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# Differential Roles of Two Tandem E2F Sites in Repression of the Human p107 Promoter by Retinoblastoma and p107 Proteins

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**Although many lines of evidence indicate that the cellular protein p107 is closely related to the retinoblastoma protein, the exact function of the p107 gene and its regulation are presently not known. To investigate the molecular mechanism controlling expression of the human p107 gene, a 5′ flanking sequence of this gene was isolated and shown to promote high-level expression of a luciferase reporter gene in cycling human 293 and Saos-2 cells. Sequencing and transcription mapping analyses showed that the human p107 promoter is TATA-less and contains a tandem, direct repeat of E2F-binding sites, with the 3′ copy overlapping the major transcription initiation site. Deletion analysis of the p107 promoter showed that a promoter DNA fragment containing only the two E2F sites together with the leader sequence could direct relatively efficient expression in 293 cells. Site-directed mutagenesis of these E2F sites revealed that although both sites were important for p107 promoter activity, mutation on the proximal, initiation site copy of the E2F site showed a stronger effect. The human p107 promoter could be repressed by the retinoblastoma protein and its own gene product. Interestingly, the repression was found to be mediated through the 5′ copy of the E2F site. These studies demonstrate for the first time differential roles of two tandem E2F sites in promoter regulation.**

It is known that eukaryotic cell cycle progression is regulated at several restricted points by both positive and negative factors. Perturbation of the expression of these factors may lead to uncontrolled growth and ultimately to cancer (for a recent review, see reference 17). One of the best-studied negative regulators is the product of the retinoblastoma susceptibility (Rb) gene (22, 23, 47), which predisposes to human retinoblastoma (44). The retinoblastoma protein (pRb) is a 105- to 110-kDa nuclear phosphoprotein (48) with tumor suppressor function (4, 32) and is believed to be a negative growth regulator (24, 63).

One of the major advances in understanding the Rb gene function was the finding that pRb is a target for the oncogene products of the small DNA tumor viruses. In cells transformed by these viruses, the adenovirus E1A protein, the simian virus 40 (SV40) large T antigen, or the human papillomavirus E7 protein forms a protein complex with pRb (10, 14, 57, 78). Genetic analysis of these viral oncoproteins showed that the regions required for binding to pRb are also required for transformation. These observations led to the hypothesis that transformation by these three groups of DNA tumor viruses may have a common mechanism, i.e., to inactivate the cellular suppressors by physical complex formation with their transforming proteins (17).

Recently, another nuclear phosphoprotein, termed p107, was found to have many features similar to those of pRb (19, 83). The p107 protein was first identified as one of the cellular proteins associated with E1A and T antigen (14, 18, 27, 80). As for pRb, the regions on the viral oncoproteins required for transformation are needed for binding to p107 (16, 38, 78). By cDNA cloning, Ewen et al. (19) found that the amino acid sequence of p107 has homology with that of pRb. The homology regions in pRb and p107 can independently bind to E1A or T and are essential for their growth suppression activities (31, 33, 38, 83). These common structural, biochemical, and biological features indicate a close relationship between p107 and pRb.

One of the biochemical mechanisms for the antiproliferative activities of pRb and p107 is believed to be mediated through the interaction with the E2F family of transcription factors (2, 3, 6, 7, 28, 39, 50, 68). Both pRb and p107 can bind to E2F and inhibit its transcription activation activity (15, 20, 29, 49, 67, 70, 77, 82). These interactions may account for the tight cell cycle-regulated transcription of several genes containing E2F-binding sites in their promoters (8, 12, 26, 30, 37, 51–53, 56, 58, 60, 61, 72).

Although numerous studies have focused on the function of p107 and its link to tumorigenesis, very little is known about the regulation of p107 gene expression. As a first step toward elucidating the mechanism controlling expression of the p107 gene, we have undertaken a direct cloning approach. In this report, we describe the isolation of a genomic clone covering the 5′ regulatory region, the first exon, and part of the first intron of the human p107 gene. We show that multiple sequences, including two tandem copies of the E2F-binding sites, one of which is located at the region overlapping the major transcription initiation site, are required for full promoter activity. By site-directed mutagenesis of these two E2F-binding sites on the human p107 promoter, we identify differential roles for these two sites in promoter expression and repression by pRb and its own gene product.

## MATERIALS AND METHODS

**Isolation of the human p107 genomic DNA sequence.** The full-length human p107 cDNA plasmid pBSKp107 (83) was kindly provided by Ed Harlow and Liang Zhu, Massachusetts General Hospital Cancer Center. A 933-bp 5′ p107 cDNA fragment was obtained by digestion of plasmid pBSKp107 with enzymes *Eco*RI and *Bsm*I. This cDNA fragment was labeled with [α-$^{32}$P]dCTP by the random primed labeling method (65) and used in screening approximately $10^6$ phages of a human lymph node genomic DNA library in vector λ Charon 4A,

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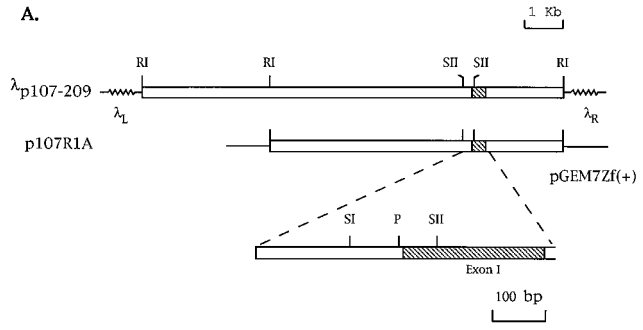


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-284  CCGCGGGCTT CATCTCCCAA GAGATGGACT ACACCTCCCA GCAGGCTCTG
-234  CGCGCGGGCT GAGGATCCCT CCGCTCTTTT TCTGTCCCGC CGGCTGGGCC
-184  CCCCGCGACC AGCCAAGGGC CAAGGACAGG TCTTTCAGAA TCTGAGGTAC
-134  ATCTTCTTAT CACATTTCCG GGGAGGGACT GCTAGGAGCT CCGGAGGAAA
 -84  AACGGACTTT TTTTGAGGAG AAAAGCGGAG GCAGACGGTG GATGACAACA
 -34  CGTCCCGCAG CTGCAGATTT TCGCGCGCTT TGGCGCAGGT GGTTGTGGGT
                                  +1
 +17  AGCGCGCCTG GGAGGGAGAA AGAAGTCGGG GGCCGTGGCG CGCAGCCCGC
 +67  GGGGCCTGAA GGGATGTTCG AGGACAAGCC CCACGCTGAG GGGGCGGCGG
+117  TGGTCGCCGC AGCCGGGGAG GCGCTACAGG CCCTGTGCCA GGAGCTGAAC
+167  CTGGACGAGG GGAGCGCGGC CGAAGCCCTG GACGACTTTA CTGCCATCCG
+217  AGGCAACTAC AGCCTAGAGg tgagcggc......
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FIG. 1. Diagram and sequence of the 5′ region of the human p107 gene. (A) Maps of the p107 genomic clone and its subclone containing the 5′ flanking sequence, the first exon, and a portion of the first intron. Abbreviations: RI, *Eco*RI; SII, *Sac*II; SI, *Sac*I; P, *Pst*I; $\lambda_R$, right arm of lambda DNA; and $\lambda_L$, left arm of lambda DNA. (B) Genomic DNA sequence of the p107 5′ flanking region. The nucleotide sequence of the p107 DNA from the 5′ upstream *Sac*II site to the beginning of the first intron is shown. The major transcription initiation site is indicated as an arrow and designated position +1. The sequences of the two tandem E2F-binding sites located at −17 to −9 and −6 to +2 are shown in boldface. The translation ATG start codon is boxed. The sequence of the first exon is underlined, and the sequence of the first intron is shown in lowercase letters.

obtained from the American Type Culture Collection (ATCC 57760). Duplicated phage lift filters were prepared from each dish, and the hybridization was performed in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate (SDS)–10% dextran sulfate–50% formamide at 42°C. After hybridization, the filters were washed twice in 2× SSC at room temperature for 15 min each time and then in 0.2× SSC–0.5% SDS at 65°C for 30 min. The hybridization signals were visualized by autoradiography.

Three positive plaques identified on duplicate filters were purified through additional rounds of screening. The DNAs from these positive phages were isolated by proteinase K treatment (65) and used in Southern blot analysis with a 73-bp *Taq*I-digested fragment of the very 5′ end of the human p107 cDNA as a probe. Two of the phages gave rise to positive hybridization signals and were confirmed to contain the 5′ flanking region of the p107 gene by sequencing. Restriction mapping experiments showed that these two phages contain overlapping p107 genomic DNA. One of these clones, designated λp107-209 (Fig. 1A), containing a larger DNA insert was chosen for further study.

**Subcloning and sequencing of the 5′ flanking region of the p107 gene.** Two *Eco*RI-digested genomic DNA insert fragments of about 7 and 3 kb were subcloned from the λp107-209 clone. Hybridization analysis showed that the 7-kb genomic DNA fragment-containing subclone, designated p107R1A, harbored the 5′ flanking region of the human p107 gene. Restriction analysis of this clone identified two *Sac*II-cutting sites on the 7-kb genomic DNA. Since the p107 cDNA sequence contains a unique *Sac*II site at the 5′ untranslated region, this 7-kb genomic DNA fragment was further subcloned by partial digestion with *Sac*II. All of the subclones were sequenced by the dideoxynucleotide chain termination method (66), and their sequences were compared with the p107 cDNA sequence (83). In addition, oligonucleotides with p107-specific sequences were synthesized and used in the sequencing reaction in order to obtain the complete 5′ flanking sequences of both strands of the p107 genomic DNA.

**Primer extension and RNase protection experiments.** Cytoplasmic RNA was isolated from human HeLa or 293 cells as described by Sambrook et al. (65). For the primer extension experiment, an avian myeloblastosis virus reverse transcriptase primer extension system (Promega) was used. Briefly, oligonucleotide p107-4, with the antisense-strand sequence from +62 to +32 relative to the major transcription start site mapped (5′-GCTGCGCGCCACGGCCCCCGACTTCTTTCTC-3′; Fig. 1), was end labeled with [$\gamma$-$^{32}$P]ATP and hybridized with 5 μg of cytoplasmic RNA at 42°C overnight. The primer-annealed RNA was converted into cDNA by avian myeloblastosis virus reverse transcriptase in the presence of 5 mM deoxynucleotides at 42°C for 45 min. The cDNA product was then analyzed on a 7.5% sequencing gel containing 8 M urea. Oligonucleotide p107-4 was also used in sequencing the p107R1A DNA to generate the sequencing ladder for reference.

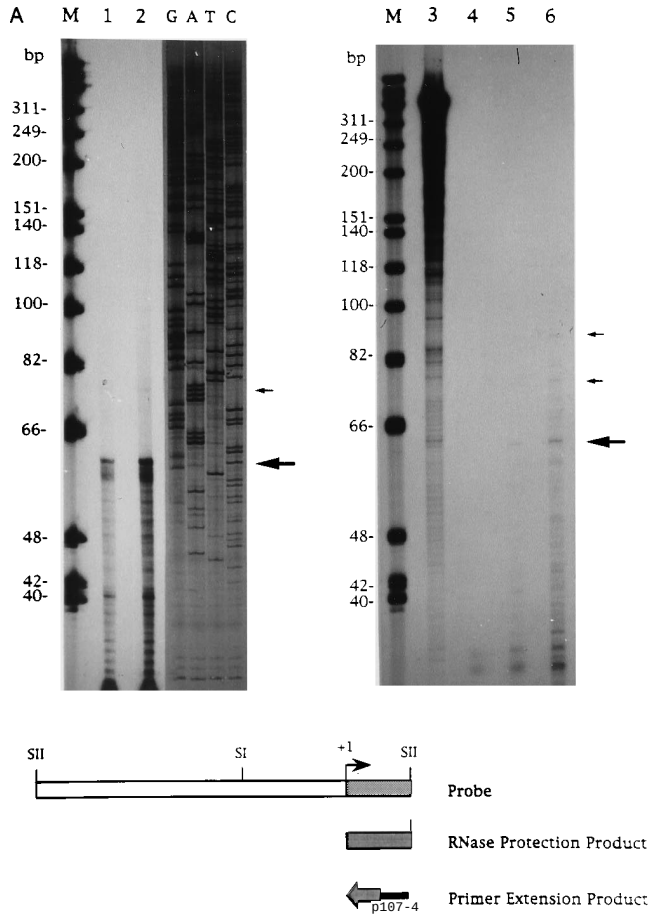
For the RNase protection experiment, a 346-bp *Sac*II fragment containing a portion of the first exon and sequence 5′ upstream of the p107 gene was subcloned into pKS(+) (Stratagene) and used to produce an antisense RNA probe, using a MAXIscript in vitro transcription kit (Ambion) in the presence of [$\alpha$-$^{32}$P]UTP. This $^{32}$P-labeled riboprobe was gel purified and used to hybridize with 10 μg of cytoplasmic RNA, using an RPA II RNase protection assay kit (Ambion). The final protected product was analyzed on a 7.5% sequencing gel. To map the 5′ end of transiently expressed p107-LUC mRNA, 293 cells were mock transfected or transfected with the p107 promoter construct with or without the pRb expression vector. At 48 h after transfection, transfected cells were harvested, cytoplasmic RNAs were prepared, and the same amount of RNA was used in the RNase protection analysis as described above.

**p107 promoter fusion constructs, mutant derivatives, and expression plasmids.** A 5.2-kb *Eco*RI-*Sac*II fragment of the human p107 promoter DNA was subcloned from plasmid p107R1A (Fig. 1A) and ligated with the firefly luciferase expression cassette in plasmid pGL2-Basic (Promega), generating the p107P5200-LUC construct (see Fig. 3A). Several promoter deletion clones were generated by dropping out the restriction fragments, using the unique restriction sites in the p107P5200-LUC DNA. One of these deletion clones, p107P19-LUC, contains only the two tandem copies of E2F-binding sites and the 5′ leader sequence of the p107 gene fused with the luciferase expression cassette. Two further deletion clones, p107P8-LUC, containing one copy of the E2F site, and the p107PI-LUC, containing the major initiation site sequence, were constructed by PCR (35). The deletion endpoint in each clone was confirmed by DNA sequencing.

Site-directed mutagenesis of the two tandem E2F-binding sites in the p107P19-LUC DNA was carried out by a PCR-based method. The primers to generate specific point mutations in the E2F sites were as follows: mutation in the 5′ copy of the E2F site (M1), 5′-GCAGCTGCAGA**TTTTCGAA**CGC**TTTGGCGC**AGGTGG-3′; mutation in the 3′ copy of the E2F site (M2), 5′-GCAGCTGCAGA**TTTTCGCGC**GC**TTTGGAA**CAGGTGGTTGTGGGTAG-3′; and mutations in both copies of the E2F sites (M12), 5′-GCAGCTGCAGA**TTTTCGAA**CGC**TTTGGAA**CAGGTGGTTGTGGGTAG-3′. The sequences in boldface represent the two E2F-binding sequences in the p107 promoter; the nucleotides underlined represent those which had been changed. The other primer used for PCR was the GL2 primer (Promega) with the sequence in the luciferase coding region. The p107P19-LUC DNA was used as the template for the PCR. The resulting PCR products were digested with *Pst*I and *Sac*II so that the DNA endpoints were identical to that of the p107 DNA in plasmid p107P19-LUC and then rebuilt in plasmid pGL2-Basic. The appropriate mutations created in these clones were also confirmed by sequencing.

The p107 or pRb expression plasmid was constructed by inserting either the full-length p107 cDNA (83) or the full-length Rb cDNA (our unpublished data), respectively, under the control of the human cytomegalovirus immediate-early promoter (41). The $pRb_{\Delta N}$ mutant was generated by digesting the Rb cDNA with *Acc*I (nucleotide position 1380 [48]) and *Nco*I (nucleotide position 1680), filling ends with the Klenow fragment of *Escherichia coli* DNA polymerase I, and then ligating the ends with an 8-mer *Sma*I linker (New England Biolabs) to create an in-frame deletion. The $p107_{DE}$ mutant was created by in-frame deletion of the sequence between the *Dra*III (nucleotide position 1287 [83]) and *Eco*RI (nucleotide position 2541) sites as described previously (83).

**Cell culture, transfection, and reporter enzyme assays.** The human osteosarcoma cell line Saos-2 (American Type Culture Collection) was grown in McCoy's 5a medium supplemented with 10% fetal bovine serum. The adenovirus-transformed human embryonic kidney cell line 293 was grown in Dulbecco's modified Eagle's medium containing 10% serum. Transfection assays were performed by the calcium phosphate precipitation method (25). Briefly, actively growing cultures were split at 20 to 24 h before transfection. Prior to transfection, the culture medium was exchanged with fresh Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The human p107 promoter-driven luciferase constructs (2 μg) were cotransfected with a control vector or an Rb or p107 expression plasmid (18 μg) into 293 or Saos-2 cells. Calcium phosphate-DNA coprecipitate mixtures were prepared and added to cells in the presence of medium. Following overnight incubation, the medium was replaced with fresh growth medium. At 48 h after transfection, cells were harvested and lysed in reporter lysis buffer (Promega). The protein concentration of the cell extract was determined by using the Bio-Rad protein assay dye reagent, and the same amount of the extract protein was used in each reporter enzyme assay. The luciferase activity was measured by using a luciferase assay system (Promega) in a Lumat LB9501 luminometer (Berthold), and the β-galactosidase activity was measured by using a Galacto-Light chemiluminescent reporter assay (Tropix). For all transfection assays, at least three independent experiments were performed. In addition, plasmid pSV2-βgal was included in the transfection experiments as an internal control for transfection efficiency.



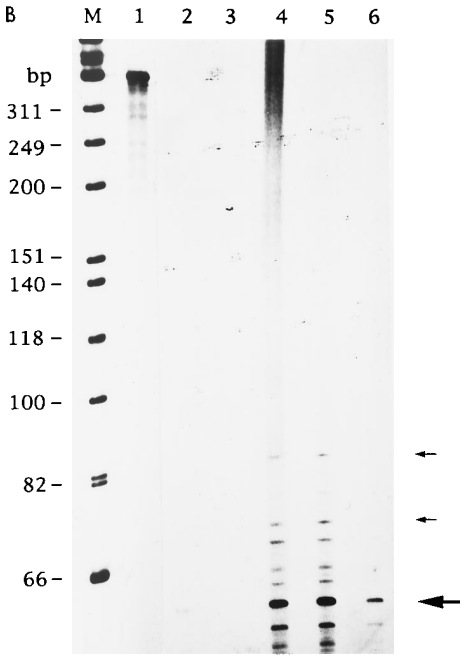




FIG. 2. Transcription mapping of the 5′ end of p107 mRNA. (A) Mapping of the 5′ end of endogenous p107 mRNA by primer extension (left panel) and RNase protection analysis (right panel). The diagram shows the RNase protection probe derived from the *Sac*II (SII) fragment of the p107 5′ flanking DNA. The relative locations of the major (large arrow) and minor (small arrows) RNase protection products and primer extension products are also shown. RNAs from both HeLa cells (lanes 1 and 5) and 293 cells (lanes 2 and 6) were used in the experiments. Yeast RNA was used as a negative control (lane 4). The riboprobe used in the RNase protection experiment is shown in lane 3. An arrow marks the major primer extension product or RNase protection product. The sequence ladders were obtained from sequencing of the p107P5200-LUC DNA with the p107-4 oligonucleotide as a primer. Molecular weight markers of *Hae*III-digested ϕX174 DNA (lane M) were used as relative size references. (B) Mapping of the 5′ end of transiently expressed p107 mRNA by RNase protection analysis. The RNase protection probe (lane 1) used for panel A was used in protection of RNA of mock-transfected 293 cells (lane 2), yeast RNA (lane 3), or RNA from 293 cells transfected with p107P280-LUC (lane 4), p107P5200-LUC (lane 5), or p107P5200-LUC plus the pRb expression plasmid (lane 6).

**Band shift experiments.** A series of double-stranded oligonucleotides derived from the human p107 promoter sequence was synthesized. The p107-E2F-12 (40-mer) oligonucleotide with the sequence 5′-GCAGCTGCAGA**TTTTCGCGC**GC**TTTGGCGC**AGGTGGTTGT-3′ contains the two tandem E2F-binding sites (shown in boldface) from the human p107 promoter (nucleotide positions −28 to +12; Fig. 1B). The p107-E2F-M1 mutant oligonucleotide contains a two-nucleotide mutation in the 5′ copy of the E2F site (5′-GCAGCTGCAGA**TTTTCGAA**CGC**TTTGGCGC**AGGTGG-3′), the p107-E2F-M2 oligonucleotide contains the mutation in the 3′ copy of the E2F site (5′-GCAGCTGCAGA**TTTTCGCGC**GC**TTTGGAA**CAGGTGGTTGTGGGTAG-3′), and the p107-E2F-M12 oligonucleotide contains mutations in both copies of the E2F sites (5′-GCAGCTGCAGA**TTTTCGAA**CGC**TTTGGAA**CAGGTGGTTGTGGGTAG-3′). The underlined nucleotides represented those which had been changed. Mutation of the underlined CG dinucleotide of the E2F site has been shown to abolish E2F binding (68, 79). Two 21-mer oligonucleotides, one (p107-E2F-1; 5′-TGCAGA**TTTTCGCGC**GCTTTG-3′; nucleotide positions −23 to −3; Fig. 1B) containing the 5′ E2F-binding site and the other (p107-E2F-2; 5′-CGCGCGC**TTTGGCGC**AGGTGG-3′; nucleotide positions −13 to +8; Fig. 1B) containing the 3′ E2F-binding site, were also prepared. As a well-defined E2F site competitor DNA, a 31-mer oligonucleotide (AdE2F-1; 5′-AAGGAC TAG**TTTCGCGC**CCTTTCTCAATTT-3′) containing the E2F-binding sequence from the adenovirus E2 promoter (45, 72) was used. In addition, a YY1-binding-site-containing oligonucleotide derived from the adeno-associated virus P5+1 sequence (69) was used as a nonspecific competitor. The oligonucleotide probes were phosphorylated with T4 polynucleotide kinase and [$\gamma$-$^{32}$P]ATP (65). A nuclear extract of HeLa cells was prepared as described by Dignam et al. (11). Band shift and competition assays (21) were performed as described by O'Connor and Hearing (59). DNA-protein complexes were separated from free DNA by electrophoresis in a 4% 30:1 (acrylamide-bisacrylamide) polyacrylamide gel run in 0.25× Tris-borate-EDTA at 4°C. For antibody perturbation experiments, 1 or 3 μl of a polyclonal rabbit antiserum against E2F (sc-512X; Santa Cruz Biotechnology, Inc.) or YY1 transcription factor (our unpublished data) was added to the reaction mixture described above, and the mixture was incubated overnight at 4°C prior to electrophoresis.

## RESULTS

**Cloning of human p107 genomic DNA containing putative promoter sequences.** To study the regulation of human p107 gene expression, we have begun to clone its promoter DNA sequence. Three independent phage clones containing genomic inserts that hybridized to a 933-bp 5′ p107 cDNA fragment (83) were isolated from a human lymph node genomic DNA library. Southern blot analysis of the phage DNAs showed that two of these clones hybridized to a 73-bp fragment of the very 5′ end of the p107 cDNA. Restriction mapping and sequencing experiments showed that these two clones contain the 5′ flanking sequence of the human p107 gene (Fig. 1). One of these clones with a longer 5′ flanking sequence, designated λp107-209 (Fig. 1A), contains a genomic insert of about 10 kb. A subclone, p107R1A, containing a 7-kb *Eco*RI fragment from the λp107-209 clone, was found to contain the 5′ end of the p107 gene. Figure 1B shows that the DNA sequence from this genomic clone matches the 5′ cDNA sequence (83) to a position 157 nucleotides 3′ of the putative ATG initiation codon and then diverges. The binucleotide sequence flanking the divergence is 5′-AGgt-3′, a perfect match to the consensus exon-intron splicing junction sequence. These results suggest that it is the end of an exon (Fig. 1B). An oligonucleotide with the cDNA sequence located immediately downstream from this exon was synthesized and used in sequencing the p107R1A

clone. No sequence was obtained, indicating that this clone does not contain any other exons of the human p107 gene.

**Identification of the human p107 promoter.** As a first step toward characterizing the p107 promoter, we carried out both primer extension and RNase protection analyses to determine the position of transcription initiation. A 31-mer oligonucleotide, p107-4, containing the antisense-strand sequence 18 to 48 nucleotides upstream from the putative ATG codon, was used in primer extension of RNAs from HeLa and 293 cells. In comparison with the sequence ladders and molecular weight markers, the major extension product was 62 nucleotides long (Fig. 2A, lanes 1 and 2). This product reached the sense-strand nucleotide G (corresponding to a C of the antisense sequence ladder) 79 nucleotides upstream of the ATG codon (Fig. 1B). The same size for this extension product was obtained in RNAs from HeLa and 293 cells (Fig. 2A, lanes 1 and 2). In addition, at least one minor extension product with a larger size was detected.

To confirm the primer extension result, RNase protection analysis was performed. A 350-bp *Sac*II fragment containing the 5′ end of the p107 gene was subcloned into pKS(+) and used in preparing an antisense probe for RNase protection analysis of RNAs from HeLa cells (Fig. 2A, lane 5) and 293 cells (Fig. 2A, lane 6). A major protected fragment with a size corresponding to a position similar to that mapped by the primer extension analysis was detected. Two slightly larger minor protected bands also were detected. These results are consistent with those of the primer extension analysis and suggest that the p107 gene is transcribed from multiple start sites. We have designated the major transcription initiation site mapped from these analyses as position +1. By reference to this position, the 5′ end of the cDNA isolated by Zhu et al. (83) begins at position +13.

The 284-bp sequence immediately upstream of the major transcription initiation site has a G+C content of 60%. There is no consensus TATA box around the −30 region relative to the major transcription initiation site. Several potential transcription factor-binding sites were identified by their similarities to consensus sequences for known transcription factor-binding sites (Table 1). These include an AP-2 transcription factor-binding site, a common factor 1-binding site, two binding sites for E2-box-binding factor class A (E2A), two E2F transcription factor-binding sites, two GC factor-binding sites, a binding site for metal regulatory element-binding factor 1, a binding site for the erythroid cell-specific nuclear factor NF-E1, a binding site for the Ets family transcription factors PU.1 and PEA3, an Sp1 transcription factor-binding site, and a binding site for the T-cell-specific transcription factor LEF/TCF-1. Of particular interest, the two E2F-binding sites are located right at or immediately upstream of the major transcription initiation site. In addition, there are two E2A-binding sites flanking each side of the E2F-binding sites (Fig. 1B and Table 1).

**Multiple DNA sequences, including two potential E2F-binding sites, are required for full promoter activity.** To test the promoter activity of the 5′ flanking sequence of the human p107 gene, the p107P5200-LUC vector, containing 5.2-kb 5′ flanking DNA (−5200 to +64 relative to the major transcription initiation site) fused with the luciferase reporter gene, was constructed and used in transfection of the adenovirus-transformed human embryonic kidney cell line 293 and the $Rb^-$ human osteosarcoma cell line Saos-2. Figure 3 shows that this 5.2-kb p107 DNA could promote high-level expression of luciferase activity in actively growing 293 cells. The activity of this p107 promoter DNA was about eightfold higher than SV40 promoter activity in 293 cells (compared with the activity of pGL-Control in Fig. 3). However, it was about 30% of the SV40 promoter activity in Saos-2 cells.

TABLE 1. Putative regulatory elements found in the 5′ flanking region of the human p107 gene[a]

| Factor | Sequence[b] | No. of binding sites | Location | Reference(s) |
|---|---|---|---|---|
| AP-2 | CCSCRGGC | 1 | | 55 |
| | CCGCGGGC | | −284 to −277 | |
| Common factor 1 | ANATGG | 1 | | 64 |
| | AGATGG | | −263 to −258 | |
| E2A | CAGNTG | 2 | | 54 |
| | GCAGCTG | | −28 to −22 | |
| | GCAGGTG | | +1 to +7 | |
| E2F | TTTSSCGC | 2 | | 45, 71 |
| | TTTCGCGC | | −16 to −9 | |
| | TTTGGCGC | | −6 to +2 | |
| GC factor | SCGSSSC | 2 | | 40 |
| | GCGCGCC | | +18 to +24 | |
| | GCGGGGC | | +65 to +71 | |
| Metal regulatory element-binding factor 1 | TGCRCNC | 1 | | 34 |
| | TGCGCGC | | −236 to −230 | |
| NF-E1 | MYWATCWY | 1 | | 74 |
| | CTTATCAC | | −129 to −122 | |
| PEA3 | AGGAAR | 1 | | 75 |
| | AGGAAA | | −90 to −85 | |
| PU.1 | GAGGAA | 1 | | 43 |
| | GAGGAA | | −91 to −86 | |
| Sp1 | CCCGCC | 1 | | 13 |
| | CCCGCC | | −199 to −194 | |
| LEF/TCF-1 | MAMAG | 1 | | 73, 76 |
| | AAAAG | | −64 to −60 | |

[a] The sequence from −284 to +79 was searched for the transcription factor-binding sites. Only those sequences showing a perfect match to consensus transcription factor-binding sites are listed. N = G, A, T, or C; R = G or A; Y = T or C; S = G or C; M = A or C; and W = A or T.

[b] The top line is the consensus sequence; the bottom line is the sequence of the indicated factor.

To determine the elements required for p107 promoter activity, a series of deletion mutants of p107P5200-LUC was constructed (Fig. 3A). These plasmids were then transfected into human cells and scored for luciferase activity as described above. Compared with the luciferase activity expressed by the p107P5200-LUC construct, the *Sma*I (−4000, p107P4000-LUC) and *Sac*II (−280, p107P280-LUC) deletion constructs resulted in only a modest reduction of luciferase activity in 293 cells (Fig. 3B) but expressed about the same level as the p107P5200-LUC construct in Saos-2 cells (Fig. 3C), suggesting that elements required for p107 promoter activity are located within this 5′ flanking DNA fragment from −280 to +64 relative to the major transcription initiation site. To examine if the transcription initiation sites determined by using the p107 promoter-LUC construct in the transient transfection assays mimic those found with the endogenous p107 gene, an RNase protection analysis of RNAs harvested from transfected 293 cells was conducted with the same antisense riboprobe derived from the *Sac*II fragment of the p107 5′ flanking DNA as described above (Fig. 2A). As shown in Fig. 2B, the same major protected fragment as detected for the endogenous p107 mRNA was seen in RNA of mock-transfected 293 cells (lane 2). Similarly, the same major protected fragment and two minor protected bands as found for the endogenous p107 mRNA (Fig. 2A) were detected in RNA from 293 cells transfected with the p107P280-LUC (lane 4) or p107P5200-LUC (lane 5) construct. Since the riboprobe protected the same 5′ region for the endogenous or transiently expressed p107 mRNA, we com-

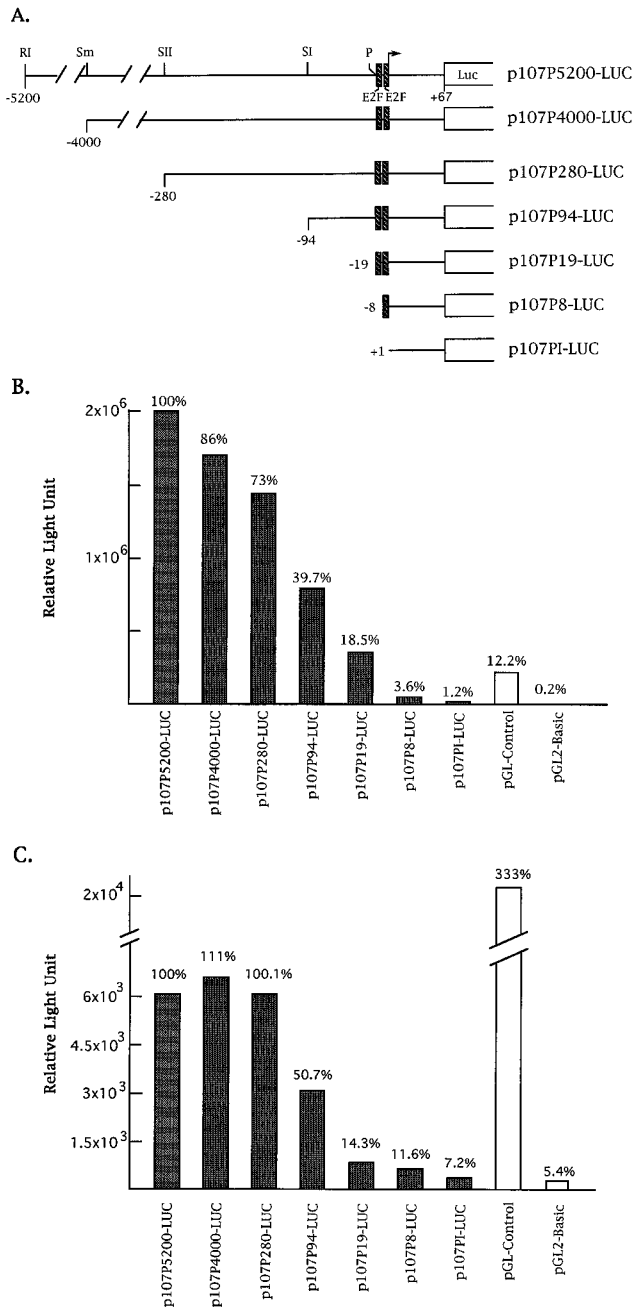



FIG. 3. Delineation of the p107 promoter by deletion and transfection analyses. (A) Schematic diagram of the p107P5200-LUC construct and its deletion derivatives. Two E2F-binding sites are shown as hatched boxes. The major transcription initiation site is designated +1 and marked with an arrow. Abbreviations: RI, *Eco*RI; Sm, *Sma*I; SII, *Sac*II; SI, *Sac*I; P, *Pst*I; Luc, luciferase expression cassette. (B) Luciferase activity expressed from various p107 promoter constructs in 293 cells. (C) Luciferase activity expressed from various p107 promoter constructs in Saos-2 cells. The activities shown are average values from three independent experiments. The SV40 promoter-containing plasmid pGL-Control (Promega) and the promoterless plasmid pGL2-Basic (Promega) were used for comparison.

pared the levels of the p107 transcript in the same amount of RNA from each transfection. The intensity of the major protected band detected in RNA from transfection of the p107P5200-LUC or p107P280-LUC construct was found to be much higher (about 20-fold higher for the p107P5200-LUC construct by densitometer scanning) than that detected in the endogenous p107 mRNA from mock transfection (Fig. 2B; compare lane 4 or 5 with lane 2). This result suggests that increased expression of the p107 transcript came from the exogenously transfected p107 promoter DNA and that the transient transfection assay accurately reflected p107 promoter usage.

Further deletion analysis showed that an additional deletion to the *Sac*I site (−94, p107P94-LUC) reduced the luciferase activity to about 40% of that produced by p107P5200-LUC in 293 cells and to about 51% of that in Saos-2 cells. Another deletion, to the *Pst*I site (−19, p107P19-LUC), decreased the luciferase activity to about 19% in 293 cells and to about 14% in Saos-2 cells. Interestingly, this p107P19-LUC construct contains only the two potential E2F-binding sites and 5′ untranslated leader sequence from the p107 promoter DNA and could express higher luciferase activity than the SV40 promoter in 293 cells. Further deletion to −8 (p107P8-LUC), leaving only one potential E2F-binding site and the leader sequence, decreased the luciferase activity to about 4% of that produced by p107P5200-LUC in 293 cells and to about 10% of that in Saos-2 cells. Construct p107PI-LUC, which contains only the p107 sequence from the transcription initiation site (+1) to +64, expressed about 1% of the activity produced by p107P5200-LUC in 293 cells. Also, this construct consistently expressed slightly higher activity than the promoterless plasmid, pGL-Basic, in Saos-2 cells. All of these results indicate that the human p107 gene is regulated through multiple DNA elements, including two potential E2F-binding sequences.

**E2F binds to the p107 promoter DNA, and both E2F sites are important for promoter activity.** To test if the sequences in the human p107 promoter were functional E2F-binding sites, we carried out a band shift-competition assay. A double-stranded 40-mer oligonucleotide (designated p107-E2F-12) containing the two E2F sites from the −28 to +12 region of the p107 promoter was $^{32}$P labeled at its 5′ end and used as a substrate in the assay using a nuclear extract prepared from HeLa cells. Figure 4A shows that specific DNA-protein complexes, labeled a and b, were detected. We noted that both a and b complexes ran as multiple bands on the gel. These DNA-protein complexes could be abolished by unlabeled p107-E2F-12 oligonucleotide DNA but not by an excess amount of the YY1-binding-site-containing oligonucleotide derived from the adeno-associated virus P5+1 sequence (69) or by an excess amount of the p107-E2F-M12 oligonucleotide containing mutations in both copies of the E2F site. Further competition experiments with the oligonucleotides in which one or the other E2F site was mutated showed that the bands in the b complex could be abolished by the p107-E2F-M1 oligonucleotide, which contains a mutation in the 5′ copy of the E2F site (i.e., the 3′ E2F site remains functional in this oligonucleotide DNA). On the other hand, the bands in the a complex were abolished by the p107-E2F-M2 oligonucleotide, which contains a mutation in the 3′ copy of the E2F site (i.e., the 5′ E2F site is functional). Some competition on the bands in the b complex by the p107-E2F-M2 oligonucleotide was also observed. An oligonucleotide (designated AdE2F-1) containing the prototype E2F-binding site from the adenovirus E2 promoter gave rise to a competition pattern similar to that found for the p107-E2F-M2 oligonucleotide. Curiously, the sequence of the E2F-binding site remaining on the p107-E2F-M2 oligonucleotide is identical to that of the AdE2F-1 oligonucleotide.

To further determine if each E2F site could form specific DNA-protein complexes, oligonucleotides containing either of

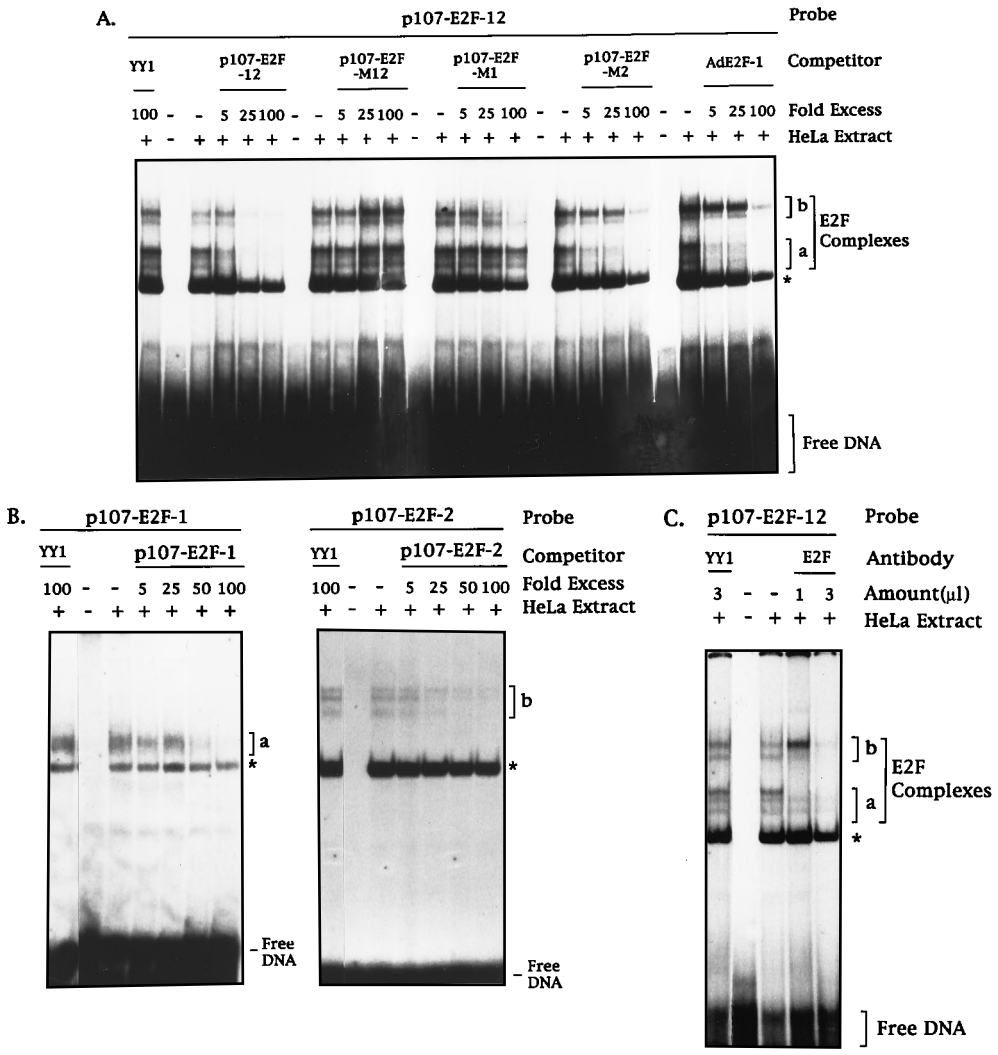



FIG. 4. The E2F transcription factor binds to both copies of the E2F site on p107 promoter DNA. (A) Gel mobility shift assay performed with HeLa cell nuclear extract and the p107-E2F-12 oligonucleotide probe containing two E2F sites of the p107 promoter. The unlabeled competitor DNAs and the amounts added are indicated. The positions of the a and b E2F complexes and of free DNA probe are marked. A nonspecific DNA-protein complex is marked with an asterisk. As a control, a YY1-binding-site-containing oligonucleotide (69) was also used in the competition experiment. (B) Gel mobility shift assay using the 5′ E2F site-containing oligonucleotide p107-E2F-1 (left panel) or the 3′ E2F site-containing oligonucleotide p107-E2F-2 (right panel) as the probe. (C) Effect of antibody on the E2F complexes bound to the p107 promoter DNA. The amount of the anti-E2F or anti-YY1 antiserum used is indicated.

the E2F sites were also used in the band shift-competition assay (Fig. 4B). When the p107-E2F-1 oligonucleotide containing the 5′ E2F site from the −23 to −3 region of the p107 promoter was used as the probe, specific DNA-protein bands similar to the a complex were detected. Conversely, when the p107-E2F-2 oligonucleotide containing the 3′ E2F site from the −13 to +8 region of the p107 promoter was used as the probe, specific DNA-protein bands similar to the b complex were detected. Finally, an antibody perturbation experiment was conducted to directly demonstrate that E2F indeed bound to the p107 promoter DNA (Fig. 4C). Addition of increasing amounts of an anti-E2F antiserum led to the disappearance of both a and b complexes when the double-E2F-site-containing oligonucleotide p107-E2F-12 was used as the probe in the band shift assay. As a control, addition of the anti-YY1 antiserum had no effect on the a and b complexes. Taken together with the sequence information (Fig. 1B) and the identity with the consensus sequence of the E2F-binding site (Table 1), these data suggest that the E2F transcription factor can bind to both E2F sites on the human p107 promoter DNA.

To determine the functional role of each copy of the E2F-binding site in regulating human p107 promoter activity, site-directed mutageneses were done to create point mutations in these sites. Previously, it was shown that the CG dinucleotide in the consensus sequence of the E2F-binding site, 5′-TTSSCGC-3′, was important for E2F binding (68, 79). Thus, these two nucleotides were mutated. Since the 3′ copy of the E2F site in the p107 promoter overlaps the major transcription initiation site, the oligonucleotides used in the mutagenesis experiment (see Materials and Methods for the oligonucleotide sequences) were designed to mutate the E2F site while maintaining a purine residue for the transcription initiation site. In addition, since this E2F site also overlapped an E2A site (Fig. 1 and Table 1), the mutation in the 3′ E2F site was designed to maintain an E2A consensus sequence. From this mutagenesis experiment, three mutated p107 promoter con-

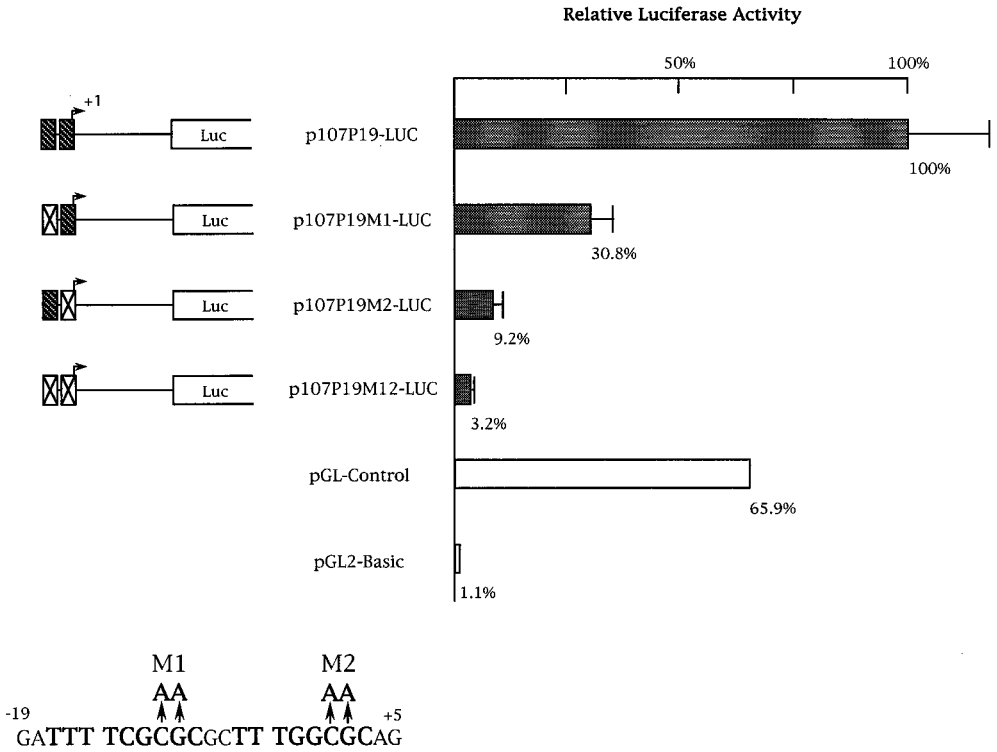



FIG. 5. Analysis of the roles of the two tandem E2F sites on p107 promoter activity by site-directed mutagenesis. The mutations in the E2F sites were created as described in Materials and Methods. At the bottom is a diagram of the p107P19-LUC construct and its mutant derivatives. The wild-type E2F site is indicated as a hatched box, and the mutated site is indicated as a crossed box. The relative luciferase activities of the wild-type and mutant E2F site-containing promoter constructs are shown; the promoter activity expressed from the wild-type p107P19-LUC construct is defined as 100%. The SV40 promoter-containing plasmid pGL-Control and the promoterless construct pGL2-Basic were used as controls.

structs, one with mutation in the 5′ copy, one with mutation in the 3′ copy, and one with mutations in both copies of the E2F-binding site, were generated. All of these mutant constructs with DNA endpoints identical to those of the p107 DNA in p107P19-LUC were rebuilt in the luciferase expression cassette and used in transfection of 293 cells. The p107P19M1-LUC construct with mutation in the 5′ copy of the E2F-binding site expressed about 31% of the luciferase activity of the p107P19-LUC construct, while the p107P19M2-LUC construct with mutation in the 3′ copy of the E2F-binding site expressed only about 9% of the luciferase activity of the p107P19-LUC construct (Fig. 5). This result indicates that although both E2F-binding sites are important for human p107 promoter activity, mutation in the 3′ copy of the E2F site has a stronger effect. Consistent with this result, mutations in both copies of the E2F sites (p107P19M12-LUC) resulted in drastic reduction of the promoter activity to about 3% of that of the p107P19-LUC construct.

**Both pRb and p107 can repress expression of the human p107 promoter through the 5′ copy of the E2F-binding site.** Since both pRb and p107 can bind to E2F and inhibit its transcription activation activity, we determined if these suppressor proteins could also inhibit p107 promoter expression. The p107P5200-LUC construct was cotransfected with either a control vector containing only the human cytomegalovirus immediate-early promoter or an expression vector carrying the full-length Rb or p107 cDNA into 293 cells and $Rb^-$ osteosarcoma Saos-2 cells. At 48 h after transfection, cells were harvested and the luciferase activity from each cotransfection was measured as before. As shown in Fig. 6, expression of the 5.2-kb p107 promoter was repressed by both pRb and p107 proteins in both cell lines. In addition, the repression of p107 promoter expression was mediated at the level of transcription, since the level of the p107 mRNA was reduced when the p107P5200-LUC construct was cotransfected with the pRb expression plasmid (Fig. 2B; compare lane 5 with lane 6).

Two mutants, one pRb-internal and one p107-internal in-frame deletion mutant, were constructed as controls (Fig. 6A). The $pRb_{AN}$ mutant lacks the amino acid sequence from positions 415 to 515 in the A-pocket region required for binding to viral oncoproteins (19, 31, 33, 83) and the E2F transcription factor (28, 29). The $p107_{DE}$ mutant lacks the amino acid sequence from position 407 in the A pocket to position 826 in the B pocket. This mutant has been shown to be defective in repression of E2F activity (83). As predicted, neither the $pRb_{AN}$ nor $p107_{DE}$ mutant repressed significantly the expression of the 5.2-kb p107 promoter in both 293 cells (Fig. 6B) and Saos-2 cells (Fig. 6C).

To identify the elements responsible for the transcriptional repression by pRb and p107, the same set of 5′ deletion mutants of the p107 promoter constructs (Fig. 3A) was used in the cotransfection analysis. Similar to that of the p107P5200-LUC construct, expression of the p107P4000-LUC, p107P280-LUC, and p107P94-LUC constructs was repressed by pRB and p107 in 293 and Saos-2 cells (Table 2). The smallest deletion clone that showed the repression effect by both suppressor proteins in 293 cells was the construct containing two E2F sites, p107P19-LUC. Further deletion of the 5′ copy of the E2F site from the p107P19-LUC construct abolished the repression effect (Table 2, p107P8-LUC). Also, construct p107PI-LUC, which contains only the major transcription initiation site and 5′ leader sequence of the p107 promoter DNA, did not show

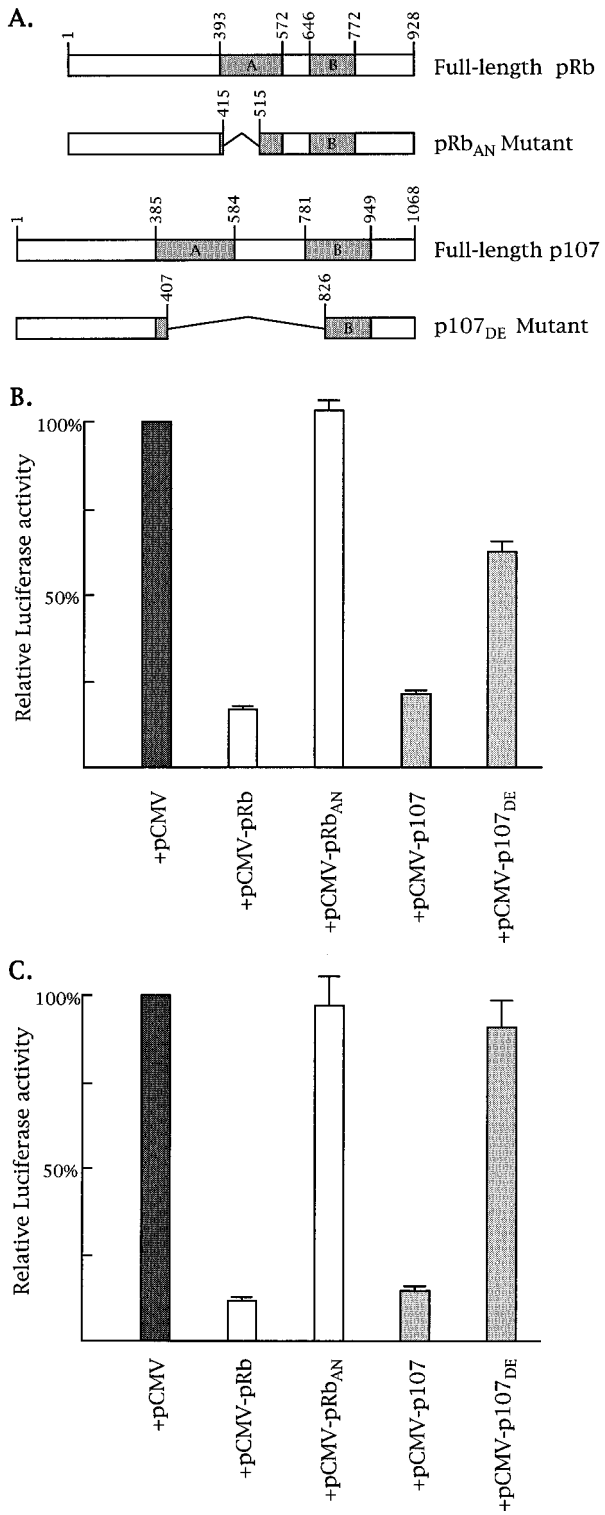



FIG. 6. Both pRb and p107 could repress the p107 promoter. (A) Diagrams of pRb, p107, and their mutant derivatives. Full-length pRb and p107 are shown schematically with the homologous A and B pockets required for interacting with viral oncoproteins and the E2F transcription factor (17). The A and B pockets are shown as shaded boxes with amino acid positions indicated. The pRb and p107 mutants were generated by deletions and in-frame fusions between two convenient restriction sites. The pRb$_{AN}$ mutant lacks the amino acid sequence from positions 415 to 515 in the A-pocket region. The p107$_{DE}$ mutant lacks the amino acid sequence from position 407 in the A pocket to position 826 in the B pocket. (B) 293 cells were cotransfected with 2 μg of the p107P5200-LUC construct and 18 μg of the pCMV control vector or a full-length or mutant pRb or p107 expression plasmid. Plasmid pSV2-βgal was included as an internal control for transfection efficiency. (C) Saos-2 cells were used in the cotransfection experiment as described for panel B. Luciferase activities were normalized to β-galactosidase activities and averaged from the results of three independent experiments.

TABLE 2. Repression of human p107 promoter expression by pRb and its own gene product[a]

| Construct | Fold repression | | | |
|---|---|---|---|---|
| | +Rb | | +p107 | |
| | 293 | Saos-2 | 293 | Saos-2 |
| p107P5200-LUC | 6.1 | 8.8 | 4.6 | 6.7 |
| p107P4000-LUC | 6.7 | 9.0 | 5.0 | 6.2 |
| p107P280-LUC | 6.0 | 7.6 | 4.5 | 6.8 |
| p107P94-LUC | 8.6 | 8.4 | 4.8 | 7.0 |
| p107P19-LUC | 8.4 | ND | 4.8 | ND |
| p107P8-LUC | 1.4 | ND | 1.2 | ND |
| p107PI-LUC | 1.7 | ND | 1.5 | ND |
| p107P280M1-LUC | 1.6 | 1.4 | 1.1 | 1.3 |
| p107P280M2-LUC | 5.5 | 6.4 | 4.7 | 6.4 |
| p107P280M12-LUC | 0.9 | 1.3 | 1.1 | 1.3 |
| p107P19M1-LUC | 1.3 | ND | 1.2 | ND |
| p107P19M2-LUC | 7.3 | ND | 4.4 | ND |
| p107P19M12-LUC | 1.3 | ND | 1.3 | ND |

[a] The human p107 promoter-driven luciferase constructs (2 μg) were cotransfected with a control vector or an Rb or p107 expression plasmid (18 μg) into 293 or Saos-2 cells together with plasmid pSV2-βgal as an internal control. Luciferase activities were normalized to β-galactosidase activities. Fold repression was calculated from the luciferase activity expressed in the absence of the cotransfected suppressor divided by the activity in the presence of the exogenously transfected pRb or p107 suppressor. ND, the repression effect was not determined because the basal promoter activities of these constructs were relatively low in Saos-2 cells.

any repression. All of these results indicate that the 5′ copy of the E2F site is responsible for the repression of human p107 promoter expression by both pRB and its own gene product.

To examine if only the 5′ copy of the E2F site is important for this repression effect, constructs with point mutations in either copy of the E2F-binding site or in both sites were analyzed in cotransfection assays as described above. Like the p107P8-LUC construct, the p107P19M1-LUC construct, which contains only the functional, 3′ copy of the E2F site, was not repressed by pRb and p107 (Table 2). In contrast, the p107P19M2-LUC construct, which contains only the functional 5′ copy of the E2F site, still showed the repression effect. Mutations in both E2F sites rendered the promoter (p107P19 M12-LUC) not repressed by pRb and p107. As shown in Fig. 3, since the p107P280-LUC construct expressed about the same level of promoter activity as the p107P5200-LUC construct, we also rebuilt the point mutations in either copy of the E2F-binding site or in both sites back into the p107P280-LUC backbone and tested for the repression effect by pRb and p107. Analogous to the finding described above, the mutation in the 5′ copy of the E2F site (p107P280M1-LUC) or in both sites (p107P280M12-LUC) abolished the repression effect, while the mutation in the 3′ copy of the E2F site (p107P280M2-LUC) did not. All of these results suggest that the 5′ copy of the E2F site is necessary and sufficient for the repression.

## DISCUSSION

We have isolated a human genomic clone containing the 5′ flanking sequence of the Rb-related p107 gene. By locating the

major transcription initiation site of this gene, we defined the location of the promoter and its upstream regulatory elements. By comparing the location of the major transcription initiation site with that of the cDNA start site, we have confirmed that the previously cloned p107 cDNA (83) contains the translational ATG start codon and therefore the complete coding sequence. Similar to the regions found in many mammalian promoters (for a review, see reference 1), the proximal promoter region of the p107 gene is G+C rich and has no apparent TATA sequence.

A 5.2-kb 5′ flanking sequence of the p107 gene, when fused to a heterologous reporter gene and transfected into actively growing human cells, possessed strong promoter activity. Deletion analysis showed that the 347-bp 5′ flanking DNA (−280 to +64) could confer most, if not all, of the promoter activity. Upon initial inspection of the sequence of this p107 promoter DNA, a unique feature, i.e., the presence of two tandem E2F transcription factor-binding sites located at or immediately upstream of the major transcription initiation site, stood out. In addition, these two E2F sites are flanked by an E2A site on each side. The locations and organization of these transcription factor-binding sites at or around the major transcription initiation site immediately prompted us to examine their functional roles in regulating p107 promoter activity. Indeed, a minimal promoter DNA fragment containing only the two tandem E2F sites together with the leader sequence could direct relatively efficient expression of a reporter gene in 293 cells. Mutating both E2F sites resulted in a drastic reduction of promoter activity. Although both E2F sites are important for p107 promoter activity, mutation of the 3′ copy of the E2F-binding site appears to have a stronger effect.

Band shift-competition analysis using synthetic oligonucleotides containing E2F-binding sequences from the p107 promoter or adenovirus E2 promoter together with the antibody perturbation experiment using the anti-E2F antibody showed that the cellular E2F transcription factor could bind to both copies of the E2F sites on the p107 promoter DNA. All of these biochemical and functional analyses indicate that E2F can interact with and regulate the human p107 promoter. There are two plausible explanations for this observation. Since E2F can bind to the transcription initiation region of the p107 gene, it may also serve as an initiator-binding protein to enhance transcription. Alternatively, as recently proposed by Javahery et al. (36), proteins such as E2F that bind at or near specific initiator elements may augment the strength of an initiator or may impart transcriptional regulation through an initiator. Nonetheless, in the case of the human p107 promoter, the presence of either one of the two tandem E2F sites could enhance transcription (Fig. 5; compare p107P19M1-LUC and p107P19M2-LUC with p107PI-LUC), while the location of the E2F site relative to the transcription initiation site may affect the level of enhancement. In the presence of both sites, a synergistic effect on transcription ensues (Fig. 5; compare p107P19-LUC with p107PI-LUC).

It should be mentioned that the promoter construct containing the major transcription initiation site plus the 5′ untranslated leader sequence of the p107 gene still maintained some transcription activity in 293 cells (Fig. 3B; compare p107PI-LUC with pGL2-Basic). Curiously, a potential E2A-binding site was found to be located at or 3′ to the transcription initiation site region. However, it is not known if this element plays any role in this promoting activity. Kim et al. (42) recently reported that the p107 gene was expressed at a relatively high level in embryonic cardiomyocytes but that its transcript level decreased markedly during the process of cardiomyocyte terminal differentiation and was very low or undetectable in adult animals. We are presently conducting experiments to examine if the B-cell- and myocyte-specific E2-box-binding factors bind to the E2A sites and regulate the transcription of p107 gene in cardiomyocytes during early embryonic development.

In addition to E2F and E2A sites, several other potential transcription factor-binding sites (only those sequences showing a perfect match to a consensus transcription factor-binding sites are listed in Table 1), including an Sp1-binding site, also were found in the p107 promoter-proximal region. The significance of the presence of these potential transcription factor-binding sites is unknown. The presence of the Sp1-binding site is common to many TATA-less promoters (1). Results from the *Sac*I deletion (which deletes the potential binding sites for AP-2, common factor 1, NF-E1, and Sp1) and the *Pst*I deletion (which deletes the potential binding sites for PEA3, PU.1, and LEF/TCF-1) showed the requirement of other additional elements for the full p107 promoter activity (Fig. 3 and Table 1). Further analysis of fine deletions and point mutations should allow us to clarify the contribution of these elements to human p107 promoter activity in cycling cells. It is noteworthy that among the potential transcription factor-binding sites identified in the p107 promoter sequence, several are for transcription factors specific to hematopoietic cells (Table 1). The human p107 gene has been previously mapped to chromosome 20q11.2 (19), and cytogenetic abnormalities in this chromosomal region have been reported in some human myelogenous leukemias (9, 46, 81). Thus, it will be interesting to examine if any of these elements are involved in controlling the expression of the p107 gene in hematopoietic cells.

Previous studies have shown that the E2F transcription factor is involved in the transcription of several cellular genes necessary for proliferation and is one of the targets of the Rb family of growth suppressors (for a review, see reference 17). The pRb, which negatively regulates cell cycle progression from $G_1$ into S phase, binds to E2F, and the functional consequence of this interaction is to prevent E2F from activating transcription. The pRb-related protein p107, implicated in cell cycle control, also associates with E2F during cell cycle progression (2, 3, 6, 7, 28, 39, 50, 68). In addition, overexpression of pRb decreases E2F-dependent transcription, and in a similar manner, overexpression of p107 leads to decreased E2F activity (15, 20, 29, 49, 67, 70, 77, 82).

By cotransfection of the human p107 promoter construct with a pRb or p107 expression vector, we found that the promoter could be repressed by both pRb and its own gene product. Intriguingly, mutational and cotransfection analyses showed that the repression effect was mediated only through the 5′ E2F site in the p107 promoter. We do not know how these two E2F sites, tandem in arrangement and overlapping the major transcription initiation site, play differential roles in this repression effect. One possibility is that the pRb- or p107-containing E2F complexes, when bound to the 5′ copy (e.g., in p107P19M2-LUC) or both copies (e.g., in p107P19-LUC) of the E2F site in the p107 promoter, prevent the RNA polymerase and other components of the transcriptional machinery from interacting with the initiator region located immediately downstream of this E2F site (5, 62). On the other hand, when the 5′ copy of the E2F site is mutated, only the 3′ E2F site, which overlaps the major transcription initiation site, is available for binding (e.g., in p107P19M1-LUC). Some other initiator-binding protein or transcription factor (for example, E2A, whose binding site is also located at the transcription initiation site region; Table 1) whose activity is not repressed by the suppressor protein may bind to this region to initiate transcription. This may explain why expression of the p107P19M1-LUC

construct was not repressed by pRb or p107. To test this possibility, in vivo and in vitro transcription analyses are now in progress, and the results should allow us to better understand how E2F and other factors regulate expression of the human p107 gene in different growth conditions and in various cell types.

## ACKNOWLEDGMENTS

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