0 cells were incubated with DRCEB probe two complexes were detected (Fig. 9, lane 2 arrows a and b) that were similar to those observed with the RCEB probe (Fig. 8). When the same assay was performed in the presence of anti-Sp1 antibody, the intensity of both bands decreased so that detection of the complex was difficult (Fig. 9A, lane 3). Anti-Rb antibody did not alter complex formation (Fig. 9A, lane 4). The same analysis was performed after the DRCEC probe was incubated with extracts from cells in G0 phase (Fig. 9A). One diffuse complex was detected (Fig. 9A, lane 2, arrow a) whose intensity was attenuated after incubation with either anti-Sp1 or anti-Rb antibodies (Fig. 9, lanes 7 and 8).

To establish the in vivo relevance of the in vitro observations, we performed a modified ChIP assay. Cells were transfected with plasmids harboring mutations in either the RCE1 or RCE2 motifs. After 24 h we performed ChIP assay with anti-Rb and anti-Sp1 antibodies. The precipitated DNA was detected by PCR using primers designed to amplify only the episomal DNA. In agreement with the in vitro assay, when RCE2 was mutated Rb did not associate with the CTα promoter (Fig. 9B). The association of Rb with the CTα promoter was not altered when the RCE1 was mutated (Fig. 9B). Incubation of extracts obtained from S phase cells with each probe generated two complexes (Fig. 10, lanes 2 and 6, arrows a and b) that were not diminished by anti-Sp1 or anti-Rb antibodies (Fig. 10, lanes 3, 4, 7, and 8). Because we did not detect Rb association with mutated RCE-2, these experiments suggest that at least the RCE-2 element is required for the association of Rb with the DNA.

**DISCUSSION**

*Rb Regulates Gene Expression of CTα through RCEs*—The Rb gene has been characterized as a tumor suppressor gene,
because it is deleted or mutated in certain types of human tumors. It has been suggested that Rb constrains cell proliferation by acting as a regulator of transcription (23, 24, 40). In this regard, regions of the human c-fos, c-myc, and transforming growth factor-β1 promoters that confer regulation by Rb to heterologous promoters were identified (23). These promoter regions share a motif termed RCE (CCACCC). In this report we demonstrate that Rb regulates CTα promoter transcription through two RCE-like sequences in the CTα proximal promoter.

Because during cell growth the requirement for PC increases and CTα expression is enhanced (19, 37), we investigated the role of the identified RCEs on CTα expression in non-proliferating cells (G0) and in growing cells (S phase). We used reporter constructs with point mutations in each of the RCEs. The results show that both RCEs are required for CTα expression. Alteration of each of these sites individually markedly decreased CTα transcription in both phases of the cell cycle (Fig. 2). This result led us to ask how Rb overexpression affects the expression profile of CTα. When each of the mutated reporter plasmids was co-transfected with a plasmid designed to overexpress Rb, the expression of the CT-luciferase reporter did not change significantly (Fig. 3), indicating that under the assay conditions intact RCEs are required to promote CTα transcription. However, when a reporter construct with intact RCEs was analyzed in the presence of Rb, two different effects were evident. During the G0 phase, Rb overexpression enhanced transcription, whereas during S phase, Rb reduced CTα expression. This effect may take place by the recruitment of transcription factor(s) or result because Rb can regulate CTα expression in opposite ways in different phases of the cell cycle. The latter possibility might be related to the regulation of Rb by phosphorylation that is known to occur during progression of the cell cycle (41–43). Phosphorylation of Rb might alter the ability of Rb to interact with other factors.

In a previous report we demonstrated that formation of a complex between Rb and histone deacetylase represses CTα transcription in quiescent cells (G0 phase). We postulated that this complex was recruited to the CTα promoter by Sp1 and E2F (20). According to this model, the induction of CTα expression by Rb might be the consequence of a competition between free Rb and Rb-histone deacetylase complexed with E2F and Sp1. In this situation excess Rb might displace the Rb-histone deacetylase complex from the anchor proteins thereby affecting CTα repression. The effect observed during S-phase is more difficult to explain, because the binding of phosphorylated Sp1 to the Sp1 binding site B drives CTα induction (37). However, Sp1 has been described as one of the RCP proteins that bind to the RCE (23). Furthermore, the proximity of RCE2 and the Sp1 binding site B might affect the binding of the Sp1-containing complex to the promoter.

The common RCE motif identified in several Rb-regulated promoter elements is a variant of the consensus Sp1 binding site (24, 44). We, therefore, examined whether or not Rb regulates Sp1 transcriptional activity specifically from the variant Sp binding site or whether the consensus Sp1 binding site also confers Rb regulation. To probe the role of the adjacent Sp1 binding site on the Rb-dependent effect on CTα expression, we used reporter constructs harboring point mutations in the Sp1 binding sites B and C, respectively. When the Sp1 site C was mutated, Rb overexpression restored CTα expression, indicating that Rb can also repress CTα transcription by an Sp1-independent mechanism (Fig. 4). These results also indicate that, in the presence of Rb, the effect of Sp1 on CTα expression can be modulated through the RCE elements. When we overexpressed Rb and evaluated the activity of the reporter construct with a mutated Sp1 site B, a normal level of expression of CTα was restored indicating that, in the absence of Sp1 site B, Rb stimulates CTα expression. Although the mechanism responsible for this effect is not completely understood, Rb might act as a Sp1 trans-activator that would induce the binding of Sp1 to the RCE elements. Nuclear factors that bind RCEs have also been shown to bind oligonucleotides that contain Sp1 binding sites (27). Taken together, our results indicate that RCEs and Sp1 binding elements cooperate to establish the CTα expression profile during the cell cycle.

Rb Requires Sp1 to Regulate CTα Expression—Cooperativity between Sp1 and Rb was clearly evident when CTα promoter activity was measured in cells that lacked Rb (SaOs-2 cells) or Sp1 (SL2 cells). CTα transcription in SaOs-2 cells was very low compared with that in SL2 and C3H10T1/2 cells and was not restored by overexpression of Rb or Sp1 (Fig. 5). However, the CTα transcript was detected by quantitative reverse transcription-PCR (Fig. 5C). This can be explained by the knowledge that these cells have alterations in the pattern of expression of the cyclins (43). As we previously reported, cyclins A and E affect CTα expression during the S phase through Sp1 phosphorylation. In SL2 cells, CTα expression was low in the absence of Sp1, a key activator (19, 38), but was induced by overexpression of Sp1 (Fig. 6). Interestingly, the maximal expression of CTα was achieved when both Rb and Sp1 were overexpressed. Rb overexpression alone had only a small effect.

Rb and Sp1 Associate with the Promoter—These observations suggest that Rb is not functioning merely by binding to and inactivating a specific transcription factor but instead is actively involved in regulating transcriptional initiation of CTα in both a positive and negative manner. It is possible that Rb interacts directly with Sp1 or with a complex that interacts with Sp1. To determine if these proteins interact with the CTα promoter, and to evaluate the role of the Sp1 and the RCEs in the binding of Rb and Sp1, we performed gel-shift and ChIP assays with wild-type and mutated RCE probes. Both Rb and Sp1 associate with the CTα promoter as we demonstrated by in vivo ChIP assays (Fig. 6). Moreover, by electrophoretic mobility shift assays, we identified two complexes that include Rb and Sp1 using RCEB and RCEC probes and G0-nuclear extract (Fig. 7). Interestingly, when we used the same extract but with a DRCEB probe whose RCE was mutated, two complexes were still observed but they did not include Rb. We did not detect any changes after incubation of the nuclear extract with anti-Rb antibody (Fig. 9), indicating that Rb does not bind to DNA when the RCE2 is mutated. When the G0 cell extract was incubated with the
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DRCEC probe, we detected only one complex instead of the two observed with the wild-type probe. This complex included both Rb and Sp1 proteins (Fig. 9).

The combined results of our studies suggest that during G0 phase the RCE elements form a complex that contains Sp1 and Rb. During S phase, one complex is formed with both probes (RCEB and RCEC) and includes Rb (Fig. 10), as was confirmed by the supershift assay. We cannot discount Sp1 as part of the complex, because Sp1 activates CTα expression during S phase (19, 37). If Sp1 had formed a complex with cyclin A/E and Cdk2 (37) the Sp1 might not be accessible to the antibody. When we found to be associated with the CTα promoter when RCE-2 was mutated (Fig. 10). Because Rb did bind to the wild-type probe, we conclude that RCEs are required for Rb to bind indirectly to the promoter. Alternatively, other as yet unidentified RCPs might bind to the probe and abolish the recognition of Rb by the antibody.

The described mechanism for CTα transcriptional regulation is far from a simple repression or induction (on/off mechanism). We speculate that CTα expression is regulated coordinately so that the cell expresses CTα at the appropriate timing during progression of the cell cycle. In an attempt to investigate the physiological consequence of this regulatory mechanism we measured CTα mRNA levels and CTα activity in cells overexpressing Rb and/or Sp1. The level of CTα mRNA was higher during S phase compared with G0 phase confirming that the reporter assay reflected the endogenous regulation. However, as expected for the low transfection efficiency of the fibroblasts, we were unable to show using qPCR that overexpression of Rb and/or Sp1 altered the level of CTα mRNA. Nevertheless, an increase in CTα activity was observed after Rb overexpression that correlated with an increased expression of CTα promoter reporters. No change in CTα activity was observed, however, when CTα transcription was decreased. These results can be explained by considering the low transfection efficiency of these cell lines. In depth study of the physiological consequences of increased Rb expression on CTα transcription will require a CTα reporter transgenic mouse.

In conclusion, we identified two RCE elements that confer Rb regulation of the CTα promoter. We have revealed another example in which Rb regulation by RCE elements requires Sp1. It is logical that genes regulated in a cell cycle-dependent manner, such as c-fos, c-myc, and Pcyta1, share common regulatory mechanisms. The studies reported herein provide the first evidence that Sp1 binding sites and RCEs interact, or cooperate, to exert transcriptional regulation of gene expression.

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