Characterization of the Human RB1 Promoter and of Elements Involved in Transcriptional Regulation

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Abstract
The retinoblastoma gene (RB1) is a recessive oncogene implicated in a number of human tumors. Although the RB1 gene is expressed in most proliferating cells, there is considerable evidence for the transcriptional regulation of this gene. Therefore, we have performed a detailed analysis of the regulatory elements in the promoter of the human RB1 gene. Deletion analysis of the 5' upstream region determined the location of the basal promoter to be between -208 and -179 nucleotides relative to the translational start. This region contains essential binding sites for the transcription elements ATF and SP1 and putatively important sites for E2F and steroid hormone responsiveness but no TATA or CAAT boxes. Primer extension and RNase protection analysis identified two initiation sites at -176 and -128 base pairs, both downstream of the promoter. Cotransfection experiments revealed repression of the RB1 promoter by its protein product p110RB1. This repression has been mapped to the core promoter region containing the E2F-binding site; however, this site is not required for autorepression.

Introduction
Loss of expression of functional RB1 gene product, p110RB1, results in a rare tumor of infants, retinoblastoma (1). Although most dividing cells contain a 4.7-kb RB1 transcript, there is evidence that RB1 expression is regulated at the transcriptional level, possibly by an autoregulatory feedback loop; a) mutant RB1 mRNA is difficult to detect in constitutional cells in the presence of functional p110RB1, whereas transcripts from the same mutant alleles are easily detected in retinoblastoma tumor cells in the absence of normal p110RB1 (2). We suggested that in the presence of normal p110RB1, there is minimal transcription from the RB1 gene, making it difficult to detect unstable mutant transcripts. In the absence of normal p110RB1, in retinoblastoma tumors, increased transcription of the RB1 gene allows accumulation of detectable levels of the unstable mutant mRNA; (b) it has been difficult to produce stable cell lines expressing p110RB1 under control of a strong heterologous promoters. Stable cell lines expressing p110RB1 under control of weak promoters can be produced, in which the level of p110RB1 may be below a critical threshold (3); (c) the RB1 promoter contains potential binding sites for four transcription factors with which p110RB1 may interact: E2F (4, 5), ATF-2 (6), SP1 (7), and the RCE binding protein (7); (d) Slack et al. (8) have shown that rapidly proliferating, undifferentiated embryonal carcinoma cells (P19) express very low levels of p110RB1 until they are induced to differentiate with RA when expression of p110RB1 increases 7-fold (8); and (e) Szekely et al. (9) have shown that during mouse embryogenesis RB1 expression is differentially regulated in different organ systems and between different cell types of the same tissue.

Understanding the mechanisms of transcriptional regulation of RB1 expression requires characterization of the promoter region and the transcriptional start sites. Initial sequencing of the 5' region of the gene did not reveal a TATA box to mark a possible promoter (10). By testing successive 5' deletions for promoter activity, Hong et al. (10) mapped the upstream boundary of the RB1 promoter to a 70-bp region located between -264 and -194 bp relative to the start codon (10). While characterizing genetic defects in RB1 in two families with low penetrance retinoblastoma, Sakai et al. (11) detected mutations in putative SP1 and ATF sites at -197 and -188 bp, which inactivated promoter activity. However, Hong et al. (10) reported a major transcription start site to be at -276 bp, which is unusual since transcription generally initiates downstream of promoters transcribed by RNA polymerase II (12). RNA polymerase II transcription of TATA-less promoters has been shown to initiate near SP1 sites with the GC-rich regions binding a factor, ETF, required to stabilize the initiation complex (13). The RB1 5' sequence contains two potential SP1 sites at -201 and -154 bp, both downstream of the published start site.

p110RB1 has been implicated in the transcriptional regulation of a number of genes including itself (14, 15). Pieterpol et al. (16) reported repression of the c-myc promoter by p110RB1 through a site bearing homology to a TGF-β control element (TCE), while Hamel et al. (15) found that c-myc repression was mediated by an E2F-binding site. The c-fos promoter required the RCE element to mediate p110RB1 repression. p110RB1 was found to regulate tran-
The transcription of IGF-II through interaction with the transcription factor SP1 (7), while activating transcription of TGF-β2 through ATF-2 (6).

We report here that the major elements of the RB1 promoter are contained within a 29-bp region including SP1, ATF, and E2F-binding elements and a potential HRE, and that the major transcription start sites are downstream of this region. We also show that, in transient expression experiments, p110KRF represses its own promoter and that this repression does not require a functional E2F-binding element.

**Results**

**Putative Transcription Elements of the RB1 5′ Sequence.**

The RB1 5′ upstream region contains other putative transcription elements in addition to the ATF and SP1 sites implicated by the mutations described by Sakai et al. (11). These elements include: a pair of 11-bp repeats which resemble a HRE of the thyroxin and RA family and E2F-binding sites (Fig. 1A). These elements and the SP1 and ATF sites are located between nucleotides -215 and -179 bp and are completely conserved in the murine and human promoters with the exception of the 5′ HRE which contains a single base difference (17). In the human RB1 promoter, there is a second potential SP1 site downstream at -154 bp, which is not conserved in the murine promoter. In addition, a pair of elements bearing homology to the RCE are located at -279 and -270 bp and are conserved between mouse and human.

**Identification of the Minimal RB1 Promoter Region.**

To accurately determine the location of the 5′ boundary of the minimal RB1 promoter, a series of RB1-CAT reporter constructs (Fig. 1A) were assayed for activity in RA-differentiated P19 cells. There was no decrease in promoter activity observed as 5′ sequence was deleted to -241 bp (Fig. 1B). Although deletion to -227 and -207 bp gave levels of CAT activity below the longer constructs, deletion of sequences downstream of -207 bp resulted in complete loss of activity, thus mapping the 5′ boundary to approximately -207 bp. In additional experiments with COS-1 cells, we used a number of RB1-luciferase reporter constructs to map both the 3′ and 5′ boundaries of the promoter (Fig. 2A). 3′ deletion of sequence upstream of -179 bp abolished activity, placing the 3′ boundary of the RB1 promoter at -179 bp. 5′ deletion of sequence downstream of -228 bp also resulted in loss of activity, supporting the results in P19 cells. These two series of experiments map the region essential for RB1 transcription to a 29-bp region between nucleotides -207 and -179 bp. To confirm the requirement for this region, we constructed a full length promoter containing an internal 17-bp deletion from -199 to -183 bp (pRBPSacCAT) which deletes the SP1-, ATF-, and E2F-binding sites. In RA-differentiated P19 cells, the deletion completely abolished activity, confirming the presence of essential promoter elements in this region (Fig. 1). An additional construct, pRBPE2CAT, containing the full length promoter but mutated in the putative E2F-binding element, showed full activity, indicating that the E2F-binding ele-
Fig. 2. Mapping the downstream boundary of the R1B1 core promoter by 3′ deletion. A, a genomic fragment of the R1B1 gene running from -677 to -36 bp was ligated upstream of the luciferase (Luc) reporter gene (RBPO.67Luc). Constructs with deletions of 5′ and 1′ promoter sequences were made from the original RBPO.67Luc plasmid. B, 2 µg of each construct was cotransfected with 1 µg of a transfection control plasmid (RSV-βgal) into COS-1 cells in triplicate. Cells were harvested on day 3 and assayed for Luc and βgal activity. Luc activity was normalized for transfection efficiency as described in Materials and Methods. Activity of RBPO.67Luc, which is designated as 100%, equaled 826 luciferase units.

ment was not required for basal transcription (Fig. 1). Taken together, these results indicate that the basal promoter is located in a 29-bp region located between -207 and -179 bp, which includes the ATF, SP1, 3′ HRE sites, and a nonessential, consensus E2F-binding motif.

**Determination of Transcriptional Start Sites.** Two previous studies (10, 18) have reported conflicting transcription initiation sites upstream and downstream of the promoter. Using 5′ nucleotide protection, Hong et al. (10) identified a start site at -276 bp. In contrast, T’Ang et al. (19) probed a Northern blot with a number of radiolabeled probes from the R1B1 5′ region to determine those able to hybridize to the 4.7-kb R1B1 transcript. They reported that a probe from -479 to -207 bp was unable to bind while probes spanning -479 to -80 bp and -244 to -80 bp detected a 4.7-kb transcript, indicating that the R1B1 transcript began downstream of -207 bp (19).

To resolve these conflicting data on the start site, we determined the transcription start by two techniques, RNase protection and primer extension. An antisense riboprobe from -228 to +170 bp was hybridized to poly(A)+ RNA from three cell lines. WIL2NS, a pre-B cell line, and HL-60, a myelocytic leukemia line, were chosen for their high expression of R1B1 transcript. The breast carcinoma line, MCF-7, was chosen for its low R1B1 expression. Hybridization to two sense probes of known structure, CD24/Hpal and RPSma/R1, gave protected fragments of the expected size (Fig. 3B, last 2 lanes). In all three cell lines, we observed two major protected fragments of 287 and 239 bp, corresponding to start sites at 150 and -102 bp, as well as three minor bands located between the major start sites. In the overexposed blot shown in Fig. 3B, it is possible to detect in WIL2NS and HL-60, a faint band of 339 bp corresponding to fully protected probe and indicative of a transcript initiating upstream of -202 (Fig. 3B). The minor bands located between 287 and 239 bp are seen to be 5-10-fold less intense than the two major bands in the MCF-7 lane. Shorter exposure times reveal the same relative intensity of bands in the HL-60 and WIL2NS lanes. The predominant start sites at -150 and -102 were shorter than expected based on previous work with the mouse promoter (17) and on the major sites identified by primer extension (see below; Fig. 4). All of the protected bands were 26 bp shorter than those generated by primer extension. Suspecting that this size discrepancy was due to digestion of a secondary structure of the probe at the 3′ end in exons 1 and 2, we constructed a second riboprobe from -276 to -58 bp, excluding this region. This probe produced specific bands corresponding to start sites at -176 and -128 with no signal at -276, although additional faint, nonspecific bands resulted due to secondary structure of the probe (data not shown).

To confirm the RNase protection results, a radiolabeled antisense oligonucleotide primer (R1B1) corresponding to nucleotide positions +1 to +29 bp was annealed to poly(A)+ RNA of the pre-B-cell line, WIL2NS, extended upstream with reverse transcriptase, and the run-off products were sized on a 6% sequencing gel. Fig. 4 shows the results of two experiments. Fig. 4C shows the entire region from -100 to -300. Two easily detected start sites were observed at -176 and -128 bp, confirming the results shown above in Fig. 3; no bands corresponding to a start at -276 were detected by primer extension. Fig. 4B shows a high resolution analysis of the start sites at -176 and -128. In addition to these major start sites, several potential minor start sites may exist as indicated by the weak bands shown in this figure. All start sites are consistent with the Northern blot analysis by T’Ang et al. (19). In both series of experiments, we have been unable to obtain evidence for the major start site at -276 previously reported by Hong et al. (10).

**The R1B1 Promoter Is Repressed by p110RB**. In a previous publication, we suggested that transcription of the R1B1 promoter may be autoregulated by p110RB, the production of the R1B1 gene (2). Hamel et al. (15) reported preliminary evidence in support of this hypothesis when they observed autorepression of the R1B1 promoter in RA-induced, differentiated P19 cells. To test the hypothesis that p110RB represses transcription of its own promoter, C3H/10T/1/2 cells were transfected with pRBP2.0CAT alone or with a p110RB expression vector. In order to eliminate possible cell cycle-dependent phosphorylation effects on the activity of p110RB, a modified protein, Δp34-HA, was used in place of the wild-type (20). This construct has been mutated at eight potential p34-H2 kinase sites and remains in a
hypophosphorylated state; the resulting mutant p110EIIa has enhanced ability to repress transcription of the EIIa
promoter (15). The full length RBP1 promoter was repressed
3-fold by Δp34-HA in C3H/10T1/2 cells (Fig. 5). It is impor-
tant to note that Δp34-HA had no effect on transcription of
β-gal driven by the pgk promoter, ruling out nonspecific
suppression of transcription by Δp34-HA.

To identify the region responsible for transcriptional re-
pression, we tested autorepression with the 5' deletion
constructs, pRBP0.28CAT and pRBP0.23CAT. Δp34-HA re-
pressed both constructs 3- to 4-fold, indicating that the
potential RCEs at -279 and -270 bp cannot be involved
and that the required region is downstream of -228 bp.
Since Hamel et al. (15) showed that Δp34-HA repression of
the c-myc promoter required the E2F-binding element, we
determined the role of the putative E2F-binding element in
RBP1 autorepression by creating a promoter construct mu-
tated in the E2F-binding element (RBP0.23ΔE2CAT, TTTC-
CCGC changed to TAAACGC). Mutation of the E2F-bind-
region increased the basal level of transcription of the

Fig. 3. Mapping of the RBP1 transcription initiation sites by RNase
protection. A, a radiolabeled antisense riboprobe was produ-
ced. In addition, two in vitro cold sense RNAs corresponding to
cDNA nucleotide positions -91 to +225 bp (CD24/HpaI), and
gene nucleotide positions -220 to +170 (RPSma/RI) were
produced. B, radiolabeled riboprobe was hybridized to 10 µg of
yeast RNA and 10 µg of poly(A)+ RNA from three cell lines,
WIL2NS, HL-60, and MCF-7, as well as in vitro sense RNAs CD24/
HpaI and RPSma/RI. After digestion by single-strand ribonu-
clases A and T1, protected probe fragments were sized versus DNA
and RNA ladders on a 6% denaturing acrylamide gel (L). Numbers
on the left refer to the calculated size of the protected RNA
bands, while numbers in parentheses refer to calculated start sites
relative to the translational start (+1), accounting for 26 bp di-
gested from the 3' end of fragments during the assay (see text). Numbers
on the right refer to the size of the protected control RNA fragments.
promoter construct but did not affect the ability of Δp34-HA to repress its transcription. This result suggests that RB1 autorepression is mediated through other elements, perhaps the SP1 or ATF sites. However, these elements are essential for basal transcription from the RB1 promoter (11, 17) and are difficult to test directly for their role in transcriptional repression.

Discussion

Characterization of the RB1 Transcription Unit. The differential phosphorylation of p110<sup>rb1</sup> during the cell cycle (21–24) suggests posttranslational regulation of RB1 gene function. As summarized in the “Introduction,” there is also evidence for transcriptional regulation of the RB1 gene. For example, the RB1 transcript and protein are barely detectable in the undifferentiated embryonal carcinoma line P19, but both rise rapidly during RA-induced differentiation to cells of the neuronal lineage, while there is no effect of RA on RB1 or p110<sup>rb1</sup> in the differentiation-defective line RAC65, which lacks functional receptors for RA (8). Slack et al. (8) also demonstrated that the RBP2.0CAT construct contains the elements required for the increased transcription response to RA; however, it is not yet clear whether the RA effect on the RB1 promoter is direct or indirect. These results, together with the results presented above, indicate that the regulation of the RB1 promoter plays an important role in development and in carcinogenesis.

We have mapped the boundaries of the RB1 promoter to a 29-bp region spanning −207 to −179 bp and containing a number of putative transcription elements. The critical role of this region was determined by both 5′ and 3′ deletions and was confirmed by an internal deletion. The results are compatible with earlier work by other groups (10, 11). Our study also complements the study of Sakai et al. (11) who found germline mutations in the ATF site and in the SP1 site in two families with low penetrance, heritable retinoblastoma. Our formal test of the promoter region and the transcriptional start sites indicates that the sites identified by Sakai et al. (11) are indeed within the major promoter of the RB1 gene. In studies on the murine promoter, we have also specifically confirmed the requirement of both the SP1 and ATF sites for full activity of the RB1 promoter (17).

Using two different techniques, RNase protection and primer extension, we detected major transcription start sites at −176 and −128 bp in three different cell lines derived from different tissues, consistent with the determination by

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Fig. 4. Mapping of the RB1 transcriptional initiation sites by primer extension. A, a radiolabeled antisense oligonucleotide corresponding to positions +1 to +29 relative to the ATG was hybridized to 10 μg RNA, WIL2NS poly(A)′ RNA, or an in vitro sense RNA control (CD24/Hpal) and extended as described in “Materials and Methods.” B and C, extension products from two different experiments were run on a 6% denaturing acrylamide gel (L) with sequence obtained by priming the CD24 cDNA construct (A) with the +1 to +29 antisense oligonucleotide. The figure indicates the major start sites at −176 and −128 nucleotides.
T'Ang et al. (19) of a start downstream of -207 bp. The finding that the major transcriptional start sites of the murine RB1 promoter correspond to the sites described in this communication for the human promoter (17) also supports our conclusion that -176 and -128 bp are the major start sites. The relative intensities of the major start sites at -176 and -128 and the three minor sites are constant in the three lines, indicating that there is no change in site usage during activated versus basal transcription. Because SP1 sites are often involved in transcription initiation in TATA-less promoters (13), it is important to note that there are conserved SP1 sites 20 and 21 bp upstream of the major transcription start sites reported here. We conclude that the minimal promoter element is a small region containing essential SP1 and ATF sites with transcription initiating 20-21 bp downstream of the SP1 site. In support of this conclusion is the finding that this region of the mouse and human promoters are exactly conserved (17).

Neither technique detected significant bands corresponding to a start site at -276 bp, as had been suggested by Hong et al. (10) using S1 protection, although RNase protection produced a weak signal consistent with a small amount of transcript initiating upstream of -228 bp; in addition, we have detected a very weak start site at -276 bp for the murine promoter (17). Because transcription starts downstream of Pol II promoters (12), a start site at -276 bp would imply a second promoter upstream. However, the absence of any SP1 sites upstream of -276 bp and the lack of homology beyond -235 bp between the murine and human genes (17) indicates this region is unlikely to contain important regulatory elements. In addition, deletion of sequences upstream of -280 (Fig. 1) had no detectable effect on promoter activity.

**Autorepression of the RB1 Promoter.** Hamel et al. (15) recently reported transcriptional repression by p110RB1 of EllaE, the adenovirus early promoter, the c-myc promoter and the RB1 promoter. For both c-myc and EllaE promoters, high level basal expression and repression by p110RB1 requires an intact E2F-binding site. These observations are consistent with previous studies showing that p110RB1 often exists in a complex with E2F (25). In the current study, we show that the RB1 gene may regulate its own transcription by repression but by a mechanism not involving E2F. The primary region of the RB1 promoter involved in this repression has been mapped by deletion analysis to be downstream of -228 bp. The construct deleted to -228 bp gives full transcriptional activity and was repressed 3-fold by exogenously expressed p110RB1, indicating that the potential pair of RCEs located at -279 bp are not required for autorepression. Surprisingly, mutation of the E2F-binding site did not eliminate autorepression in CHF10T1/2 cells, and we conclude that p110RB1 does not repress its promoter through either an E2F-binding element or an RCE. These results were also observed using differentiated P19 cells. However, the E2F-binding site may play a role in regulation of RB1 transcription since deletion of this site reproducibly increased the basal level of transcription.

Other investigators have identified additional elements through which p110RB1 may regulate transcription. Repression of the c-myc promoter by p110RB1 in keratinocytes occurred through a TGF-β responsive element located upstream of the P1 start site (16). In addition to interacting with E2F, p110RB1 has been shown to bind the c-myc protein (26) and myoD (27), suggesting the possibility that p110RB1 is involved in multiple transcriptional regulatory pathways. Kim et al. (6, 7) have demonstrated an interaction between p110RB1 and the transcription factors SP1 and ATF2 which are involved in transcriptional regulation of the IGF-II and TGF-β promoters, respectively. Given the importance of the SP1 and ATF sites in the RB1 promoter (17), they must be considered possible targets for autorepression. However, because the SP1 and ATF sites are essential for basal transcriptional activity, we were unable to test the role of these sites by direct mutational analysis. Recently Shan et al. (28) demonstrated that transcription of the RB1 promoter increased approximately 10-fold following transient expression of exogenous E2F. This activation, which appears to be mediated by the potential E2F-binding site, is partially suppressed by coexpression of p110RB1. However, mutation of this site resulted in activity equal to that of the wild-type promoter in the absence of exogenous E2F, similar to the results obtained in this study. In addition, while Shan et al.

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5 R. M. Gill, unpublished data.
(28) were able to demonstrate repression of the RB1 promoter, they have not shown that mutation of the E2F-binding element eliminates RB1 autorepression in the presence or absence of excess E2F.

Materials and Methods

Cell Culture. Mouse P19 embryonal carcinoma cells were cultured in Iscove’s media containing 7.5% bovine serum and 2.5% fetal calf serum. P19 cells were induced to differentiate for 6 days with 300 nM RA as described (29). MCF-7, a human breast carcinoma (30), was cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. HL-60, a human myelocytic leukemia line (31), and WIL2NS, a human pre-B-cell line, (32) were cultured in RPMI 1640 containing 10% fetal calf serum. COS-1 cells (33) were cultured in Dulbecco’s modified Eagle’s medium containing 10% bovine calf serum. C3H/10T1/2 (34) cells were cultured in a minimal essential medium containing 10% fetal calf serum.

RB1-CAT Expression Vectors. A BamHI/EcoRI genomic fragment of the RB1 gene corresponding to -1.9 to +2.0 kb relative to the start of translation was ligated into pGEM7. A BamHI/XhoI fragment from the resulting plasmid was ligated into the BamHI/XhoI sites of the pTKCAT expression vector, replacing the TK promoter with the RB1 5′ upstream region plus exon 1 and part of intron 1. Exon 1 and intron 1 sequences were removed by cutting with BssHII (site at -56) and XhoI and religating. The resulting vector, pRB2.0CAT, was mutared by a series of 5′ deletions by cutting with specific restriction enzymes, blunt-ending, and religating. All 5′ deletions are named according to the number of kilobases retained upstream of the ATG start codon. An additional construct with a 24-bp internal deletion (pRBPSaccAT) was made by cutting pRB2.0CAT with SacII (sites at -205 and -182) and religating. An E2F-binding element mutant (pRBPl2CAT) was made by replacing the SacI1 fragment of pRB2.0CAT with a pair of oligonucleotides containing a 3-bp change in the E2F-binding element, mutating it from TTTCGGGC to TAAAAGCC. One 5′ deletion, RBp.28CAT, was made by replacing the sequence upstream of the Apal site at -232 bp with a pair of synthetic oligonucleotides corresponding to sequence from -280 bp to the Apal site. The structure of all plasmids was confirmed by sequencing.

RB1-Luc Expression Vectors. A DpnI/BssHII genomic fragment of the RB1 gene corresponding to -680 to -56 kb relative to the start of translation was ligated into the HindIII/BglII sites of the pTKLuc expression vector, replacing the TK promoter with the RB1 5′ sequences. In the resulting vector, pRBp.67Luc, 5′ and 3′ deletions were created by cutting with specific restriction enzymes, blunt-ending, and religating. The structure of all constructs was confirmed by sequencing.

RNase Protection. A Smal genomic fragment of the RB1 gene spanning from -228 to +170 bp was subcloned into the pGEM7 vector (RBP5mal) and linearized with HindIII. A radiolabeled antisense riboprobe was made from the T7 promoter and purified on a 6% denaturing acrylamide gel. An aliquot of probe containing 10^6 cpm was hybridized with 10 μg poly(A)^+ RNA in 80% formamide at 60°C for 20 h, and annealed RNA was digested with 50 units RNase T1 and 2.5 μg RNase A for 1 h at 29°C. The size of the resulting protected fragments was determined on a 6% denaturing acrylamide gel beside a DNA ladder made by priming the

RBP0.18CAT plasmid with an antisense oligonucleotide corresponding to nucleotides -111 to -86 bp, using a DNA to RNA conversion factor of 0.92. The calculated size of fragments produced from cellular RNA hybridizations do not include 26 bp, which is digested from the 3′ end of exon 1. The probe was also annealed to two unlabeled control sense RNAs; RBP5mal/R1 sense RNA was made from the SP6 promoter of the plasmid RPSmal linearized with EcoRI, and CD24/HpA1 was made from the T7 promoter of HpAl-linearized CD24 plasmid containing RB1 cDNA from -91 to +225 bp cloned into pGEM7.

Primer Extension. Total cellular RNA was isolated from MCF-7, WIL2NS, and HL-60 cell lines by the guanidium isothiocyanate method as described (4). Poly(A)^+ RNA was purified by oligo-dT chromatography. A 5′ end-labeled oligonucleotide (RB^+1), corresponding to nucleotide positions +1 to +29 bp, was annealed to 10 μg of either trNA, WIL2NS poly(A)^+ RNA, or an in vitro sense control RNA initiating at -120 bp (CD24/HpAl) and extended with Superscript RNase H^- reverse transcriptase (Bethesda Research Laboratories). The resulting labeled DNA fragments were separated on a 6% denaturing acrylamide gel beside a sequencing ladder generated from the CD24 plasmid (RB1 cDNA from -91 to +225 bp cloned into pGEM7) primed with an oligonucleotide corresponding to sequence from +30 upstream to +1 bp.

Transfections and Assays. Using the calcium phosphate method, 2.0 μg of RB1-CAT reporter were cotransfected with 5 μg of plasmids expressing a functional but phosphorylation-defective p110^pho(Bp34) (20) or unrelated plasmid DNA and 3 μg of a β-gal expression vector (pgk-βgal) to monitor transfection efficiency. The recipient cells were C3H/10T1/2 cells plated 24 h previously at 2–3% confluence. Transfections were performed in triplicate at least twice. Forty-eight h following transfection, cells were harvested and lysed by a freeze-thaw procedure. Lysates were assayed for CAT activity using the quantitative ethyl acetate extraction procedure (35) and for β-gal activity. COS-1 cells were cotransfected with 10 μg of RB1-Luc reporter and 2 μg of a β-gal expression vector (RSV-βgal) by the lipofectin procedure. Cells were harvested 48 h after transfection and assayed for luciferase and β-gal activity. RA-treated P19 cells were cotransfected with 0.5 μg of RB1-CAT reporter with 3 μg of β-gal expression vector (pgk-βgal) by the calcium phosphate method. CAT and luciferase assays were evaluated in their linear ranges. CAT assays always converted less than 20% chloramphenicol, and luciferase assays did not exceed 1000 units. CAT and luciferase activities were normalized to β-gal activity to correct for differences in transfection efficiency, and results are presented as a ratio of CAT or luciferase activity, divided by β-gal activity. Results presented in Fig. 1 are a combination of two experiments with promoter activity normalized to full length (RBP2.0CAT) in each case.

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References


