The Human Retinoblastoma Susceptibility Gene Promoter Is Positively Autoregulated by Its Own Product*

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The product of the retinoblastoma susceptibility gene is a 105-kDa protein that has properties of a cell cycle regulatory factor. Previous reports indicated that two distinct DNA-binding factors, RBF-1 and ATF, play an important part in the transcription of the human retinoblastoma gene (Rb). Recently, we demonstrated that pRb activates expression of the human transforming growth factor-β2 gene through ATF-2. Since the human Rb gene promoter also contains an ATF-2-like binding site, we examined whether pRb can regulate its own expression through ATF-2. Here we report that overexpression of Rb stimulates Rb promoter activity through the ATF binding site in a variety of different cell types. Mutation of the ATF binding site of the Rb promoter abolishes the Rb autinduction. We have also determined that the carboxyl-terminal domain of pRb is responsible for the Rb autinduction through ATF-2. Rb autinduction may be important for maintaining the action of pRb during cell growth, and loss of autoregulatability may contribute to retinoblastoma.

Inactivation of the retinoblastoma gene product (pRb) has been associated with the etiology of a subset of human tumors (Friend et al., 1986, 1987; Pung et al., 1987; Lee et al., 1987, 1988; Harbour et al., 1988; Horowitz et al., 1989, 1990). The protein product of the retinoblastoma susceptibility gene (pRb) thus appears to play an important role in the negative regulation of cell proliferation. It has been suggested that pRb can cause cell cycle arrest in mid- to late G1 phase (Goodrich et al., 1991). Whereas underphosphorylated forms of pRb are the primary species seen in the G0 and G1 phases of the cell cycle, the protein undergoes phosphorylation at multiple sites as cells traverse the G1/S boundary (Mihara et al., 1989; Ludlow et al., 1990; Laiho et al., 1990). This suggests that it is the underphosphorylated form of pRb that is involved in negative growth control. Moreover, products of certain DNA virus oncogenes bind to the underphosphorylated form of pRb.

Recent studies have demonstrated that pRb binds cellular transcription factors (Rustgi et al., 1991; Defeo-Jones et al., 1991; Gerin et al., 1991; O'Reilly et al., 1992a, 1992b; Gu et al., 1993; Hagemeier et al., 1993). Most of these interactions have been demonstrated in vitro, but three, E2F-1, and Myc, have also been observed in vivo (Helin et al., 1992; Kaelin et al., 1992; Wang et al., 1993; Gu et al., 1993). These results suggest that pRb might act as a transcriptional regulator. pRb inhibits the transcription of genes involved in growth control (Wagner and Green, 1991) and can regulate expression of several genes, including transforming growth factor-β1 (TGF-β1) and -β2, e-fos, and insulin-like growth factor II genes (Robbins et al., 1990; Kim et al., 1991, 1992a, 1992b). Because TGF-β2 inhibits proliferation of many cell types and arrest growth in the late G1 phase of the cell cycle, induction of TGF-β2 by pRb may play an important role in cell cycle control.

Recently, two naturally occurring point mutations in the promoter region of Rb genes have been identified which result in decreased expression of the Rb gene (Sakai et al., 1991). These mutations are associated with hereditary retinoblastoma, suggesting that a quantitative decrease in the expression of the Rb gene can contribute to the development of retinoblastoma. These mutations occurred in the promoter regions that bind RBF-1, Sp1, and ATF5, suggesting that these transcription factors might play an important role in the expression of the human Rb gene.

Since pRb is a transcriptional regulator that can regulate activity of both Sp1 (Kim et al., 1992a) and ATF-2 (Kim et al., 1992a), it is possible that pRb can regulate its own promoter. In this report, we provide evidence that Rb promoter activity is directly stimulated by its own gene product through the ATF binding site. This positive autoregulatory loop is likely to amplify responses to growth signals.

MATERIALS AND METHODS

Plasmids—Chimeric promoter/CAT plasmids, pRB1, pRB2, pRB3, pRB4, pRB5, pRB6ATFm, pRB6, and pRB7, were constructed by ligating human Rb promoter fragments (Sakai et al., 1991), produced by polymerase chain reaction, into the promoterless CAT-containing plasmid, pCAT (Promega). The 3' oligonucleotide used in all amplifications corresponded to the 21-base pair sequence starting at -48 from the ATG codon of the human Rb gene to which an XbaI site and four random nucleotides were added. Using these oligonucleotides, fragments were amplified according to the standard protocol of the GeneAmp kit (Perkin-Elmer). Correct sequence of the polymerase chain reaction-amplified fragments was verified by DNA sequence analysis.

One additional chimeric promoter/CAT plasmid was constructed by ligation of the ATF site of the human Rb promoter, produced by polymerase chain reaction amplification, to the region of the human TGF-β2 promoter corresponding to the 531-base pair sequence between nucleotides -68 and +75, and then into the Smal site of pGEM4-SVOCAT (O'Reilly et al., 1992) and verified as above.

The human Rb expression vector pRH and control plasmid have been described (Robbins et al., 1990). Rb deletion constructs were also
were grown in Dulbecco's modified Eagle's medium-12 medium in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing human cervical carcinoma) cells were grown ATF-1 and pECE-ATF-2 were kindly provided by Michael Green.

described previously (Qian et al., 1992). The expression plasmids pECE-ATF-1 and pECE-ATF-2 were kindly provided by Michael Green.

Cell Culture—CCL-64 (mink lung epithelium), Saos-2 (human osteogenic sarcoma), and C-33A (human cervical carcinoma) cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal bovine serum. PC-3 (human prostate adenocarcinoma) cells were grown in Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 10% fetal bovine serum. PLC/PRF/5 (human hematoma) cells were grown in minimal essential medium supplemented with 10% fetal bovine serum.

Transfections were performed by the calcium phosphate precipitation method. Following incubation with calcium phosphate precipitates and glycerol shock, the cells were incubated for 48 h. After 48 h, cells were harvested, and extracts were assayed for CAT activity according to Gorman et al. (1982). CAT enzyme activity was normalized for transfection efficiency by cotransfection of 1.0 µg of a human growth hormone expression plasmid, pSVGH, and determination of secreted growth hormone in the medium, prior to harvesting for CAT activity (Nichols Institute, San Juan Capistrano, CA).

Mobility Shift Assays—GST-ATF1 and GST-ATF2 fusion proteins were kindly provided by Susan Wagner and Michael Green. Purified E2F protein was provided by Hans Huber. Binding reaction mixtures contained 50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 100 µM ZnCl2, and 10,000 cpm of 32P-labeled probe. The oligonucleotide probes used in the gel shift analysis were prepared by annealing the complementary strands and end labeling using large fragment DNA polymerase I. The analysis of binding complexes was done by electrophoresis on a 5% 0.5 X TBE (50 mM Tris, 50 mM boric acid, 1 mM EDTA) polyacrylamide gel. Gels were dried directly and autoradiographed.

RESULTS

Induction of Rb Transcription by the Rb Protein—To determine if Rb protein is able to regulate its own transcription, we examined pRb-mediated regulation of the human Rb promoter in five different cell lines. Regulation of human Rb-CAT gene constructs was monitored following transfection of a human Rb promoter construct containing sequences between -197 and -48 into cells together with either vector plasmid DNA (p30l) or this same plasmid carrying the human Rb cDNA (pRb) (Fig. 1, lanes 1–10). In three independent experiments, we observed an average 3–6-fold increase in CAT activity with pRb, depending on the cell types. A previous report (Hamel et al., 1992) showed that in differentiated p19 (embryonic carcinoma) cells, overexpression of Rb represses Rb promoter activity. However, we have not observed repression of the human Rb promoter in the cell lines. To identify the specific sequences in the human Rb promoter responsible for its stimulation by pRb, a series of human Rb deletion constructs was cotransfected into CCL-64 cells with a human Rb expression plasmid or control plasmid. Basal expression of pRb1 was low to undetectable, suggesting that sequences between -686 and -586 might contain a negative element. The activity of pRb2 (−586 to −48) was induced 7-fold by pRb (Fig. 2). The transcription of all 5′ deletion constructs down to −197 was stimulated by pRb, whereas the pRb inducibility dropped almost to the basal level when the 5′ deletion extended to −186, suggesting that the pRb-responsive element is located between −197 and −186. The human Rb promoter contains a consensus ATF binding site (5′-GTGACGTCA-3′) at positions −193 to −185 (Sakai et al., 1991). To determine if this ATF site is involved in pRb-mediated transcriptional regulation, we mutated the site by making a 3-base substitution (5′-GTGCCCTT-3′) in the pRb5 promoter. This mutant promoter (pRb5 ATF mt) was not activated by pRb (Fig. 3, lane 4). This result suggests that pRb stimulates transcription of the human Rb promoter through an ATF binding site.

To demonstrate further the involvement of the ATF binding site in the autoinduction of human Rb, the human Rb ATF sequence was ligated to the region of the human TGF-β2 promoter between −68 and +63, which contains the TATA sequence but not the TGF-β2 ATF sequence (pRbATF-pB2-68) (Fig. 4; O’Reilly et al., 1992). We demonstrated previously that pRb activates transcription of the human TGF-β2 gene through the transcription factor ATF-2 (Kim et al., 1992a). The ATF binding site (5′-GCACCTCA-3′) in the TGF-β2 gene is located at positions −74 to −67 (Kim et al., 1992). We transfected pRbATF-pB2-68, the TGF-β2 promoter constructs, pRb2-77, which contains an ATF binding site as a positive control and pB2-40, which does not contain an ATF sequence, into CCL-64 cells with or without the Rb expression plasmid. Both pB2-77 and pRbATF-pB2-68 showed an increase in CAT activity by pRb,

![FIG. 1. Inducibility of human Rb/CAT chimeric gene by human Rb.](image1)

![FIG. 2. Mapping of the promoter element mediating Rb autoinduction.](image2)
whereas little increase in CAT activity was observed when pB2-40 was cotransfected with an Rb expression plasmid. This suggests that the Rb promoter sequence is functioning analogously to the TGF-β2 ATF-2 binding site.

The ATF Site in the Human Rb Promoter Is a Relatively High Affinity Binding Site for ATF-2—ATFs are a family of transcription factors that possess homologous DNA binding domains (Hai et al., 1989). We demonstrated previously that pRb can interact with the ATF-2 protein in nuclear extracts and that pRb can specifically stimulate ATF-2-mediated transcription. To investigate whether ATF-2 is also involved in the autoinduction of the human Rb promoter, we examined whether ATF-2 is able to bind with high affinity to the Rb-ATF site. Using equal amounts of bacterially expressed GST-ATF1 and GST-ATF2 fusion proteins, we demonstrated in a mobility shift assay that both fusion proteins bound to the ATF site of the human Rb promoter (Fig. 5A, lanes 1, 2, 5 and 6). However, the GST-ATF2 fusion protein bound to the ATF site of the human Rb promoter (Fig. 5A, lanes 5 and 6) with higher affinity than a GST-ATF1, indicating that the ATF site in the human Rb promoter is a relatively high affinity binding site for ATF-2. The 3-nucleotide substitution mutant (−199/−183 mt) failed to bind either GST-ATF1 or GST-ATF2 (Fig. 5A, lanes 4 and 8).

To further investigate the role of ATF-1 and ATF-2 in the transcription of the human Rb promoter, cotransfection experiments with expression plasmids containing ATF-1 or ATF-2 cDNAs were performed. CCL-64 cell lines were transfected with pRbB5 in the presence or absence of pECE-ATF-1 or pECE-ATF-2 (Fig. 5D). Cotransfection with ATF-2 resulted in a modest 3-4-fold increase in CAT expression in CCL-64 cells, whereas cotransfection with ATF-1 resulted in a decrease in CAT expression.

The ATF-2 binding site in the human Rb promoter is directly upstream from a putative E2F binding site. pRb has been demonstrated to bind to E2F in vivo to regulate its activity negatively. Thus ATF-2 and E2F that are positively and negatively regulated by pRb, respectively, bind to adjacent sequences. To investigate whether E2F protein binds to the E2F site in the human Rb promoter, we repeated the mobility shift assay with a purified E2F protein. Fig. 6 shows that purified E2F protein bound with similar affinities to the E2F sites of the human Rb and the adenovirus E2 promoters (Fig. 6, lanes 1 and 6), showing that the Rb E2F site is a high affinity binding site for purified E2F protein. This binding was specifically competed for by the adenovirus-E2F and Rb-E2F competitors, but not by the Rb ATF/Spl (−205 to −186) and the Rbp53 (−63 to −88) competitors. This result suggests that E2F could be involved in mediating negative regulation by Rb in certain cell types (Hamel et al., 1992).

The Carboxyl-terminal Region of the Retinoblastoma Protein Is Responsible for the Autoinduction—Recently, pRb has been shown to interact with E2F, and two regions of pRb are required for E2F binding (Qian et al., 1992). To investigate if pRb induces its own transcription through the same regions of pRb
The Rb probe is derived from sequences from -199 to -183 of the human high affinity binding site (filled bar). Cotransfections with similar results.

The values represent ratios of the activity obtained with the Rb promoter/CAT reporter construct (pRb5) and the pRb5 promoter, including each of the amino-terminal mutants, affecting codons 37–614, and the "spacer" separating the two E1A binding domains. One mutant, deleting codons 775–817, is essentially devoid of pRb5 inducing activity. This mutant is located immediately following the second E1A binding domain and contains several sites of phosphorylation of pRb (Lees et al., 1991). Two other mutants of the carboxyl terminus of pRb, deleting codons 614–662 (DL11) (within the second E1A binding domain), and the other deleting codons 839–892 (DL15), were found to be consistently diminished but partially active in pRb5 induction. These three mutants were previously found to be unable to complex to the E2F transcription factor (Qian et al., 1992). Three other mutants, affecting either the first or part of the second E1A binding domains, or codons 817–839, were fully able to induce pRb5, despite the fact that they were previously found to lack binding to E2F and to be unable to induce growth arrest in transfected cells (Qian et al., 1992). The contrast between protein sequences important for E2F transcription factor binding and transcriptional stimulation of pRb5 demonstrates that autostimulation of the pRb5 promoter by pRb does not require direct interaction between pRb and E2F. Further, this result suggests that different transcription factors interact with pRb through distinct sequences.

**DISCUSSION**

The retinoblastoma susceptibility gene product Rb is a negative regulator of cell growth whose inactivation is associated with the etiology of a subset of human tumors. How Rb functions to constrain cell proliferation is unclear, but it has been demonstrated that Rb can function as a transcriptional regulator. The observed transcriptional regulation by Rb is mediated through the regulation of activity of specific transcription factors. Rb has been shown to interact specifically with certain transcription factors to modulate their activity in either a positive or negative manner. In particular, Rb has been shown to bind and repress E2F-mediated transcription, whereas it can bind to and stimulate ATF-2-mediated transcription. Given that Rb is a transcriptional regulator, it was of interest to
determine if Rb was able to regulate its own expression. Thus we have examined the ability of the product of the retinoblastoma susceptibility gene to autoregulate its own promoter activity using a transient cotransfection assay.

We have demonstrated that in five different cell types, coexpression of Rb stimulated Rb promoter activity 3-6-fold. The stimulation was observed both in cells containing a normal Rb (CCL-64) or a defective Rb (Saos-2). Deletion analysis of the Rb promoter has identified an ATF binding site in the promoter as important for the stimulation by Rb. Several different lines of evidence suggest that it is ATF-2 that functionally interacts with Rb-ATF site. First, the Rb ATF binding site is similar to the ATF binding site in the TGF-β2 promoter that we have demonstrated previously to bind to ATF-2 (Kim et al., 1992a). Second, insertion of the Rb ATF-2 site in place of the TGF-β2 ATF binding site still allows for activation of the TGF-β2 promoter by Rb. Third, ATF-2 binds to the Rb ATF binding site with high affinity. Fourth, expression of ATF-2 in vivo stimulates Rb promoter activity. Taken together these results directly implicate ATF-2 as an important factor for mediating positive autoregulation by Rb.

It has been reported previously that Rb is able to regulate its expression in P19 cells negatively (Hamel et al., 1992). Although we have not observed negative regulation of the Rb promoter by Rb, we have demonstrated that a putative binding site for E2F directly adjacent to the ATF-2 binding site in the Rb promoter indeed binds E2F with high affinity. This E2F site may be important for conferring negative regulation by Rb in certain cell types, whereas the ATF-2 might be involved in positive regulation by Rb in other cell types. Similar to the Rb promoter, we have identified ATF, SP1, and E2F binding sites in the TGF-β1 promoter that is positively regulated by Rb in certain cell types and negatively regulated in others (Kim et al., 1991).

We have mapped the domains of Rb responsible for conferring activation of the Rb promoter through ATF-2 using a panel of Rb mutants (Qian et al., 1992). Our finding that the carboxyl terminus of Rb is sufficient to induce the ATF-2-mediated transcription correlates with functions of Rb shown by two other groups. Goodrich et al. (1991) reported that a bacterially expressed fusion protein encoding the carboxyl terminus of Rb is able to inhibit growth of microinjected cells. Qin et al. (1992) have recently reported that a carboxyl-terminal fragment of Rb expressed by the strong cytomegalovirus promoter is sufficient to reduce colony formation in transfected Saos-2 cells. Our results also suggest that the amino-terminal fragment of Rb is not required to induce the Rb promoter activity.

We demonstrated recently that Rb regulates Sp1 transcription (Kim et al., 1992b). Our unpublished data also demonstrate that the carboxyl-terminal portion of the pRb is required for the regulation of Sp1 transcription. Similarly, the carboxyl terminus has been shown to bind the D-type cyclin, c-myc, myoD, and Elf-1 in vitro (Dowdy et al., 1993; Ewen et al., 1993; Rustgi et al., 1992; Gu et al. 1993; Wang et al., 1993). These results suggest that the carboxyl terminus of pRb is the general target for transcriptional regulatory factors.

Several growth factors including platelet-derived growth factor (Paulsson et al., 1987), TGF-α (Coffey et al., 1998), and TGF-β1 (Van Obberghen-Schilling et al., 1988; Kim et al., 1990) also autoregulate the expression of their mRNAs, resulting in increased secretion of the respective peptides. Such autoinduction can amplify responses to these growth factors during development or in disease processes such as carcinogenesis. The transcription factor jun proto-oncogene is also positively auto-

**Fig. 7. Mapping of the region of the human Rb protein responsible for the autoinducibility.** A schematic representation of deletion mutants of pRb expression vectors used in this study is shown at the top. The construction of these plasmids was described elsewhere (Qian et al., 1992). The average CAT activity after normalization to growth hormone obtained from CCL-64 cells is graphically represented. The values represent ratios of the activity obtained with the pRb5 and are an average of three to four experiments with similar results.
regulated by its product, Jun/AP-1 (Angel et al., 1988). This positive regulatory loop is likely to be responsible for prolonging the transient signal. Rb autoregulation may also be responsible for amplification of negative growth signals. Many observations indicate that Rb must act in some way as a transducer of signals that cause the cell to stop growing. Since Rb is a nuclear protein, it may regulate a bank of responder genes including c-fos, c-myc, and TGF-βs whose expression somehow influences growth and differentiation. Therefore, an autoregulatory loop may be very important for Rb to maintain sufficient levels to transduce the negative growth signals efficiently. The finding that oncogenic germ line mutations in Sp1 and ATF sites in the human Rb gene also resulted in hereditary retinoblastoma because of a quantitative decrease in the expression of the Rb gene (Sakai et al., 1992) suggests that autoinduction of the Rb through an ATF site might be important for the biological function of Rb.

Acknowledgments—We thank M. Sporn, A. Roberts, and D. Romeo for discussions and reading of the manuscript. We also thank R. Allison and L. Mullen for oligonucleotide synthesis.

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