Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 11002–11006, November 1994
Biochemistry

Down-regulation of Rous sarcoma virus long terminal repeat promoter activity by a HeLa cell basic protein

(transcription/transformation)

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Contributed by Aaron J. Shatkin, July 19, 1994

ABSTRACT **We have previously isolated a HeLa cell cDNA encoding a 21-kDa polypeptide that is 48% similar to transcription factor IIS. To explore the possibility that p21 plays a role in transcriptional regulation *in vivo*, we tested the effect of p21 expression on the synthesis of reporter chloramphenicol acetyltransferase (CAT) in transfected COS-1 cells. CAT formation under control of the Rous sarcoma virus long terminal repeat (RSV LTR) promoter was decreased nearly 20-fold in cells coexpressing p21. In contrast, CAT production under control of other sequence elements was only slightly reduced (human immunodeficiency virus type 1 LTR, simian virus 40 early promoter), unaffected (human heat shock protein of 70-kDa promoter, adenovirus major late promoter TATA box), or increased (terminal deoxynucleotidyltransferase initiator element, c-*fos* promoter) by p21 coexpression as compared to cells cotransfected with the parental vector. The abundance of steady-state CAT transcripts from RSV LTR was also decreased by p21 expression in a dose-dependent manner, suggesting that transcription of RSV LTR/CAT is under negative control by p21. Consistent with an effect on transcription, p21 was localized in nuclei of transfected cells. Deletion analysis of p21 indicated that the sequences essential for inhibition of RSV LTR function include the previously identified Arg/Ser-rich region and zinc finger-like motif. Proliferation of chicken embryo fibroblasts transfected with an infectious molecular clone of RSV was diminished by p21 expression, which also resulted in fewer transformed foci.**

It is well established that the expression of most genes is regulated at the level of transcription by a combination of cis control elements and the availability of transcription factors (1, 2). Both general transcription factors and specific DNA-binding proteins contribute to the regulation of individual promoters. Those promoters that can respond to numerous cellular regulators, like the long terminal repeat (LTR) elements of some retroviruses, usually direct high levels of gene expression. Comparative transcriptional studies of several retrovirus LTRs have demonstrated that Rous sarcoma virus (RSV) LTR is among the strongest promoters and mediates high levels of transcription (3). The high steady-state level of RSV RNAs presumably results from efficient transcription by host cell RNA polymerase II as well as the positive effects of multiple cellular factors on the LTR (4).

The DNA sequences responsible for RSV LTR promotion of transcription have been delineated by deletion mutagenesis (5, 6). Cis-acting elements within the LTR region consist of a mosaic of several different sequence motifs that bind cellular factors, resulting in activated transcription. The sequence located between the 5′ end of the LTR and an *Sph* I site at −141 from the RSV transcription start site includes binding targets for cellular factors EFII (7), C/EBP-like protein (8), and FI (9). Located downstream of the *Sph* I site are two inverted CCAAT boxes that bind cellular protein EFI (10) and a CArG motif, defined as 5′-CC(A/T)GG-3′, which binds the EFIII protein (11). The nature of these cellular factors involved in the regulation of viral gene expression is largely unknown. While many cellular factors augment LTR activity, very few have been shown to down-regulate LTR-promoted gene expression.

In this report, we demonstrate that overexpression of a HeLa cell basic protein p21 (12) exerts a specific inhibitory effect on RSV LTR promoter activity in transfected COS-1 cells and chicken embryo fibroblasts (CEFs). The inhibition apparently occurs at the RNA level and appears not to involve direct binding of p21 to the target sequence. Instead, p21 may exert its effects on the RSV LTR via protein–protein interactions with other transcriptional regulators.

MATERIALS AND METHODS

Materials. Restriction endonucleases and DNA modifying enzymes were purchased from New England Biolabs. Oligonucleotides used for PCR mutagenesis and gel shift assays were synthesized in the DNA Synthesis Laboratory at University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School. All radionucleotides were obtained from Amersham.

Plasmid Construction. The chloramphenicol acetyltransferase (CAT) reporter gene constructs pR-CAT (13), pSV-CAT (14), pAdMLP TATA-CAT, pTdTinr-CAT (15), phsp70-CAT (16), and pc-fos-CAT (17) and the infectious molecular clone of RSV SRA-2 (18) have been described. pAdMLP TATA-CAT and pTdTinr-CAT contained identical simian virus 40 (SV40) 21-bp repeats and differed downstream in having either the adenovirus major late promoter (AdMLP) TATA box or the terminal deoxynucleotidyltransferase gene initiator (TdTinr) sequence to direct the initiation of transcription. pHIVLTR-CAT and pSV-Tat were kindly provided by A. B. Rabson (Center for Advanced Biotechnology and Medicine). The cDNA encoding p21 (12) was subcloned from pBluescript into the pBC12BI parental expression vector (13) between the *Xho* I and *Xba* I sites. For hemagglutinin (HA) tagging, the p21 cDNA was first fused with an influenza virus HA epitope DNA (19) by PCR and then subcloned into pBC12BI via *Xho* I and *Eco*RV sites. Three deletion mutants of the resulting vector for expression of HA-tagged p21 (p21HA) were constructed by oligonucleotide-directed mutagenesis to generate unique *Pvu* II or *Dra* I sites, followed by digestion with the corresponding restriction enzymes and religation. All plasmids were isolated on Qiagen columns as recommended by the manufacturer.

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Abbreviations: RSV, Rous sarcoma virus; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; CEF, chicken embryo fibroblast; SV40, simian virus 40; HA, hemagglutinin; HTH, helix–turn–helix; pd, putative domain.

Transient Transfection and CAT Assays. COS-1 cells were cotransfected with the indicated amounts of reporter and effector plasmids by the DEAE-dextran method with chloroquine and dimethyl sulfoxide (DMSO) treatments as described (13, 14). CAT was assayed 48 hr posttransfection as follows. Cells on 60-mm dishes were washed three times with phosphate-buffered saline (PBS) and scraped into 0.75 ml of TEN (40 mM Tris·HCl, pH 7.8/1 mM EDTA/150 mM NaCl). Cells were collected by brief centrifugation, resuspended in 0.15 ml of 250 mM Tris·HCl (pH 7.8), and disrupted by freeze–thawing four times. After clearing by centrifugation at 4°C for 1 min at 12,000 × *g*, cell extracts were serially diluted for optimal detection of the CAT protein by ELISA as recommended by the supplier (5 Prime → 3 Prime, Inc.).

Northern Blot Analyses. Total cellular RNA was isolated from duplicate plates (14). RNA (10 μg) was electrophoresed through a 1% agarose gel containing 2.2 M formaldehyde, transferred to a nitrocellulose filter (Schleicher & Schuell), and hybridized to a random-primed ^{32}P-labeled 0.77-kb CAT DNA probe, which was generated by *Hin*dIII/*Bam*HI excision from pR-CAT. Hybridizations were carried out at 68°C for 1 hr in ExpressHyb hybridization solution (Clontech), and the membrane was washed at room temperature with 2× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.05% SDS for 40 min followed by 40 min at 50°C with 0.1× SSC/0.1% SDS. As an internal control, the same blot was stripped and reprobed with a random-primed ^{32}P-labeled β-actin probe.

Immunological Assays. To detect p21HA expression, COS-1 cells were transfected with the indicated amounts of p21HA DNA or the parental pBC12BI by the DEAE-dextran method (14). Total cell lysates were then subjected to SDS/PAGE on a 12% gel followed by Western blot analysis with anti-HA monoclonal antibody (19) and detection by enhanced chemiluminescence (Amersham).

For immunofluorescence, COS-1 cells transfected with p21HA DNA or the parental plasmid were grown on glass coverslips, fixed with methanol for 15 min at −20°C, and washed with PBS. The cells were then incubated for 1 hr at 37°C with a 1:200 dilution of anti-HA antibody (19), washed extensively in PBS, incubated with rhodamine-labeled anti-mouse IgG antibody (1:100 dilution, 37°C, 1 hr), and examined by fluorescence microscopy.

CEF Transfections. One day before DNA transfection by the calcium phosphate precipitation technique (18), primary CEFs were seeded at a density of 4 × 10^5 cells per 60-mm plate in 5 ml of ETF2C2 medium containing 80% Dulbecco's modified Eagle's medium (DMEM; GIBCO), 20% tryptose phosphate broth, 2% calf serum, and 2% fetal bovine serum. Five micrograms of cloned SRA-2 DNA (18) was mixed with an equal amount of calf thymus DNA, p21HA expression vector, or pBC12BI parental vector in 0.5 ml of Hepes-buffered saline (HBS; 20 mM Hepes, pH 7.0/137 mM NaCl/5 mM KCl/7 mM Na_2HPO_4/6 mM glucose). To precipitate the DNA, 0.1 vol of 1.25 M $CaCl_2$ was added dropwise. After 30 min, 0.5 ml of the mixture was applied directly to the medium (5 ml) in each 60-mm culture plate. After 4–6 hr at 37°C, the medium was decanted, and the cell monolayers were treated with 1 ml of 30% DMSO in HBS for 3 min, washed once with ETF2C2 medium, and subsequently maintained in fresh medium at 37°C. Cell proliferation and transformed foci were visualized after 6 and 12 days by Giemsa staining.

RESULTS

Inhibition of RSV LTR-Directed CAT Expression by p21. HeLa cell p21 is a basic protein that possesses significant sequence homology to transcription factor TFIIS (12). We therefore asked whether p21 overexpression would have any effect on transcription *in vivo*. To test this possibility, COS-1 cells were cotransfected with a reporter plasmid containing the CAT gene driven by the RSV LTR (or a different promoter) and a second plasmid for expression of p21 (or p21 tagged with the influenza HA epitope) as an effector. Extracts of cells transfected with 0.5 μg of pR-CAT and 5 μg of p21 or p21HA DNA contained nearly 20-fold less CAT protein compared to transfection with either pR-CAT alone or in combination with the parental vector pBC12BI (Fig. 1*A*). Repression of pR-CAT expression was progressively greater with increasing amounts of either p21 or p21HA effector plasmid (Fig. 1*B*). This decrease apparently was not due to nonspecific effects of p21 on the RSV LTR—e.g., squelching (20)—because expression of CAT under control of human immunodeficiency virus type 1 LTR (either with or without Tat) or the SV40 early promoter was decreased by less than 2-fold (Fig. 1*C*). In addition, p21 had little or no effect on CAT production from plasmids containing human hsp70 or adenovirus major late promoter TATA sequences (Fig. 1*C*). By contrast, CAT synthesis from terminal deoxynucleotidyltransferase initiator sequence and c-*fos* promoter was increased by p21 coexpression (Fig. 1*C*). These results demonstrate that p21 expression in COS-1 cells strongly and specifically inhibits RSV LTR promoter function.

Decreased Levels of RSV LTR/CAT Steady-State Transcripts in p21-Expressing Cells. To test whether the inhibition of RSV LTR/CAT expression occurs at the RNA level, Northern blot analyses were performed with a CAT-specific probe. In agreement with the CAT protein synthesis results, transfection with 0.5 μg of pR-CAT and 4 μg of p21 DNA decreased steady-state CAT transcript levels by 5- to 6-fold compared to levels in cells cotransfected with 4 μg of pBC12BI (Fig. 2*A*, compare lanes 1 and 2). Lower amounts of p21 DNA resulted in a dose-dependent recovery of CAT RNA levels (lanes 2–4). At 1 μg of p21 DNA there was 2- to 3-fold more CAT RNA than in cells cotransfected with 0.5 μg of pR-CAT and 4 μg of parental vector, presumably due to promoter competition at the higher DNA concentration (14). These effects were not due to differences in RNA loading as indicated by the levels of endogenous β-actin transcripts (Fig. 2*B*). The results suggest that p21 expression decreases pR-CAT transient expression at the RNA level in transfected cells.

Nuclear Localization of p21. In an effort to establish more clearly a functional link between p21 and the concentration-dependent inhibition of pR-CAT shown in Fig. 1*B*, expression levels and subcellular localization of p21 were determined in COS-1 cells transfected with p21HA expression plasmid. Western blot analysis was performed with total cell extracts of COS-1 cells transfected with increasing amounts of p21HA or the parental pBC12BI DNA. As shown in Fig. 3*A*, the monoclonal anti-HA antibody reacted with a major band of 31 kDa, the HA-tagged p21 protein, only in extracts of p21HA DNA-transfected cells, and p21 expression increased in parallel with input DNA levels as quantitated by a PhosphorImager. It should be noted that untagged p21 migrates in a position corresponding to 27 kDa (C.-H.Y., unpublished results), possibly due to an intrinsic property such as the extremely basic nature of the protein (12). The minor, higher molecular weight band present in all lanes apparently corresponds to a cross-reactive endogenous protein. Immunofluorescent staining of transfected cells demonstrated that the HA-tagged p21 polypeptide is present predominantly in nuclei, while parental vector-transfected cells displayed some background cytoplasmic staining probably resulting from the cross-reactive protein detected in the Western blot (Fig. 3*B*).

Both the Arg/Ser-rich Region and the Zinc Finger-like Motif in p21 Are Necessary for Inhibition of RSV LTR Activity. We next mapped the region(s) in p21 necessary for its effects on CAT expression under control of RSV LTR. Examination of the primary sequence of p21 (12) revealed three putative

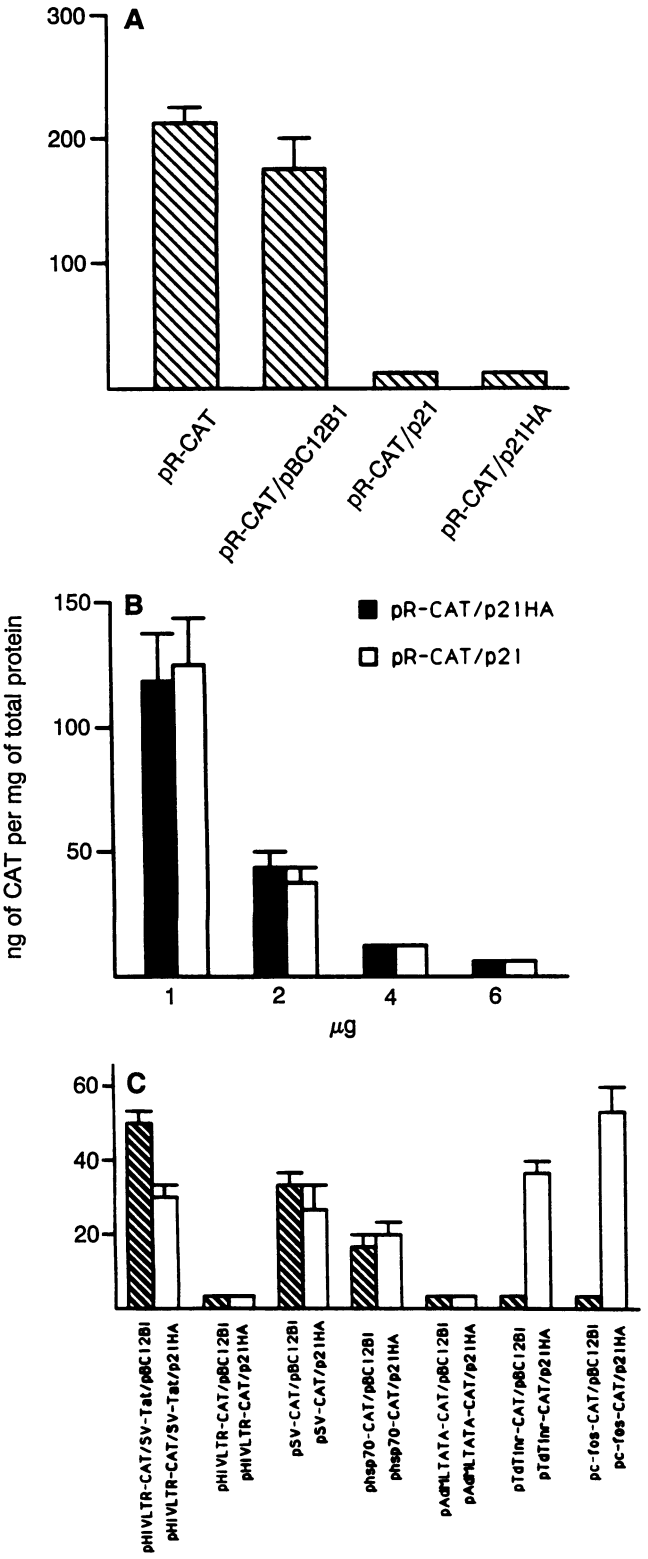

FIG. 1. Decreased RSV LTR-directed CAT expression in cells expressing p21 or p21HA. (*A*) COS-1 cells were transfected with 0.5 μg of pR-CAT alone or with 5 μg of each of the indicated expression plasmids. At 48 hr after transfection, cell extracts were assayed for CAT expression by ELISA. (*B*) pR-CAT (0.5 μg) and the indicated amounts of p21 or p21HA DNA were cotransfected into COS-1 cells, and CAT protein was measured by ELISA in cell extracts prepared 48 hr posttransfection. (*C*) As described for *A*, with the indicated CAT reporter DNAs (0.2 μg) and 2 μg of pBC12BI or p21HA DNA, except for pHIVLTR-CAT (0.2 μg) and SV-Tat (1 μg) where 1 μg of parental vector or p21HA DNA was used. Values represent means ± SD for three experiments.

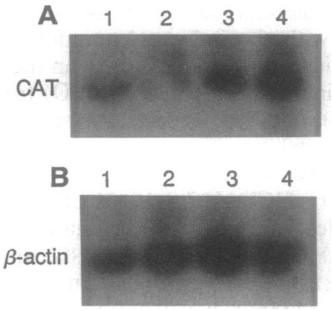

FIG. 2. Effect of p21 expression on CAT RNA levels. (*A*) Northern blot analysis of 10 μg of RNA isolated from COS-1 cells transfected 48 hr earlier with 0.5 μg of pR-CAT and 4 μg of pBC12BI parental vector (lane 1) or 4 μg (lane 2), 2 μg (lane 3), or 1 μg (lane 4) of p21 expression plasmid. The filter was hybridized with a ^{32}P-labeled CAT-specific probe followed by autoradiography. Relative levels of CAT transcript in lanes 1–4, measured by PhosphorImager and normalized for loading, were 1.0, 0.2, 1.3, and 3.3, respectively. (*B*) The same blot was stripped and rehybridized with a β-actin probe as an internal control for RNA loading.

functional domains (Fig. 4*A*). Close to the N terminus is an Arg/Ser-rich stretch (amino acids 4–72) that possesses 80% sequence homology to the RS domain of chicken and human pre-mRNA splicing factor SC35 (21) and also carries multiple Ser-pair clusters likely to be targets of phosphorylation (12, 22). The central region of p21 (amino acids 58–93) contains a zinc finger-like motif (C-X_2-C-X_{26}-C-X_4-H), followed by a C-terminal helix–turn–helix (HTH) structure (amino acids 95–148) (23). We used cotransfection assays to test the effect on CAT expression of p21 mutant proteins deleted in each of the three putative domains: pd12–49, pd50–100, and pd101–149. To correct for potential differences in mutant p21 expression and/or stability, HA-tagged products were used and protein levels were determined by ELISA with anti-HA antibody. As shown in Fig. 4*B*, p21HA expression decreased CAT levels from pR-CAT by >15-fold, while the same amount of mutant protein deleted in either the Arg/Ser-rich domain (pd12–49) or the zinc finger-like motif (pd50–100) had dramatically reduced inhibitory effects. In contrast, the HTH mutant pd101–149 retained inhibitory activity and decreased expression from pR-CAT to an extent similar to that observed with the same amount of p21HA (Fig. 4*B*). These deletion analyses indicate that the inhibitory effects of p21 on pR-CAT require the presence of both the Arg/Ser-rich region and the zinc finger-like motif.

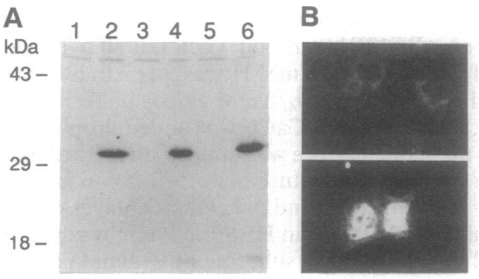

FIG. 3. Expression and subcellular localization of p21HA. (*A*) COS-1 cells were transfected with 1, 2, and 4 μg of p21HA expression plasmid (lanes 2, 4, and 6, respectively) or pBC12BI parental vector (lanes 1, 3, and 5, respectively). Equal amounts of whole cell extracts were assayed by SDS/PAGE followed by a Western blot with monoclonal antibody against an influenza HA epitope. Relative levels of p21HA in lanes 2, 4, and 6 as determined by PhosphorImager were 1.0, 1.8, and 3.3, respectively. (*B*) Immunofluorescence staining of COS-1 cells transfected with either the pBC12BI (*Upper*) or p21HA expression plasmid (*Lower*). Cells were fixed and treated with anti-HA antibody 48 hr posttransfection.

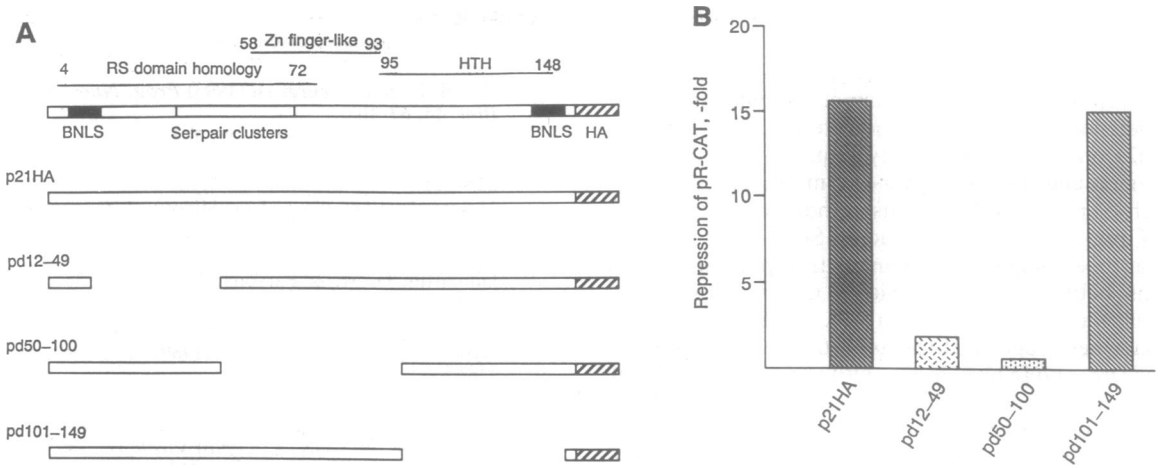

FIG. 4. Regions required for p21 inhibition of RSV LTR. (*A*) Schematic of p21HA and three deletion mutants. p21 contains 157 amino acids including two bipartite nuclear localization signals (BNLS), multiple Ser-pair clusters, and three putative functional domains: the Arg/Ser-rich sequence, the zinc finger-like motif, and the HTH structure (12). Influenza virus HA epitope was added to the C terminus. (*B*) Repression of pR-CAT expression (-fold) in cotransfected COS-1 cells by each of the p21HA derivatives shown in *A*. Values were normalized for p21HA and mutant protein concentrations as determined by ELISA. Repression was calculated from the CAT protein levels obtained in the presence of p21HA or the indicated deletion mutant proteins as compared to pBC12BI parental vector. The calculated -fold decreases in CAT measured by ELISA for p21HA, pd12–49, pd50–100, and pd101–149 were 16, 3.2, 1.1, and 16, respectively.

p21 Expression Diminishes Proliferation and Transformation of CEFs by RSV cDNA. Primary CEFs grown to near confluence in tissue culture consist of nondividing, quiescent cells that can be induced to proliferate and form foci of transformed cells by introduction of the viral oncogene v-*src* under control of the LTR in the RSV genome. To assess the biological significance of the p21 inhibition of RSV LTR observed in COS cells, we studied the effect of p21 on transformation of CEFs by v-*src*. Primary CEFs were cotransfected with SRA-2, an infectious replication-competent molecular clone of RSV, and either p21 expression plasmid or its parental vector DNA. At 6 days after transfection, stained cell cultures appeared similar, and cell proliferation was not obvious (Fig. 5*A*). However, by 12 days cultures transfected with SRA-2 DNA alone or with SRA-2 plus parental vector pBC12BI were proliferating and contained many foci of transformed cells. Cultures cotransfected with SRA-2 and p21HA DNA grew less and contained smaller, fewer foci, while mock-transfected cells were essentially unchanged at 12 days posttransfection (Fig. 5*A*). To verify that p21 expression continued in transfected CEFs, cultures were radiolabeled with [^{35}S]methionine for 4 hr 12 days after transfection with p21HA. Immunoprecipitation of cell extracts with HA-directed antibody detected p21HA expression only in the cell culture that had been transfected with p21HA DNA (Fig. 5*B*).

DISCUSSION

We have shown that expression of p21, a TFIIS-related protein, can down-regulate RSV LTR promoter activity. The inhibitory effect exerted by p21 may involve interactions with other cellular factors rather than directly with DNA since gel mobility shift assays with *in vitro* synthesized p21 and DNA fragments containing RSV LTR and upstream sequences, including the SV40 *ori*, failed to detect direct binding of p21 to the template (data not shown). Transcription of specific genes can be down-regulated in various ways by transcriptional inhibitors: for example, some can compete with specific transcriptional activators for DNA-binding sites (24); others form protein complexes with transcription activators, thereby altering or reducing their DNA-binding activity (25); and certain DNA-binding transcriptional repressors down-regulate transcription directly rather than inhibit specific activators (26). In addition, alterations in chromatin structure

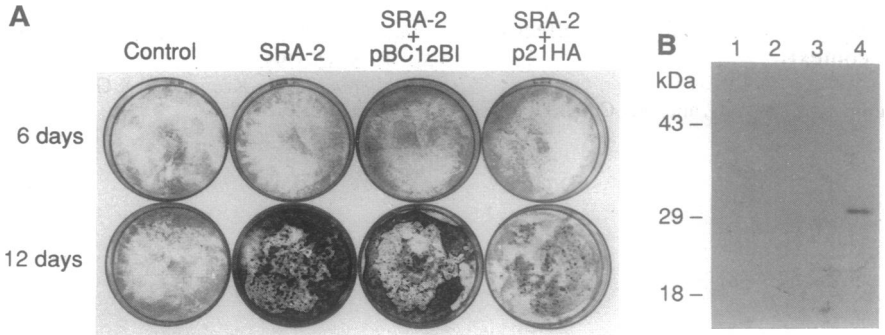

FIG. 5. Effect of p21 expression on RSV-mediated transformation of CEFs. (*A*) Primary CEFs were transfected by the calcium phosphate procedure with 10 μg of calf thymus carrier DNA (control) or 5 μg of carrier plus 5 μg of SRA-2 DNA, 5 μg of SRA-2 plus 5 μg of pBC12BI, or 5 μg of SRA-2 plus 5 μg of p21HA. Plates were fixed and stained with Giemsa after 6 and 12 days. Number of transformed foci in control, SRA-2, SRA-2 plus pBC12BI, and SRA-2 plus p21HA was 0, 66, 52, and 28, respectively, and in another experiment it was 0, 41, 31, and 13, respectively. (*B*) At 12 days posttransfection as in *A*, cultures were labeled for 4 hr with [^{35}S]methionine. Cell lysates were immunoprecipitated with anti-HA antibody, and the immunoprecipitated complexes were analyzed by SDS/12% PAGE. Lanes: 1, control; 2, SRA-2 DNA; 3, SRA-2 plus pBC12BI DNA; 4, SRA-2 plus p21HA plasmid.

or nucleosome repositioning can also mediate decreases in transcription (27).

Although the mechanism of transcriptional repression by p21 remains to be determined, an important clue may come from the deletion studies, which indicate that the inhibitory effect of p21 on RSV LTR activity requires both the Arg/Ser-rich region and the zinc finger-like motif. The Arg/Ser-rich stretch of p21 displays extensive homology to the RS domain of pre-mRNA splicing factor SC35 (21). The RS domain has been implicated in mediating protein–protein interactions between several splicing factors (28) and is reminiscent of a regulatory domain (29). Furthermore, basic residues have been reported to be important for the repressor function of *Drosophila* protein Eve (30) and several artificial repressors (31). Although the requirement of a zinc finger-like motif for p21 inhibitory activity may reflect an adverse effect of its deletion on protein folding, it may also suggest that p21 can bind DNA indirectly after contacting other protein(s). For example, the inhibition by p21 could be the result of p21 counteracting the effect of an activator either by forming an inactive heterodimer with the putative factor or by replacing it on the DNA with a p21-containing protein complex.

It is important to note that p21 is homologous to TFIIS, notably in the RNA polymerase-binding site (12), but the second TFIIS domain is absent from p21. In this regard, it is possible that p21, like the TFIIS mutant Δ230–280 (32), may inhibit transcription by competing with TFIIS for binding to engaged RNA polymerase II and impairing its ability to read through potential pause sites within the CAT gene in pR-CAT but not in some other CAT constructs. Examples of promoter-dependent determinants of transcription elongation and termination have been described (33–37). It will be important to determine whether p21 interacts either positively or negatively with other factors that can recognize the RSV LTR promoter.

The ability of p21 to influence expression of the CAT reporter constructs described in this report appears to be promoter context dependent—e.g., p21 decreased CAT expression from human immunodeficiency virus type 1 LTR and SV40 early promoter by <2-fold as compared to 20-fold for RSV LTR. Comparison of nucleotide sequences from these promoters revealed limited if any homology. Moreover, examination of the effect of p21 expression on several cellular promoters revealed diverse results: terminal deoxynucleotidyltransferase initiator sequence and c-*fos* promoter, as measured by expression of a transfected CAT reporter, were activated by >10-fold by cotransfection of a p21 expression vector. By contrast, p21 expression had no significant effect on CAT synthesis under control of human hsp70 promoter. From these results, it appears that p21 contains both activating and repressing potential, the predominant effect determined by promoter context.

We thank Dr. L. A. Laimins for plasmids, and Drs. Céline Gélinas, Arnold Rabson, and Danny Reinberg for critical reading of the manuscript and valuable discussion.

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