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Association of p107 with Sp1: Genetically Separable Regions of p107 Are Involved in Regulation of E2F- and Sp1-Dependent Transcription

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The retinoblastoma-related protein p107 has been shown to be a regulator of the transcription factor E2F. p107 associates with E2F via its pocket region and represses E2F-dependent transcription. In this study, we provide evidence for a novel interaction between p107 and the transcription factor Sp1. We show that p107 can be found endogenously associated with Sp1 in the extracts of several different cell lines. Moreover, in transient transfection assays, expression of p107 represses Sp1-dependent transcription. This repression of Sp1-dependent transcription does not require the DNA-binding domain of Sp1. Transcription driven by a chimeric protein containing the Gal4 DNA-binding domain and the Sp1 activation domains is inhibited by p107. Interestingly, unlike the repression of E2F-dependent transcription, the repression of Sp1-dependent transcription does not depend on an intact pocket region. We show that distinct regions of p107 are involved in the control of Sp1 and E2F.

p107 is a member of the retinoblastoma family of proteins. Like the retinoblastoma protein (Rb), p107 is a target of the DNA tumor virus oncoproteins (12, 14–18). The adenovirus E1A, simian virus 40 (SV40) T-antigen, and papillomavirus E7 proteins associate with p107; these associations coincide with the immortalization function of these oncoproteins (11, 12, 15, 18). p107 forms specific complexes with several cell cycle-regulatory proteins such as cyclins and cyclin-dependent kinases. Cyclin E and cyclin A (16, 19) associate with p107 via a unique sequence within the spacer region of the protein (16), and the cyclin-regulated kinase cdk2 associates with p107 in conjunction with cyclin E or cyclin A (33).

p107 forms complexes with the cellular transcription factor E2F (5, 6, 13, 47). E2F has been shown to be involved in the transcription of several cell cycle-regulated genes, including the n-*myc*, c-*myc*, *myb*, thymidine kinase, E2F-1, *dhfr*, cdk2, and DNA polymerase α genes (1, 18, 37). Interaction of p107 with E2F correlates with a repression of E2F-dependent transcription (44, 49, 52, 53). p107 forms two distinct complexes with E2F; a p107-E2F-cyclin E-cdk2 complex is detected in late G_1 (33), and a p107-E2F-cyclin A-cdk2 complex is detected in S phase (13, 47). However, the functions of these cell cycle-specific E2F complexes are not well understood.

p107 shares properties with the tumor suppressor protein Rb, including complex formation with E2F (15, 18). However, the E2F-Rb complex is distinct from the E2F-p107 complex in that different members of E2F multigene family are involved in the interaction with Rb and p107. E2F-1, E2F-2, and E2F-3 bind Rb (24, 26, 46), whereas E2F-4 binds p107 (3, 20). In transient-transfection assays, both Rb and p107 repress E2F-mediated transcription (5, 44, 49, 52, 53). Recent experiments have shown that overexpression of p107 in responsive cell lines results in growth arrest in the G_1 phase of the cell cycle (53).

The transcription factor Sp1 plays a major role in the expression of numerous viral as well as cellular genes, including constitutive housekeeping genes and inducible genes (9). Sp1 binds to a GC-rich promoter element and stimulates transcription from the promoters containing this consensus element (29). Structure-function analysis of Sp1 by mutagenesis has revealed the presence of three separate regions involved in its transcriptional regulatory functions: (i) a DNA-binding domain consisting of three zinc finger motifs; (ii) multiple activation domains consisting of two glutamine-rich subdomains, A and B, and a third subdomain, C, with no outstanding motif (8, 39); and (iii) a carboxyl-terminal domain, D, that is involved in multimerization and cooperative transactivation (39). Sp1 forms heteromeric complexes with several cellular proteins. The TATA-binding protein-associated protein TAF110 binds Sp1 and functions as a coactivator in Sp1-dependent transcription (25). Sp1 interacts with the cellular protein YY1 (32, 45) and with the RelA subunit of NF-κB (48). The bovine papillomavirus E2 protein has also been shown to associate with Sp1 (35). Recent experiments have shown that the activity and synthesis of Sp1 are subject to a variety of regulations. Sp1 expression is increased during SV40 infection of the CV1 cells (27, 43). Rb either activates or represses Sp1-dependent transcription in a cell-type-specific manner (7, 30, 50, 51). The level of expression of Sp1 protein varies widely among different cell types in the mouse, and an increased expression of Sp1 has been associated with late stages of differentiation (42). Sp1 is modified by both phosphorylation and O glycosylation; however, the role of these posttranslational modifications in Sp1-dependent transcription is not clear (27, 28).

Interestingly, several cell cycle-regulated genes, including the *dhfr*, E2F-1, cyclin E, *cdk2*, and c-*myc* genes, contain Sp1- and E2F-binding sites in their regulatory regions (1). While the E2F sites have been shown to be involved in cell cycle regulation, the role of the Sp1 sites in the cell cycle regulation is unclear. As described in this report, we observed that the cell cycle-regulatory protein p107 can be found endogenously associated with the transcription factor Sp1 in several different cell lines. We also show that in cotransfection assays, p107 specifically represses the Sp1-dependent transcription. More-

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over, the mechanism of repression through the Sp1 site is distinct from that involved in the repression through the E2F site.

MATERIALS AND METHODS

Cells and extracts. Saos-2 osteosarcoma cells, 293 cells, COS cells, L cells, F9 teratocarcinoma cells, and HeLa cells were obtained from the American Type Culture Collection and were grown in Dulbecco's modified Eagle medium (GIBCO/BRL) supplemented with 10% fetal bovine serum. Spinner cultures of mouse L cells were grown in minimum essential medium (S-MEM; GIBCO-BRL) supplemented with 5% calf serum. Whole cell extracts were prepared by a previously described procedure (41).

Plasmids and DNA probes. Plasmid BCAT-2, containing the bacterial chloramphenicol acetyltransferase (CAT) gene downstream of two Sp1-binding sites and the E1B-TATA element (39) was a kind gift from R. Tjian. Wild-type and mutant cytomegalovirus (CMV)-p107 expression plasmids (53) were kindly provided by Liang Zhu and Ed Harlow. JDG[Gal4(1-147)] and JDG + Sp1N (Gal4-Sp1) were kindly provided by S. Sif and T. D. Gilmore (48). Gal4-HNF3 was provided by R. Costa (Department of Biochemistry, University of Illinois at Chicago). The reporter constructs phTG7 (CAT gene driven by the transforming growth factor β1 [TGF-β1] promoter [10]), SV40-CAT (8), G5BCAT (36), E2F(−) E2 CAT (10), and E2 CAT (2) have been previously described. To obtain plasmid E2F(−)Sp1 CAT, an oligonucleotide corresponding to the Sp1 consensus sequence 5′GATCGGGGCGGGGC3′ was subcloned into the *Bgl*II site of plasmid E2F(−) E2 CAT. The chimeric construct E2F(−)Sp1 CAT contained one copy of the oligonucleotide in correct orientation, which was confirmed by sequencing. Consensus oligonucleotides containing Sp1, AP2, and NF1 binding sites were obtained from Promega. The plus-strand sequence for the Sp1 oligonucleotide is 5′ATTCGATCGGGGCGGGGCGAGC3′, that of the AP2 oligonucleotide is 5′GATCGAACTGACCGCCCGCGGCCCGT3′, and that of the NF1 oligonucleotide is 5′CCTTTGGCATGCTGCCAATATG3′.

Antibodies. p107 monoclonal antibodies SD-4, SD-6, SD-9, and SD-15 as hybridoma supernatants were kindly provided by N. Dyson. p130 monoclonal antibody Z11 was a gift from Peter Whyte. p300 monoclonal antibody NMII was a kind gift of E. Moran. The Sp1 antibody (against residues 520 to 538 of Sp1 protein), the Egr1 antibody (against a 14-amino-acid carboxyl-terminal peptide), and the Rb monoclonal antibody (IF8) were from Santa Cruz Biotechnology. The E1A monoclonal antibody Ab-1 was from Oncogene Science. The p107 polyclonal antibody was raised against glutathione *S*-transferase (GST)–p107 protein by S. Ray in P. Raychaudhuri's laboratory. The immunoglobulin G fractions from different antibodies were purified by either protein A-Sepharose or protein G-Sepharose chromatography.

Immunoprecipitation and electrophoretic mobility shift assays. Mouse L-cell extracts were fractionated by heparin-agarose chromatography with KCl gradient elution from 0.1 to 0.6 M as described previously (41). Column fractions (between 0.28 to 0.35 M KCl) containing the majority of the Sp1-like activity (which were identified by electrophoretic mobility shift assay) were pooled and used for the immunoprecipitation experiments. Sixty-microliter aliquots of different antibodies (0.1 mg of immunoglobulin G per ml) against Egr1, Sp1, p130 (Z11), p300 (NM11), Rb (IF8), or p107 (polyclonal) were incubated with 25 μl of protein G-Sepharose beads at 4 C for 1.5 h. Four hundred micrograms of the pooled heparin-agarose fraction was added to the antibody-bound protein G-Sepharose beads after dilution with an equal volume of buffer A (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 0.1% Nonidet P-40, 0.1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol) supplemented with 1 μg each of aprotinin, leupeptin, and pepstatin per ml and 10 μM phenylmethylsulfonyl fluoride. The mixtures were incubated at 4°C for 2 h. The immune complexes bound to protein G-Sepharose beads were washed five times with 1 ml of buffer A containing 0.15 M KCl. The bound proteins were eluted from the beads with 25 μl of buffer A containing 0.4 M KCl and 0.5 mg of bovine serum albumin (BSA) per ml. The eluted proteins were subjected to the gel shift assay as described below.

Mobility shift assays were performed as previously described (39), with the following modifications. Different amounts of proteins and antibodies (as indicated in the figure legends) were mixed with 0.1 to 0.5 ng of ^{32}P-labeled DNA probe and 1 μg of poly(dI-dC) in 20 μl of reaction buffer and incubated at room temperature for 20 min. An aliquot of each reaction mixture (7.5 μl) was analyzed by a gel retardation assay using a 4% native polyacrylamide gel containing 0.25× Tris-borate-EDTA and 0.1% Nonidet P-40. Oligonucleotide 5′GATCTGGGTGGGGC3′, containing the TGF-β1 promoter sequence between positions −90 and −80, was subcloned into the *Bam*HI site of pBluescript II SK+. The DNA fragment was excised by *Xba*I and *Hin*dIII digestion, labeled with Klenow enzyme and [^{32}P]dATP, and used as a probe for the gel retardation assays. For NF1 and AP2 probes, double-stranded oligonucleotides containing the respective consensus binding sites (Promega) were labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. For competition experiments, a 100-fold molar excess of unlabeled oligonucleotides was added to the reaction mixture prior to the addition of proteins. Sp1 and AP2 oligonucleotides (Promega) were used as competitors.

Western blot (immunoblot) analysis. Immunoprecipitated proteins from L-cell extracts (as described above) with appropriate antibodies were separated by a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (7.5% polyacrylamide gel) and transferred to nitrocellulose membranes. The membranes were blocked by incubation in 0.1% Nonidet P-40 in phosphate-buffered saline (PBS-N) containing 5% dry milk at 4°C. The membranes were then incubated with either an Sp1 antibody or p107 antibodies (monoclonal SD cocktail, a mixture of SD-4, SD-6, SD-9, and SD-15) diluted in PBS-N containing 1% dry milk for 3 h at room temperature. For the p107 monoclonal antibodies, a second antibody, rabbit antimouse immunoglobulin G (1:1,500 dilution), was used. The blots were then probed with ^{125}I-protein A and subjected to autoradiography.

Southwestern (DNA-protein) analysis. The immunoprecipitated proteins from L-cell extracts (as described above) with the appropriate antibodies were eluted from the protein G-Sepharose beads by boiling the beads with an equal volume of 2× SDS-PAGE sample buffer. The eluted proteins were separated by SDS-PAGE (10% polyacrylamide gel) and transferred to a nitrocellulose membrane. The nitrocellulose blot was subjected to denaturation and renaturation cycles, using binding buffers (25 mM HEPES [pH 7.9], 40 mM KCl, 3 mM $MgCl_2$, 0.2% Nonidet P-40, 1 mM dithiothreitol) containing decreasing concentrations of guanidine-HCl (6, 3, 1.5, 0.75, 0.38, and 0.19) and finally using binding buffer only. All washes were performed at 4°C, each for 5 min. The blot was blocked by incubation at room temperature for 2 h in binding buffer containing 5% nonfat dry milk. The blot was prehybridized in binding buffer containing 100 μg of poly(dI-dC) per ml, 50 μg of tRNA per ml, and 0.25% milk for 30 min at room temperature. A ^{32}P-labeled double-stranded multimerized oligonucleotide (10^6 cpm/ml) representing the sequence between positions −90 and −80 of the TGF-β1 promoter (5′GATCTGGGTGGGGC3′) was added to the prehybridization mixture, and the mixture was incubated for 2 h. The blot was washed three times with binding buffer containing 0.25% milk and once with binding buffer and then subjected to autoradiography.

V8 protease digestion. HeLa cell extracts were immunoprecipitated with the p107 antibody as described above. After an extensive wash with 0.15 M KCl-containing buffer, the proteins bound to the protein G-Sepharose beads were eluted with buffer A containing 0.4 M KCl and 0.5 mg of BSA per ml. The eluates were diluted with an equal volume of buffer A containing no KCl and digested by V8 protease at three different concentrations, 0.1, 0.5, and 2 μg, at 25°C for 2 h. One footprinting unit of recombinant Sp1 (Promega) was digested under identical conditions. Digested proteins were separated on an SDS–12% polyacrylamide gel and then transferred to a nitrocellulose membrane. The membrane was probed with the Sp1 antibody as described above.

Transfections and CAT assays. Saos-2 cells were transfected at 40% confluence, using the calcium phosphate coprecipitation method as described previously (10, 21). DNA precipitates were removed 14 h after transfection, and the cells were replenished with fresh medium. Cells were harvested 30 h later, and the CAT activity was determined. Two micrograms of CMV–β-galactosidase plasmid was also cotransfected to monitor transfection efficiency. All transfections were repeated a minimum of three times, and the standard deviations were calculated.

RESULTS

Association of an Sp1-related DNA-binding protein with p107. The Sp1-cognate element is found in the promoter regions of several cell cycle-regulated genes such as the *dhfr*, c-*myc*, thymidine kinase, cyclin E, and E2F-1 genes. These promoters also contain E2F-binding sites. While the E2F sites have been shown to be the targets of several cell cycle regulators, the role of the Sp1 sites in the regulated expression of these genes has remained elusive. Recent studies indicated that Rb could modulate transcription through the Sp1 sites (7, 30, 50, 51). However, the mechanism of Rb regulation through the Sp1 site has not been clearly established. To investigate a relationship between Sp1 and the retinoblastoma family of proteins, we assayed for an endogenous interaction between these proteins. Antibodies against Rb, p107, and p130 were used to detect coimmunoprecipitation of Sp1 with these proteins. Partially fractionated mouse L-cell extracts containing the majority of the Sp1-like activity along with an antibody against Rb, p107, p130 or p300 were used in the immunoprecipitation experiments. The immune complexes were pooled with protein G-Sepharose beads and washed extensively with buffer containing 0.15 M KCl, and the associated proteins were eluted with buffer containing 0.4 M KCl. The DNA-binding activity in the 0.4 M KCl eluate was assayed by a gel retardation assays using a DNA probe containing an Sp1-cognate

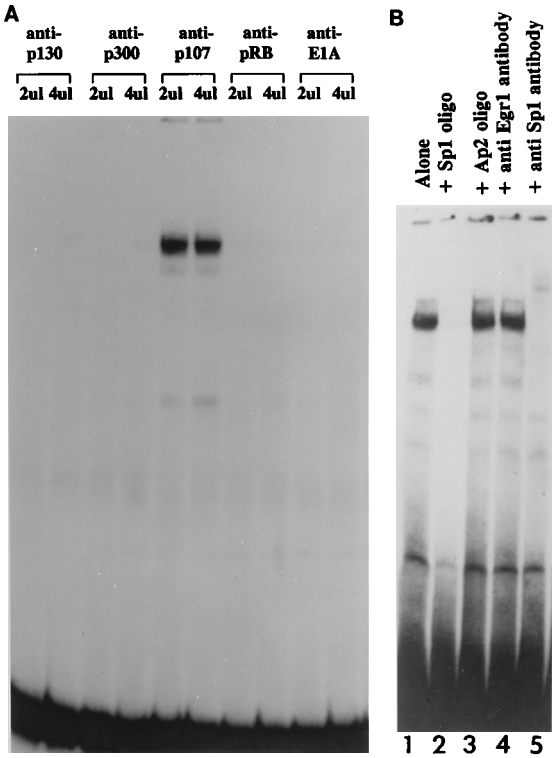

FIG. 1. The p107 antibody coprecipitates an Sp1-like DNA-binding protein. (A) An L-cell extract was fractionated by heparin-agarose chromatography as described in Materials and Methods. An aliquot of the heparin-agarose fraction containing the majority of the Sp1-like activity (400 μg) was immunoprecipitated with an antibody against p107, p300, p130, Rb, or E1A. The proteins associated with the immunoprecipitates were eluted with 0.4 M KCl-containing buffer (25 μl). Aliquots (2 and 4 μl) of the 0.4 M KCl eluates were analyzed in a gel retardation assay using ^{32}P-labeled oligonucleotide containing an Sp1-binding site as a probe. (B) Two-microliter aliquots of the eluted DNA-binding protein(s) from the immunoprecipitations with the p107 antibody were assayed in the presence of a 100-fold molar excess of unlabeled oligonucleotides containing either an Sp1-binding site (lane 2) or an AP2-binding site (lane 3). In lanes 4 and 5, the reaction mixtures were incubated with either an Egr1 antibody (lane 4) or an Sp1 antibody (lane 5) prior to gel retardation analysis.

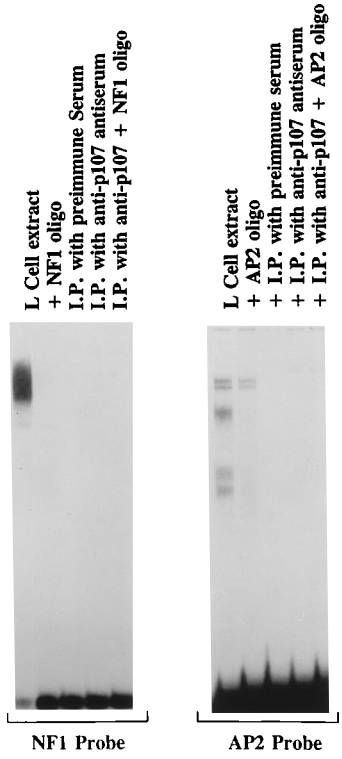

FIG. 2. The p107 antibody does not coprecipitate NF1 or AP2 activity. L-cell extract proteins (400 μg) were immunoprecipitated with either a control or p107 antibody. The proteins associated with the immunoprecipitations (I.P.) were eluted with 0.4 M KCl-containing buffer (25 μl). Aliquots of 4 μl were subjected to gel retardation assays using a ^{32}P-labeled oligonucleotide containing either an NF1- or AP2-binding site as a probe. The levels of NF1- and AP2-binding activity in an L-cell extract (10 μg) are shown.

element (5′TGGGTGGGGC3′) (10). As shown in Fig. 1A, only the p107 antibody coprecipitated a DNA-binding activity that specifically bound to the DNA probe. The specificity of the gel-shifted complex was determined by oligonucleotide competition assays (Fig. 1B). Oligonucleotides containing an Sp1-binding site (lane 2), but not an AP2-binding site (lane 3), competed for the immune complex-associated DNA-binding protein. To further investigate the relatedness of the immune complex-associated DNA-binding protein with Sp1, an Sp1 antibody was used in the gel retardation assay. The DNA-binding protein associated with the p107 immune complex was recognized by the Sp1 antibody (lane 5) but not by an antibody against another GC-rich promoter-binding transcription factor such as Egr1 (lane 4). Thus, the p107-associated DNA-binding protein is antigenically related to Sp1. We did not detect coprecipitation of the Sp1-like activity with p300, p130, or Rb. However, this negative result does not rule out the possibility that some of these proteins interact with the Sp1-like activity. The antibodies or the protein fractions used in this experiment detected only interactions with p107.

To determine the specificity of the immunoprecipitation, we looked for coprecipitations of other DNA-binding proteins, which bind to GC-rich promoter elements such as NF1 and AP2, with p107. Using ^{32}P-labeled NF1 and AP2 probes in gel retardation assays, we found that L-cell extracts contained significant levels of both NF1 and AP2 activities (Fig. 2). However, in coprecipitation assays with the p107 antibody, we did not detect any coprecipitation of NF1 or AP2 activities with p107 (Fig. 2). These results clearly suggested that the coprecipitation of the Sp1-like factor with the p107 antibody was specific.

Identification of the Sp1-related protein in the p107 immunoprecipitate as the transcription factor Sp1. Several DNA-binding proteins have been shown to bind GC-rich promoter elements (4, 31). To further characterize the DNA-binding protein in p107 immunoprecipitates, we performed Western blot analysis of the immunoprecipitates obtained with antibodies against different proteins of the retinoblastoma family. As shown in Fig. 3A, the immunoprecipitates obtained with both the p107 antibody (lane 4) and the Sp1 antibody (lane 2) contained a 105-kDa protein which was specifically recognized by the Sp1 antibody. Lane 1 of Fig. 3A shows immunoblotting of the recombinant Sp1 protein. To determine what other Sp1-related proteins coprecipitate with p107, we performed Southwestern analysis of these different immunoprecipitates, using the multimerized ^{32}P-labeled Sp1 oligonucleotides as the probe. Pooled heparin fractions (between 0.28 and 0.35 M KCl eluate) used for the immunoprecipitations were found to contain multiple proteins that were recognized by the Sp1 oligonucleotide (Fig. 3B, lane 1); however, among these proteins, only the 105-kDa Sp1-related protein was coprecipitated with the p107 antibody (lane 6). A mutant Sp1 oligonucleotide in

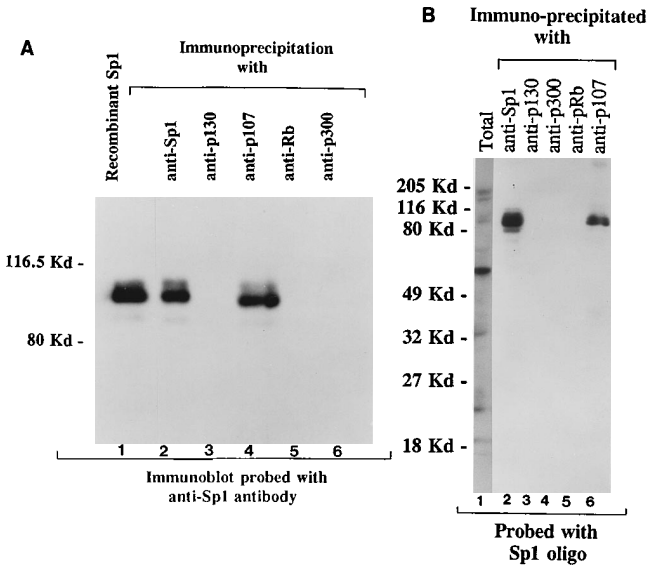

FIG. 3. Detection of an Sp1-like protein in the p107 immunoprecipitates by immunoblot and Southwestern analyses. (A) Immunoblot analysis. Pooled heparin-agarose column fractions of L-cell extracts were immunoprecipitated with an antibody against Sp1, p130, p107, Rb, or p300. Immunoprecipitates were separated by SDS-PAGE (7.5% polyacrylamide gel) and subjected to Western blot analysis. The blot was probed with the Sp1 antibody. Lane 1 contained 1 footprinting unit of recombinant Sp1 as a positive control. (B) Southwestern analysis. The immunoprecipitates from the experiment shown in panel A were separated by SDS-PAGE (10% polyacrylamide gel), transferred to nitrocellulose, and probed with ^{32}P-labeled oligonucleotides containing the Sp1-binding site in a Southwestern assay as described in Materials and Methods. An assay of the heparin-agarose fraction (0.28 to 35 M KCl) before immunoprecipitation is shown in lane 1.

the same assay did not recognize the 105-kDa protein (data not shown).

To further investigate the relatedness of the p107-associated protein with Sp1, we compared their partial proteolytic maps. The p107-associated proteins were purified from HeLa cell extracts by immunoprecipitation with the p107 antibody followed by elution with 0.4 M KCl-containing buffer. The eluted proteins as well as the purified recombinant Sp1 protein (Promega) were digested with different concentrations of V8 protease. Digested peptides were then separated by SDS-PAGE (12% polyacrylamide gel), transferred to nitrocellulose, and probed with the Sp1 antibody. As can be seen in Fig. 4, the Sp1 antibody-reactive peptides obtained from the immunoprecipitated proteins were identical to those obtained from the recombinant Sp1. This result strongly supports the notion that the p107-associated DNA-binding protein is indeed the transcription factor Sp1.

The p107 antibody does not cross-react with Sp1. To examine if the p107 antibody could directly interact with Sp1, we checked for cross-reactivity of the p107 antibody with partially purified Sp1 protein in Western blot assays (Fig. 5A). We purified Sp1 from L-cell extracts by sequential column chromatographies using heparin-agarose and DEAE-Sepharose. The Sp1 activity, which was eluted from the DEAE-Sepharose column by 0.1 M KCl, was further purified by sequence-specific DNA affinity chromatography and wheat germ agglutinin chromatography (28, 29). Panel 1 of Fig. 5A shows a silver-stained gel of the Sp1-containing fractions obtained by sequence-specific DNA-affinity chromatography (lane 1) and that obtained by wheat germ agglutinin chromatography (lane 2). Immunoblot analysis of these fractions with the Sp1 antibody clearly detected the Sp1 protein (Fig. 5A, panel 2), while the p107 antibody did not show any cross-reaction (Fig. 5A, panel 3). We also performed immunoprecipitation of ^{35}S-labeled-Sp1 protein synthesized in rabbit reticulocyte lysate, using Sp1 and p107 antibodies. As shown in Fig. 5B, while the Sp1 antibody clearly precipitated the ^{35}S-labeled Sp1 (lane 2), the p107 antibody did not immunoprecipitate Sp1 (lane 1). On the basis of these observations, we conclude that the p107 antibody does not cross-react with Sp1.

The Sp1 antibody coprecipitates p107. We carried out the reciprocal experiment to determine whether an Sp1 antibody can coimmunoprecipitate p107. In this experiment, we first immunoprecipitated L-cell extracts with an Sp1 antibody, and the immunoprecipitates were collected on protein G-Sepharose beads. The immunoprecipitates were then separated by SDS-PAGE (7.5% polyacrylamide gel) and assayed for the p107 protein by immunoblot analysis. A cocktail of monoclonal antibodies against p107 was used to probe the blot. As shown in Fig. 6A, the Sp1 antibody coimmunoprecipitated from mouse L-cell extracts a 120-kDa protein that was recognized by the p107 monoclonal antibodies (lane 3). In a parallel immunoprecipitation experiment with an Egr1 antibody, no coprecipitation of p107 was detected (lane 4). The coimmunoprecipitated protein recognized by the p107 antibodies was not Rb but p107, because we did not detect any interaction of the monoclonal p107 antibodies with GST-Rb in this assay (lane 1). Cross-reactivity of the Sp1 antibody with p107 was checked by an immunoprecipitation assay. As can be seen in Fig. 6B (lane 2), the Sp1 antibody did not immunoprecipitate the ^{35}S-labeled p107 protein synthesized in rabbit reticulocyte lysate, whereas the p107 antibodies immunoprecipitated p107 as expected (lane 3).

An endogenous Sp1-p107 complex can be detected in the extracts of several cell lines. Next we analyzed whether the association of Sp1 and p107 was ubiquitous or specific for mouse L cells and HeLa cells. Cell extracts from five different cell lines (HeLa, COS, 293, F9, and L) were immunoprecipitated with either a preimmune serum or the p107 antiserum. The immunoprecipitates were extensively washed with 0.15 M KCl buffer, and the associated proteins were eluted with 0.4 M KCl-containing buffer. The 0.4 M KCl eluates were assayed for

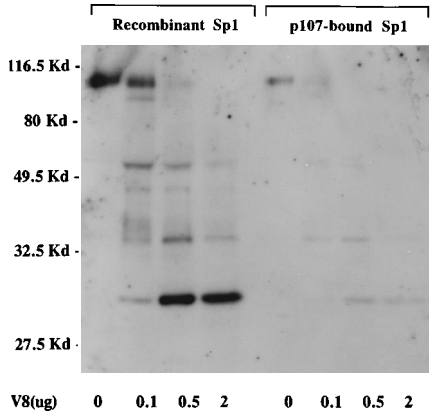

FIG. 4. V8 protease Cleveland mapping of the p107-associated DNA-binding protein and recombinant Sp1. HeLa cell extracts (0.5 mg) were immunoprecipitated with the p107 antibody. The immunoprecipitated proteins were digested by 0.1, 0.5, and 2 μg of V8 protease as described in Materials and Methods. Recombinant Sp1 protein (1 footprinting unit) was digested under the same conditions. Digested proteins were separated by SDS-PAGE (12% polyacrylamide gel) followed by blotting to a nitrocellulose membrane. The blot was probed with the Sp1 antibody and developed with ^{125}I-protein A.

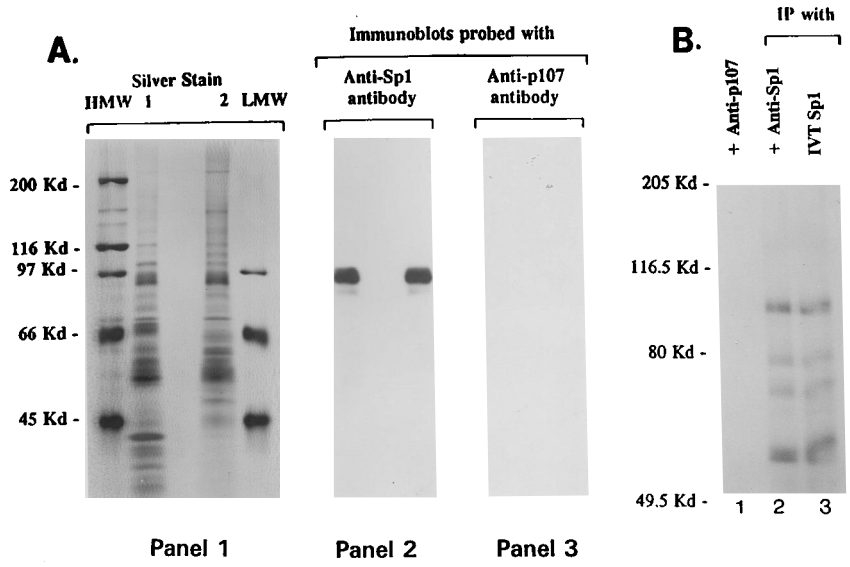

FIG. 5. The p107 antibody does not cross-react with Sp1. (A) Panel 1, partial purification of Sp1. Sp1 proteins from L cells were purified by either immunoaffinity chromatography (lane 1) or wheat germ agglutinin chromatography (lane 2) as described in Materials and Methods. Aliquots of the partially purified fractions were analyzed by SDS-PAGE (7.5% polyacrylamide gel) and visualized by silver staining. HMW, high-molecular-weight marker proteins; LMW, low-molecular-weight marker proteins. Panels 2 and 3, immunoblot analysis of the partially purified Sp1 proteins. The purified fractions containing Sp1 proteins were subjected to immunoblot analysis using the Sp1 antibody (panel 2) or p107 antibody (panel 3). The blots were developed with ^{125}I-protein A. (B) Immunoprecipitation analysis. ^{35}S-labeled Sp1 protein made in rabbit reticulocyte lysate (10 μl) was immunoprecipitated (IP) with the Sp1 or p107 antibody. The immunoprecipitated proteins were separated by SDS-PAGE (7.5% polyacrylamide gel) followed by autoradiography. In the lane marked IVT Sp1, 2.5 μl of the in vitro-translated ^{35}S-labeled product was directly applied.

Sp1 activity by the gel retardation assay. We detected Sp1 activity in the p107 immunoprecipitates obtained from the extracts of all cell lines tested (Fig. 7). Extracts from two additional cell lines (NIH 3T3 and Jurkat) were also found to contain an endogenous Sp1-p107 complex (data not shown). The 293 cell extract contains endogenous E1A proteins, and the COS cell extract contains endogenous T antigen. Both T antigen and E1A have been shown to associate with p107. Interestingly, we immunoprecipitated the Sp1 activity from both 293 and COS cell extracts by the p107 antibody. Thus, it is possible that neither E1A nor T antigen dissociates the complex between Sp1 and p107.

p107 represses Gal4-Sp1-activated transcription. After establishing an interaction between Sp1 and p107, we set out to determine the functional consequences of the interaction. The cell cycle-regulatory protein p107 has been shown to possess transcriptional regulatory properties (22, 49, 52, 53). For example, p107 associates with the transcription factor E2F and represses E2F-dependent transcription (49, 53). To determine the effect of p107 on Sp1-dependent transcription, we carried out cotransfection experiments with p107- and Gal4-Sp1-expressing plasmids. We used the Gal4-Sp1 construct JDG-Sp1N, in which the Sp1 cDNA sequence between amino acids 83 and 621 was fused in frame with the Gal4 DNA-binding domain (48). The fusion protein Gal4-Sp1 does not contain the DNA-binding domain of Sp1. We analyzed the effect of p107 expression on transcription driven by the Gal4-Sp1 construct in cotransfection assays. Saos-2 cells were transfected with a p107 expression plasmid, the Gal4-Sp1 plasmid, and the reporter construct G5BCAT. The G5BCAT construct contained five Gal4 DNA-binding sites in front of a TATA motif (36). As shown in Fig. 8B, expression of p107 showed significant repression of Gal4-Sp1-activated transcription. This p107-mediated repression was specific for Sp1-dependent transcription, because in a similar assay, transcription driven by Gal4-HNF3 was not affected by p107 (Fig. 8C). We analyzed the effects of several p107 mutants in a cotransfection assay with the Gal4-Sp1 construct. As shown in Fig. 8B, repression of Gal4-Sp1-activated transcription was dependent on the N terminus of the p107 protein. The N-terminus deletion mutant p107N385, which carries a deletion of amino acids 1 to 385, was found to be

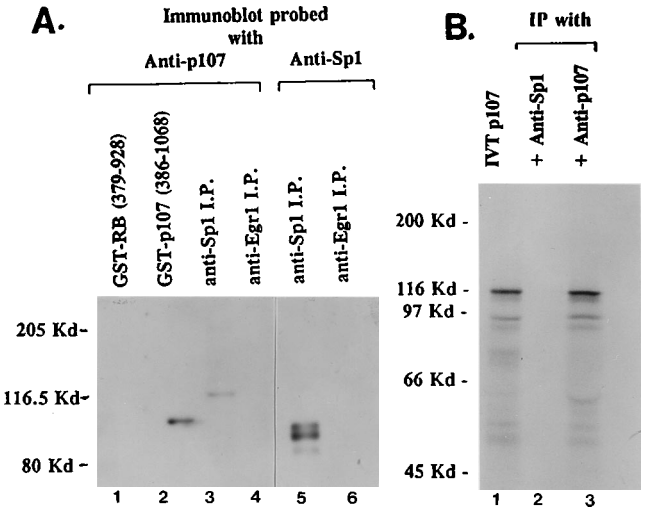

FIG. 6. (A) The Sp1 antibody coprecipitates p107. Pooled heparin-agarose fractions from an L-cell extract (400 μg) were immunoprecipitated with either the Sp1 or Egr1 antibody. The immunoprecipitates (I.P.) were eluted with SDS sample buffer and subjected to Western blot analysis. The Sp1 and Egr1 immunoprecipitates were immunoblotted with the p107 monoclonal antibodies (lane 3 and 4). One hundred-nanogram aliquots of GST-p107 (amino acids 386 to 1068) (lane 2) and GST-Rb (amino acids 379 to 928) (lane 1) were used as controls to determine the specificity of the p107 antibodies. The Sp1 and Egr1 immunoprecipitates were probed with the Sp1 antibody (lanes 5 and 6). (B) The Sp1 antibody does not cross-react with p107. ^{35}S-labeled p107 (10 μl) made in rabbit reticulocyte lysate was immunoprecipitated (IP) with the Sp1 antibody (lane 2) or p107 antibody (lane 3). The immunoprecipitated proteins were separated by SDS-PAGE (10% polyacrylamide gel) followed by autoradiography.

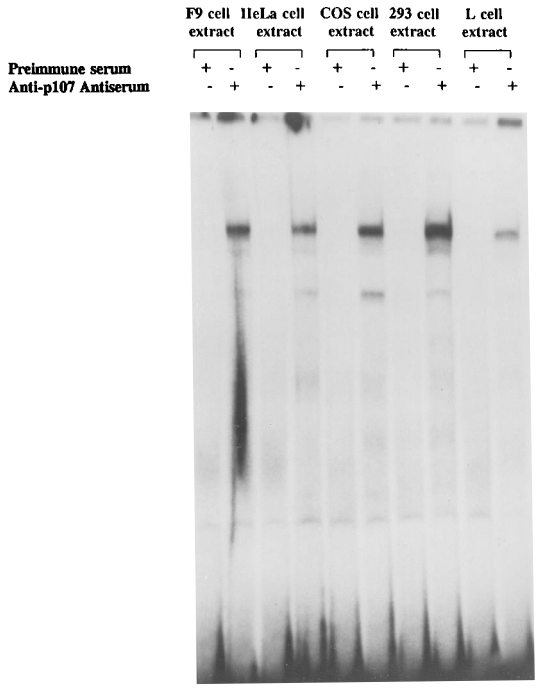

FIG. 7. Detection of an endogenous Sp1-p107 complex in the extracts of a variety of cell lines. Whole cell extracts were prepared from the indicated cell lines and equal amounts of the extracts (500 μg) were immunoprecipitated with a control antibody or the p107 antibody as described in Materials and Methods. The immunoprecipitates were eluted with 0.4 M KCl-containing buffer (25 μl). Aliquots of 4 μl were analyzed for Sp1-binding activity in a gel shift assay using a ^{32}P-labeled oligonucleotide containing an Sp1-binding site as a probe.

totally impaired in the repression of Sp1-activated transcription. Other p107 mutants carrying deletions in the pocket domain (p107DE) or in the C terminus (p107EC) of the protein showed no significant impairment. Both the pocket domain and the carboxyl terminus of p107 have been shown to be involved in the protein's growth suppression function (53). Involvement of the N terminus of p107 alone in the repression of Sp1-dependent transcription further indicates that this repression is a specific effect, not a reflection of inhibition of proliferation in p107-transfected cells. Rb and p107 have extensive sequence homologies in the pocket domains and C-terminal regions (17, 18). We analyzed the effect of the pocket domain and C terminus of the Rb protein in cotransfection assays using the Gal4-Sp1 construct in Saos-2 cells. Interestingly coexpression of Rb (between residues 379 and 928) showed no significant effect on Sp1-dependent transcription in this cell line (Fig. 8B).

p107 has also been shown to repress E2F-dependent transcription. This repression of E2F-dependent transcription required the pocket region of p107; the p107 mutant harboring an N-terminal deletion (pp107N385) showed no significant impairment in the repression of E2F-dependent transcription (53). We compared the repressions mediated wild-type and N-terminus mutant p107 on E2F- and Sp1-dependent transcription. In parallel assays, when cells were transfected together with the wild-type p107 expression plasmid, both Sp1- and E2F-dependent transcriptions were significantly repressed (Fig. 9A). However, when cells were cotransfected with the N-terminus mutant of p107 (p107N385), only the E2F-dependent transcription exhibited the same level of repression as that obtained with wild-type p107. The Sp1-dependent transcription, on the other hand, was not significantly repressed by

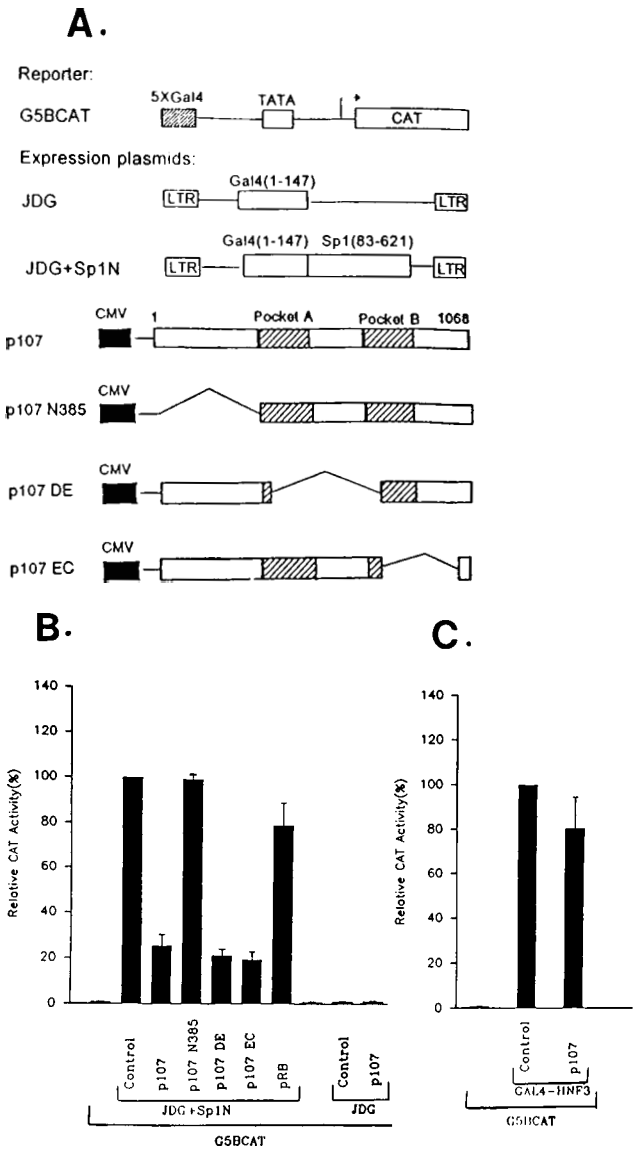

FIG. 8. p107 represses Gal4-Sp1-activated transcription. (A) Schematics of the CAT gene reporter construct (G5BCAT), Gal4-Sp1 construct, and p107 expression constructs. (B) Saos-2 cells were transfected with the CAT gene reporter plasmid G5BCAT (2 μg), which contains five Gal4 DNA-binding sites, along with the plasmid JDG (5 μg), which expresses the Gal4 DNA-binding domain only, or plasmid JDG+Sp1N (5 μg), which expresses a fusion protein containing the Gal4 DNA-binding domain and Sp1 amino acids 83 to 621. Where indicated, the transfection mixture also contained a control plasmid (pGEM), one of the p107 expression plasmids (4 μg), or an Rb expression plasmid (CMV Rb 379-928; 4 μg). Transfection and the CAT activity assays were performed as described in Materials and Methods. Relative CAT activities and standard deviations are shown; 100% activity represents 26% ± 4% acetylation. (C) The CAT gene reporter construct G5BCAT (2 μg) and a Gal4-HNF3 expression plasmid (5 μg) were cotransfected in Saos-2 cells together with a control plasmid (pGEM) or the p107 expression plasmid (4 μg). Relative CAT activities and the standard deviations are shown; 100% activity represents 33% ± 6.5% acetylation. For each experiment, transfections were repeated three times.

expression of the N-terminal mutant of p107. This result was reproduced three times in independent transfection experiments. We compared the level of expression of the p107 mutant p107N385 with that of the wild-type p107 protein in transfected Saos-2 cells by Western blot analysis. As shown in Fig. 9B, in transfected Saos-2 cells, the levels of expression of the

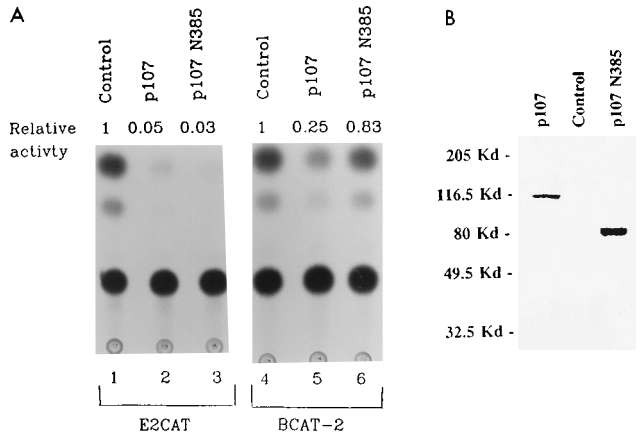

FIG. 9. Repression of Sp1-dependent transcription and repression of E2F-dependent transcription involve different regions of p107 polypeptides. (A) The CAT reporter gene constructs driven by the adenovirus early E2 promoter (E2 CAT; 1 μg) and that driven by two Sp1-binding sites (BCAT-2; 3 μg) were transfected in Saos-2 cells with either a wild-type p107 expression plasmid (4 μg) or the mutant p107 expression plasmid p107N385 (4 μg). Transfections and CAT gene activity assays were performed as described in Materials and Methods. Transfections were repeated four times, and the relative activities were calculated for each experiment. The means and standard deviations were calculated as follows: for plasmid E2 CAT based on 1.0 in lane 1, 0.05 ± 0.01 in lane 2 and 0.03 ± 0.00 in lane 3 (relative to 1.0 in lane 1); for plasmid BCAT-2, 0.25 ± 0.05 in lane 5 and 0.8 ± 0.12 in lane 6 (relative to 1.0 in lane 4). (B) Assay of p107 in transfected Saos-2 cells. Soluble proteins (200 μg) were extracted from Saos-2 cells 30 h after transfection with wild-type p107 (CMVp107) or mutant p107 (N385p107) expression plasmids. The proteins were analyzed in a Western blot assay using p107 monoclonal antibodies (SD cocktail). Mobilities of the molecular weight markers are shown on the left.

wild-type protein and the N-terminus deletion mutant of p107 were comparable. Thus, clearly, different regions of p107 are involved in the repression of E2F- and Sp1-dependent transcription.

p107 represses cellular and viral promoters containing Sp1 sites. We analyzed the effects of p107 expression on two natural promoters, the SV40 early promoter and human TGF-β1 promoter, that carried multiple Sp1-binding sites. In cotransfection assays, expression of wild-type p107 resulted in significant repression of transcription driven by both promoters (Fig. 10). Additionally, introduction of an Sp1-binding site to an otherwise nonresponsive promoter [E2F(−) E2 promoter] conferred p107 responsiveness to that promoter (Fig. 10). Taken together, the results that the cell cycle-regulatory protein p107 specifically associates with the transcription factor Sp1, with a consequent repression of the Sp1-dependent transcription.

DISCUSSION

The cellular protein p107 is a potent inhibitor of cell cycle progression, and overexpression of p107 arrests cells in the G_1 phase of the cell cycle (53). p107 has been shown to interact with several cellular proteins, including the transcription factor E2F (5, 6, 47, 49), the c-Myc protein (22), cyclin A (33), cyclin E (33), and cdk2 kinase (13, 33). The significance of p107's interaction with E2F has been studied in detail, and the formation of a p107-E2F complex has been shown to coincide with a repression of E2F-dependent transcription (49, 52, 53). Furthermore, the repression of E2F correlates with the growth suppression properties of p107. Our studies showed that p107 associates with Sp1 as well as with E2F. Using p107- and Sp1-specific antibodies, we found evidence for an endogenous interaction between p107 and Sp1. We observed that in immunoprecipitation experiments, the transcription factor Sp1 coprecipitated with p107 and vice versa. Interaction between p107 and Sp1 was detected in extracts of a variety of cell lines. However, we were unable to detect a p107-Sp1 complex that bound DNA. Thus, it is quite likely that the complex containing p107 and Sp1 does not bind DNA, at least under the conditions of the gel retardation assays. Our attempts to reconstitute the p107-Sp1 complex by using recombinant Sp1 and p107 polypeptides were also unsuccessful. It is possible that the interaction depends on posttranslational modifications that are missing in the recombinant proteins. Again, involvement of other polypeptides in the p107-Sp1 interaction cannot be ruled out.

Both E1A and T antigen have been shown to bind the pocket region of the p107 polypeptide and have been shown to dissociate complexes between E2F and p107 (6, 40). Interestingly, the interaction between p107 and Sp1 was detected in cell extracts that contained endogenous E1A and T antigen, which suggests that either the p107-Sp1 complex is more abundant than these oncoproteins in these extracts or the pocket region of p107 is not involved in the interaction with Sp1.

We also show that p107 is a potent regulator of Sp1-activated transcription and that a mutant of p107 lacking the N-terminal 385 amino acids (p107N385) is impaired in the repression of Sp1-activated transcription. This N-terminus mutant efficiently repressed the E2F-dependent transcription. Thus, separate regions of p107 are involved in the repression of E2F- and Sp1-dependent transcription. The amino-terminal region of p107 has been reported to be highly conserved among various species, including humans, mice, and *Xenopus laevis* (53). However, the N terminus of p107 has not been found to be involved in any known function of p107. Thus, the results presented here have clearly identified a novel function for the N terminus of p107. The N terminus of p107 has extensive sequence homology with p130, another Rb-related protein (23, 34). However, in our immunoprecipitation assay using one p130 monoclonal antibody, we did not detect any endogenous interaction between p130 and Sp1. Further analysis using different antibodies against p130 will be needed to confirm this observation. The growth suppression function of p107 has been analyzed in colony suppression assay using Saos-2 cells (53). These studies indicated that the pocket region and the carboxyl terminus are important for the growth

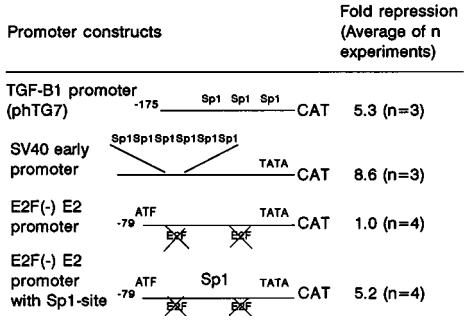

FIG. 10. The CAT reporter gene constructs driven by the TGF-β1 promoter (phTG7; 2 μg), SV40 early promoter (pCAT-promoter [Promega]; 4 μg), E2F(−) E2 promoter (10 μg), or E2F(−) Sp1 promoter (10 μg) were cotransfected in Saos-2 cells with the wild-type p107 expression plasmid (6 μg) or a control plasmid (pGEM-4). Transfections and CAT gene activity assays were performed as described in Materials and Methods. The fold repression was calculated by comparison with the CAT activity of the control plasmid for each transfection experiment. The average fold repression values from several independent transfection are presented.

suppression function of p107 and that the N terminus of p107 is dispensable. It is possible that regulation of the Sp1-dependent transcription by the N terminus of p107 by itself is not sufficient to suppress cell proliferation in colony suppression assays. It is also possible that the effect of p107's N terminus is cell type specific. Clearly, further investigation will be needed to determine the biological functions of the N terminus of p107.

Ironically, many cell cycle-regulated genes contain both E2F- and Sp1-binding sites in their promoters; such genes include the *dhfr*, thymidine kinase, thymidylate synthetase, DNA polymerase α, c-*myc*, E2F-1, and cdk2 kinase genes (1, 37). It is possible that cell cycle-regulated transcription of these genes involves both of these factors. Previous reports have established an association between E2F and cell cycle-regulatory proteins p107, p130, and Rb. Our results provide evidence for a specific association between Sp1 and the cell cycle-regulatory protein p107. It is quite possible that p107 regulates these cellular genes through both E2F- and Sp1-binding sites. It has been shown that the accumulation of p107 protein reaches the highest level in the S phase of the cell cycle (7a). It is likely that this heightened expression of p107 has a regulatory function in S phase. We speculate that in S phase, p107 turns off the above-mentioned proliferation-associated genes that are turned on during the G_1-to-S transition and that the Sp1-p107 interaction plays a role in this process.

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