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Inhibition of E2F Activity by the Cyclin-Dependent Protein Kinase Inhibitor p21 in Cells Expressing or Lacking a Functional Retinoblastoma Protein

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p21$^{Sdi1/WAF1/Cip1}$ inhibits cyclin-dependent protein kinases and cell proliferation. p21 is presumed to inhibit growth by preventing the phosphorylation of growth-regulatory proteins, including the retinoblastoma tumor suppressor protein (pRb). The ultimate effector(s) of p21 growth inhibition, however, is largely a matter of conjecture. We show that p21 inhibits the activity of E2F, an essential growth-stimulatory transcription factor that is negatively regulated by unphosphorylated pRb. p21 suppressed the activity of E2F-responsive promoters (dihydrofolate reductase and cdc2), but E2F-unresponsive promoters (c-*fos* and simian virus 40 early) were unaffected. Moreover, the simian virus 40 early promoter was rendered p21 suppressible by introducing wild-type, but not mutant, E2F binding sites; p21 suppressed a wild-type, but not mutant, E2F-1 promoter via its autoregulatory E2F binding site; p21 deletion mutants showed good agreement in their abilities to inhibit E2F transactivation and DNA synthesis; and E2F-1 (which binds pRb), but not E2F-4 (which does not), reversed both inhibitory effects of p21. Despite the central role for pRb in regulating E2F, p21 suppressed growth and E2F activity in cells lacking a functional pRb. Moreover, p21 protein (wild type but not mutant) specifically disrupted an E2F–cyclin-dependent protein kinase 2–p107 DNA binding complex in nuclear extracts of proliferating cells, whether or not they expressed normal pRb. Thus, E2F is a critical target and ultimate effector of p21 action, and pRb is not essential for the inhibition of growth or E2F-dependent transcription.

Eukaryotic cells can exist in a reversibly quiescent growth state, G_0, from which they can be stimulated to proliferate by mitogens. The transition from G_0 to the S phase of the cell cycle entails the sequential induction or activation of growth stimulators. It also entails the repression or inactivation of growth inhibitors (54).

Among the events that stimulate the G_0-to-S transition is the sequential activation of cyclin-dependent protein kinases (Cdks). Cdks comprise a family of serine kinases whose activation requires the binding of a regulatory cyclin. Several Cdk catalytic subunits and cyclin regulatory subunits have been identified (17, 45, 47, 53). The cell type specificity, regulators, and components of Cdk-cyclin complexes are incompletely understood. Nonetheless, a broad picture of how Cdks regulate entry into S phase has recently emerged.

During the G_0-to-S transition, Cdk-cyclin complexes are sequentially activated: Cdk4- or Cdk6-cyclin D in early to mid-G_1, Cdk2-cyclin E in mid- to late G_1, and Cdk2-cyclin A in late G_1 to early S (47, 53, 55, 64). Active Cdks are presumed to phosphorylate regulatory proteins, thereby triggering events that are essential or permissive for G_1 progression. In fact, Cdk substrates whose phosphorylation is critical for the onset of S phase are largely unknown. An exception is the retinoblastoma tumor suppressor protein, pRb. pRb is underphosphorylated in quiescent cells, where it is thought to suppress growth. As cells approach S phase, pRb is progressively phosphorylated, at least in part by Cdks, which extinguishes its growth-suppressive properties (3, 5, 10, 46).

Underphosphorylated pRb binds several proteins. One of these, E2F, is a heterodimeric transcription factor composed of one DP and one E2F protein, of which several family members are known. E2F proteins bind pRb and/or the pRb-related proteins p107 and p130 (2, 4, 6, 24, 30, 31, 33, 37, 41, 42, 56, 58, 61, 62, 70). Of the five known E2F proteins, E2F-1 is not expressed by quiescent cells, is induced in mid-G_1, and appears to be essential for the induction of several genes just before S phase (11). Underphosphorylated pRb binds E2F-1. pRb phosphorylation releases E2F-1, permitting active E2F-1-DP complexes to form. One model of G_1 progression suggests that sequential Cdk activation leads to progressive pRb phosphorylation, which leads to E2F-1-dependent E2F activity and the transcription of genes needed for DNA synthesis (11, 22, 32, 41, 51, 73). In support of this model, E2F-1 expression is sufficient to induce DNA synthesis in quiescent rat fibroblasts (36, 39, 57), although other cells may require additional factors (16). In addition, E2F-1 is repressed in irreversibly G_1-arrested senescent cells, and the cells lack E2F complexes associated with the promoter for dihydrofolate reductase (DHFR), a late-G_1 gene (16).

Recently, a family of Cdk inhibitors was identified (65). These proteins inhibit Cdk-dependent phosphorylation, and they arrest cell proliferation when highly expressed. The first Cdk inhibitor to be discovered is a 21-kDa protein alternatively termed p21, Sdi1, WAF1, and Cip1. The p21 cDNA was independently cloned by virtue of encoding an overexpressed mRNA in senescent human fibroblasts (52), a p53-inducible transcript (19), and a Cdk2-associated protein (26, 28, 75). p21

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inhibits the activity of Cdk-cyclin complexes and the onset of DNA replication.

p21 is presumed to block DNA replication at least in part by inhibiting pRb phosphorylation. This inhibition should in turn inhibit E2F activation in late G_1. It is not yet known whether p21 inhibits E2F activity, much less whether pRb is central to this inhibition. Here, we show that E2F is indeed a critical downstream target of p21. Surprisingly, our results also indicate that there is a pRb-independent pathway for this activity of p21.

MATERIALS AND METHODS

Cell culture. A31 cells are clonally derived, immortal but nontumorigenic, BALB/c murine 3T3 fibroblasts, previously described (44). U2OS human osteosarcoma cells, C33a human cervical carcinoma cells, and HTB-9 (5637) human bladder carcinoma cells were from the American Type Culture Collection (Rockville, Md.). A31 and U2OS cells express wild-type pRb, whereas C33a and HTB-9 cells lack a functional pRb (6, 25, 69, 70). Cells were grown in 10% calf (A31) or fetal calf (U2OS, C33a, and HTB-9) serum, as described previously (16, 44).

Vectors. CMV-p21 expressed full-length human p21 (49, 52), and CMV-p21Δs expressed p21 with internal deletions (49), both from the human cytomegalovirus early promoter (CMV). CMV1, CMV-E2F1, CMV-T, CMV-β-gal, DHFR-CAT, and cdc2-CAT have been described before (16). CMV-T[K1] was made by inserting into CMV1 the *Bam*HI fragment of pSG5-K1 (from J. DeCaprio) encoding a pRb-p107-p130-binding-defective T antigen (Glu-107→Lys-107) (9, 20). CMV-T[p] was made by inserting into CMV1 the *Bam*HI-*Bgl*I fragment of Tdl434-444 (from J. Tevethia) encoding a p53-binding-defective T-antigen (amino acids 434 to 444 were deleted) (38). pSV-CAT and pCAT-basic were from Promega (Madison, Wis.), and c-fos-CAT (63) was from J. Fridovich-Keil. Wild-type (pGL2-AN) and mutant (pGL2-ANΔE2FA+B) E2F-1 promoters (50), from W. Kaelin, were subcloned into pCAT-basic. To make SV/E2F-CAT and SV/E2Fm-CAT, three copies of the DHFR E2F binding region (16, 22, 66, 71) were inserted into the *Bgl*II site of pSV-CAT. The E2F binding sites were wild type in SV/E2F-CAT ([binding site is underlined] 5′ CCCGACTGCAATTTCGCGCCAAACTTGGG 3′) and mutant in SV/E2Fm-CAT ([mutant bases are indicated by lowercase letters] 5′ CCCGACTGCAATTTCGatCCAAACTTGG G 3′). Human E2F-4 was cloned from our fibroblast cDNA library in λZap-Express (Stratagene; La Jolla, Calif.), using primers derived from the published sequence (1, 24) and a PCR probe; the phage was converted to plasmid, as instructed by the supplier (Stratagene), to generate CMV-E2F4.

Transfection. Cells were plated at 0.5×10^4 to 1×10^4/cm^2 on 35-mm-diameter dishes for DNA synthesis and immunostaining (containing coverslips for immunostaining) or on 60-mm-diameter dishes for reporter activity. Sixteen to twenty-four hours later, the cells were proliferating and were transfected with the indicated expression, reporter, and/or CMV-β-gal normalization vectors with Lipofectamine (Gibco-BRL; Gaithersburg, Md.), as previously described (16).

Transactivation. Cells were scraped into phosphate-buffered saline (PBS) 60 to 72 h after transfection and collected by centrifugation and lysed; duplicate lysates were assayed for β-galactosidase (β-Gal) by spectrophotometry or luminescence, to control for transfection efficiency, and for chloramphenicol acetyltransferase (CAT) or luciferase reporter activity, by ethyl acetate extraction or luminescence, as described previously (16, 27, 50).

DNA synthesis. Cells were given 10 μCi of [*methyl*-^{3}H]thymidine (60 to 80 Ci/mmol) 24 h after transfection; after an additional 24 h, they were fixed and stained for β-Gal activity (43), to identify transfected cells, or processed for immunostaining and then were processed for autoradiography to score DNA synthesis (59). Cells were viewed by bright-field microscopy at ×100 to ×200 magnification, and 100 to 500 cells per datum point were scored for β-Gal activity and labeled nuclei.

Immunostaining. Twenty-four hours after transfection, cells were labeled with [^{3}H]thymidine for 24 h, fixed in 4% paraformaldehyde–PBS for 20 min at room temperature, and permeabilized, as described previously (12). Rabbit polyclonal immunoglobulin G (IgG) against human p21 (amino acids 2 to 21) or mouse monoclonal IgG against human E2F-1 (amino acids 342 to 386) (Santa Cruz Biotechnology, Santa Cruz, Calif.) was diluted 1:20 in 0.2% bovine serum albumin–PBS and applied for 1 h at room temperature. Coverslips were washed, incubated with biotinylated anti-rabbit or anti-mouse IgG followed by fluorescein isothiocyanate- or Texas Red-conjugated streptavidin (12), and processed for autoradiography (59); the emulsion was developed after 6 to 10 h. Cells were viewed by epifluorescence with filters for fluorescein isothiocyanate or Texas Red and bright-field microscopy.

Electrophoretic mobility shift assays. Nuclear extracts were prepared by a modification of the method of Dignam et al. (13), as previously described (14, 16). Glutathione *S*-transferase-p21 fusion proteins were purified from *Escherichia coli* cells expressing pGEX-2T (Pharmacia Biotech; Piscataway, N.J.) into which human p21 cDNAs were cloned (49), as described previously (67). Extracts were incubated for 10 min at room temperature with 1 μg of fusion proteins for 1 h on ice with 1 μl of anti-Cdk2 antiserum (from E. Leof) or 1 μg of anti-p107 IgG (Santa Cruz Biotechnology). E2F binding was detected by using the E2F binding sequence conserved in the hamster, mouse, and human DHFR promoters; specificity was confirmed by competition with wild-type or mutant oligonucleotides; and DNA-protein complexes were separated by electrophoresis, as previously described (14, 16), except that the gel was run for 5 h, rather than 2 h, to better resolve DNA-protein complexes.

TABLE 1. p21 suppresses transcription from E2F-responsive (DHFR and cdc2), but not E2F-unresponsive (SV and c-*fos*), promoters[a]

Reporter vector	Expression vector	DNA (μg)	Normalized activity[b]	% Activity[c]	% Inhibition[d]
DHFR-CAT	CMV1	2	9,462	100	0; 0
	CMV-p21	1	8,196	87	13; 43
		2	2,613	28	72; 66[e]
		4	1,757	19	81; 89
cdc2-CAT	CMV1	2	3,304	100	0; 0
	CMV-p21	1	1,664	50	50; 60
		2	2,000	60	40; 55[e]
		4	2,008	61	39; 63
SV-CAT	CMV1	2	3,620	100	0; 0
	CMV-p21	1	3,743	103	0; 3
		2	3,828	106	0; 5
		4	3,366	93	7; 7
c-fos-CAT	CMV1	2	16,927	100	0
	CMV-p21	2	16,273	96	4; 10

[a] Proliferating A31 cells were transfected with 2 μg of reporter vector, 200 ng of CMV-β-gal, 0 to 4 μg of CMV-p21, and CMV1 to equalize the amount of transfected DNA. Cell lysates were assayed for CAT and β-Gal activities and normalized, as described in Materials and Methods.

[b] CAT activity (in counts per minute), normalized for β-Gal activity, from one experiment.

[c] Normalized CAT activity, divided by normalized CAT activity in cells receiving CMV1, × 100. Shown in this column are the results from the experiment whose results are reported under Normalized activity.

[d] The first of each pair of values is the result from the experiment whose results are reported under Normalized activity and % Activity; the second value is the result from an identical, independent experiment which gave similar results.

[e] The effects of 2 μg of CMV-p21 on the DHFR and cdc2 promoters were investigated three additional times. Shown are the average values.

RESULTS

p21 inhibits transcription driven by E2F-responsive promoters. We obtained preliminary evidence that p21 suppresses E2F activity by cotransfecting exponentially growing murine A31 fibroblasts with a human p21 cDNA expression vector (CMV-p21) and a CAT reporter gene driven by one of several promoters. We used two E2F-responsive promoters: one from the hamster DHFR gene and the other from the human cdc2 gene. DHFR and cdc2 are expressed in late G_1, at least in part because of transcription that depends on E2F binding sites in their 5′ regulatory regions (7, 16, 66). We also used two promoters that have not been reported to be E2F responsive—the simian virus 40 early promoter (SV) and the cellular c-*fos* promoter.

The p21 expression vector, but not a control vector (CMV1), suppressed both DHFR and cdc2 promoter activities (Table 1). The DHFR promoter, which contains two E2F binding sites (66, 71), was on average inhibited somewhat more strongly than the cdc2 promoter, which contains a single E2F binding site (7). At the vector-to-cell ratios used here, p21 inhibited DHFR and cdc2 promoter activities to roughly the same extent that it inhibited DNA replication (compare, for example, Table 1 and Fig. 3). In sharp contrast to its effect on the DHFR and

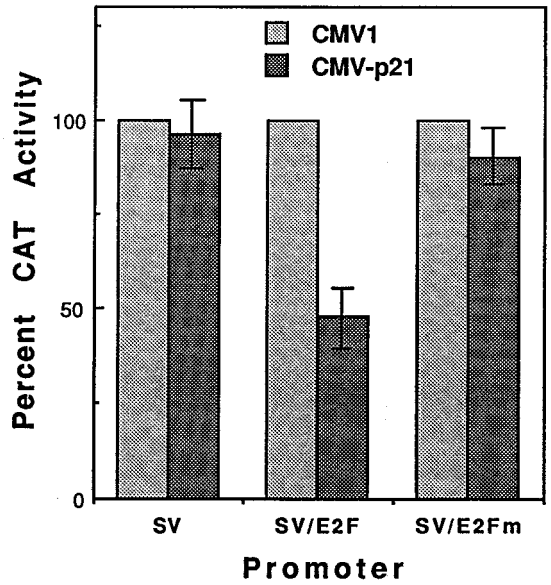

FIG. 1. Inhibition of transcription by p21 depends on an E2F binding site. Proliferating A31 cells were transfected with 2 μg of CMV-p21 or the control (CMV1) vector, 200 ng of CMV-β-gal, and 2 μg of CAT vector driven by either the SV promoter or the SV promoter into which three tandem wild-type (SV/E2F) or mutant (SV/E2Fm) E2F binding sites were inserted; CAT activity was determined and normalized for β-Gal activity, as described in Materials and Methods. Reported is the normalized CAT activity in each CMV-p21-transfected sample, divided by the normalized CAT activity in the appropriate control sample, × 100. Shown are the average, and range of, values from two independent experiments (SV promoter) or three independent experiments (SV/E2F and SV/E2Fm promoters). The normalized CAT activities (in counts per minute) for each control transfection were as follows: experiment 1, 3,620 for SV, 7,758 for SV/E2F, and 10,640 for SV/E2Fm; experiment 2, 3,826 for SV, 26,284 for SV/E2F, and 36,264 for SV/E2Fm; experiment 3, 6,055 for SV/E2F and 7,244 for SV/E2Fm.

cdc2 promoters, p21 expression had little or no effect on either the SV or c-*fos* promoter (Table 1).

These results suggested that overexpression of p21 in proliferating cells may selectively inhibit transcription driven by E2F-responsive promoters.

p21 inhibits transcription mediated by an E2F binding site. To more critically test the idea that p21 suppresses E2F-mediated transcription, we tested its effect on promoters containing either wild-type or mutant E2F binding sites. First, we inserted three tandem wild-type or mutant E2F binding sites into the SV promoter. We compared the p21 sensitivities of these modified promoters (SV/E2F and SV/E2Fm) with that of the unmodified SV promoter and with each other (Fig. 1).

Both the SV/E2F and SV/E2Fm promoters were severalfold more active than the SV promoter in proliferating cells (see the legend to Fig. 1). The reason for this hyperactivity is not known. It may be due to fortuitous, E2F-independent stimulation by the insertion (see Discussion). This altered control activity notwithstanding, p21 nonetheless suppressed the SV/E2F, but not the SV/E2Fm, promoter (Fig. 1). Thus, E2F binding sites rendered the SV promoter p21 suppressible, and the suppression depended on a wild-type, but not a mutant, E2F binding site.

p21 suppresses E2F-1 promoter activity. Because the SV/E2F and SV/E2Fm promoters are artificial, we also determined the p21 sensitivity of a natural promoter, namely that driving E2F-1 expression. E2F-1 is a cell cycle-regulated E2F component. It is induced in mid- to late G_1 (16, 62, 66) at least in part because of a stimulation of transcription that depends on two upstream E2F binding sites (35, 50). We asked whether E2F-1 promoter activity was suppressed by p21 and, if so, whether suppression depended on the E2F binding sites. We tested two cell lines, A31 murine fibroblasts and U2OS human osteosarcoma cells. We also tested wild-type p21 and a p21 mutant (p21m, amino acids 17 to 52 deleted) that expresses a stable protein in cells but does not inhibit cell proliferation (49).

Proliferating cells were transfected with the control or a p21 expression vector, together with vectors in which CAT was expressed from either a wild-type E2F-1 promoter or one in which the E2F binding sites were deleted (50). In both A31 and U2OS cells, p21 suppressed the wild-type E2F-1 promoter (Fig. 2A). By contrast, p21 had little effect on the mutant E2F-1 promoter (E2F binding sites deleted) (Fig. 2B). In addition, the p21 mutant had little effect on either the wild-type or mutant E2F-1 promoter (Fig. 2). Thus, overexpression of wild-type, but not mutant, p21 in proliferating cells suppressed E2F-1 promoter activity, and this depended on the E2F binding sites in the promoter.

Together, the data on the SV and E2F-1 promoters suggest that p21 can inhibit E2F-dependent transcription. The behavior of the p21 deletion mutant further suggests that p21's ability to inhibit E2F activity is linked to its ability to inhibit DNA replication.

Inhibition of E2F activity is linked to inhibition of DNA synthesis in p21 mutants. To explore the idea that p21 may inhibit DNA synthesis at least in part by inhibiting E2F activity, we examined additional p21 mutants for both properties (Fig. 3). p21 proteins with deletions of amino acids 24 to 29 (mutant Δ24-29), 53 to 58 (Δ53-58), or 17 to 52 (Δ17-52; p21m in Fig. 2) have been shown to accumulate to a range of levels and to inhibit DNA replication to varying extents, in immortal human fibroblasts (49). The Δ24-29 mutant protein accumulates to the lowest level in cells but, in fact, has near-wild-type biological activity (inhibition of DNA synthesis). The Δ17-52 mutant has little or no biological activity but accumulates to near-wild-type levels (49). The Δ53-58 mutant is intermediate for both accumulation and inhibition of DNA replication (49).

Expression vectors encoding wild-type or mutant p21 proteins were transfected into proliferating A31 cells, and DNA synthesis and DHFR promoter activity were monitored. There was very good correspondence among these mutants in their relative abilities to inhibit DNA synthesis and suppress DHFR-driven transcription (Fig. 3). The ability to inhibit DNA synthesis and DHFR promoter activity also correlated well with the mutants' abilities to bind Cdks (49). Moreover, the Δ17-52 p21 mutant failed to inhibit DNA synthesis in both A31 (Fig. 3) and U2OS cells (data not shown), failed to inhibit E2F-1 promoter activity in both cell lines (Fig. 2) and, as shown below, failed to disrupt a specific E2F-Cdk DNA binding complex.

We conclude that the ability of p21 to inhibit DNA synthesis and the ability to suppress E2F-mediated transcription correlate well, supporting the idea that inhibition of E2F activity is an effector of p21-mediated growth inhibition.

Effects of E2F-1, E2F-4, and wild-type or mutant T antigens on inhibition of DNA synthesis and E2F activity by p21. E2F-1 is induced a few hours before S phase (16, 62, 66) and is bound and inhibited by underphosphorylated pRb (56). Following pRb phosphorylation in late G_1, E2F-1 is released, whereupon it induces genes needed for DNA synthesis (11, 16, 30, 37, 62, 66). If p21 suppresses E2F activity and DNA synthesis by inhibiting pRb phosphorylation, E2F-1 and proteins that inactivate pRb should overcome the effects of p21 on DNA syn-

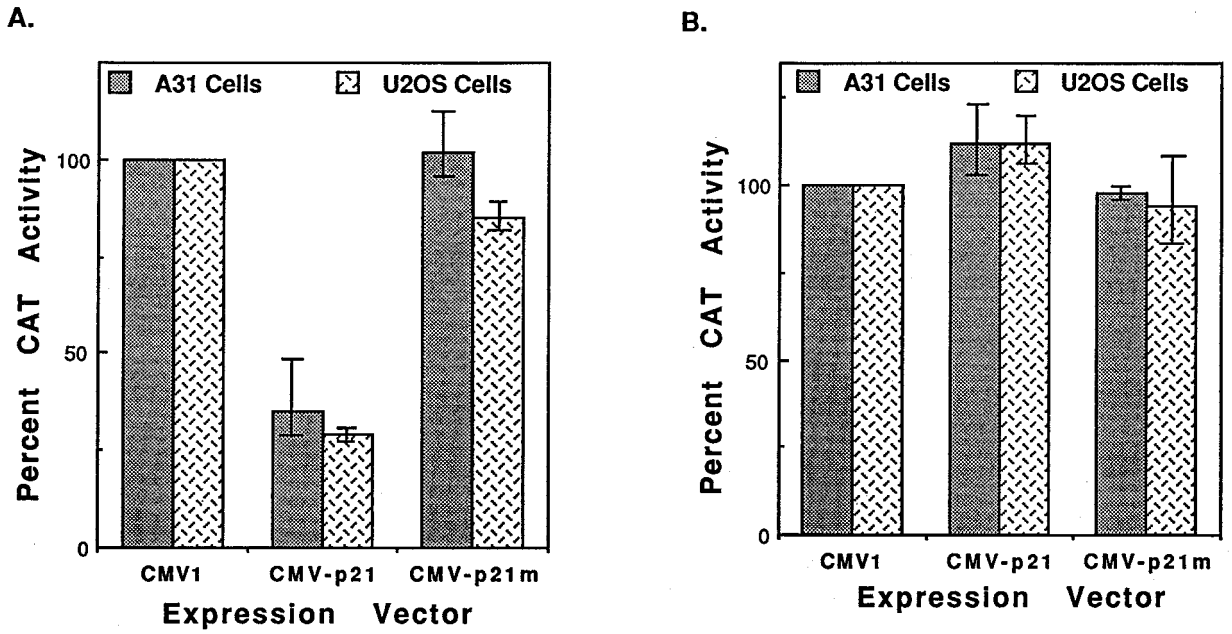

FIG. 2. Wild-type, but not mutant, p21 suppresses E2F-1 promoter activity. Proliferating A31 (gray bars) and U2OS (stippled bars) cells were transfected with CMV-β-gal and CMV1, wild-type p21 (CMV-p21), or mutant p21 (CMV-p21m; Δ17-52 deletion) expression vectors and a CAT vector driven by E2F-1 promoters that were either wild type (A) or mutant (B) in the E2F binding sites (48), as described in the legend to Fig. 1 and Materials and Methods. The normalized CAT activity for each condition was calculated. Shown are the average, and range of, values from two independent experiments (wild-type promoter in U2OS cells and mutant promoter in A31 and U2OS cells) or three independent experiments (wild-type promoter in A31 cells). The normalized CAT activities (in counts per minute) for each (CMV1) control were as follows: U2OS, wild-type promoter, 11,967 and 7,590; U2OS, mutant promoter, 8,777 and 6,035; A31, wild-type promoter, 9,875, 2,764, and 15,161; A31, mutant promoter, 9,307 and 3,684.

thesis and E2F activity. E2F proteins that do not bind pRb should be less effective in this regard.

To test this idea, we transfected E2F-1 (CMV-E2F1) and p21 expression vectors into proliferating A31 cells and monitored DNA synthesis. E2F-1 reversed the growth inhibition by p21 (Table 2). By contrast, E2F-4 was nearly inactive in this regard (Table 2). Unlike E2F-1, E2F-4 does not bind pRb; rather, it binds the pRb-related proteins p107 and p130 (2, 24, 70). The E2F4 vector was capable of relieving the inhibition of DNA synthesis caused by p107 overexpression (Table 3).

Simian virus 40 T antigen also reversed p21-mediated growth inhibition (Table 2). T antigen binds and inactivates pRb as well as p107 and p130 (21). This activity was important for the reversal of p21-mediated growth inhibition: a pRb-p107-p130-binding-deficient T antigen (9) was only 15 to 20% as active as wild-type T antigen. T antigen also binds and inactivates p53 (21), but a p53-binding-deficient T antigen (38) had near (65 to 70%) wild-type activity in counteracting p21 (Table 2). The functions of the T-antigen mutants were confirmed by introducing them into senescent human fibroblasts, which initiate DNA synthesis in response to wild-type, but neither mutant, T antigen (15). In this assay, mdm2, a cellular inactivator of p53, complemented the p53-binding-deficient T antigen (unpublished data), and E7, a viral inactivator of pRb-p107-p130, complemented the pRb-p107-p130-binding-deficient T antigen (27).

In U2OS cells, as in A31 cells, wild-type but not mutant (Δ17-52) p21 inhibited DNA synthesis (Table 2). In addition, E2F-1 reversed the p21-mediated growth arrest in U2OS cells. E2F-4 was less effective than E2F-1 in this regard but did show some activity in these cells, in contrast to its minimal activity in A31 cells (Table 2). In both cell lines, E2F-4 partially reversed the p21-mediated growth arrest at higher E2F-4/p21 ratios (e.g., 2:1 versus 1:1), but E2F-4 was always less effective than E2F-1.

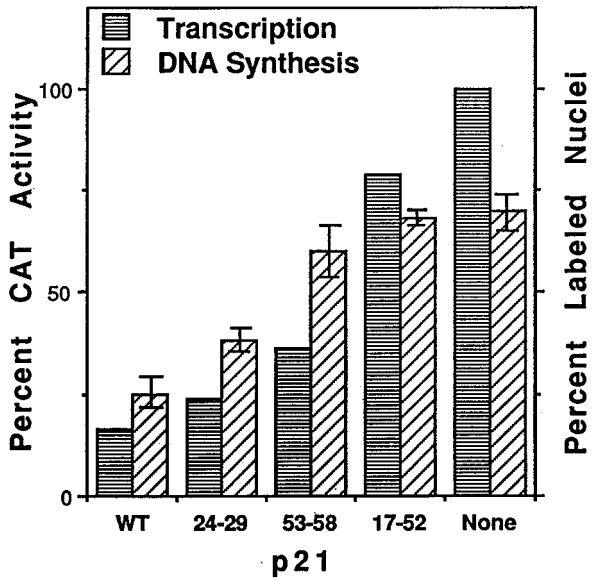

FIG. 3. Inhibition of DNA synthesis and transcription by p21 deletion mutants. Proliferating A31 cells were transfected with 2 μg of p21 expression vectors or CMV1 (None) and 200 ng of CMV-β-gal. p21 cDNAs encoded either wild-type protein (WT) or proteins in which amino acids 24 to 29, 53 to 58, or 17 to 52 were deleted. For DNA synthesis, cells were radiolabeled and stained for β-Gal, and transfected cells were scored for labeled nuclei, as described in Materials and Methods. Shown are the results of a single experiment, which agree with previously published results (47). For transactivation, 2 μg of DHFR-CAT was included in the transfection; cells were assayed for CAT and β-Gal activities, as described in Materials and Methods. Reported are the normalized CAT activity, as described in the legend to Fig. 1 and Materials and Methods, and the average, and range of, values from two independent experiments.

TABLE 2. E2F-1 and wild-type T antigen, but not E2F-4 or a Rb-binding-deficient T antigen, overcome p21-mediated growth inhibition[a]

Cell line	Expression vector	% Labeled nuclei[b]
A31	CMV1 (control)	100
	CMV-p21	34 (20–46)
	CMV-p21 + CMV-E2F1	92 (88–96)
	CMV-p21 + CMV-E2F4	39 (31–47)
	CMV-p21 + CMV-T	85 (82–87)
	CMV-p21 + CMV-T[K1]	19 (14–23)
	CMV-p21 + CMV-T[p]	64 (56–72)
U2OS	CMV1 (control)	100
	CMV-p21	27 (25–28)
	CMV-p21m	92 (88–95)
	CMV-p21 + CMV-E2F1	85 (77–96)
	CMV-p21 + CMV-E2F4	50 (46–54)

[a] Proliferating cells were transfected with 200 ng of CMV-β-gal and 1 μg (each) of expression vector or 2 μg of CMV1 (control). Where only CMV-p21 was added, 1 μg of CMV1 was included. Cells were radiolabeled, assayed for β-Gal activity, and processed for autoradiography, as described in Materials and Methods.

[b] Percentage of β-Gal-positive cells with labeled nuclei in plates receiving expression vectors, divided by the percentage of β-Gal-positive cells with labeled nuclei in control plates, × 100. In control plates, the percentage of β-Gal-positive cells with labeled nuclei ranged from 70 to 85%. Shown are the average, and range of, values from two independent experiments (A31 plus CMV-T, CMV-T[K1] or T[p] and U2OS, all conditions), or three independent experiments (A31 plus CMV-E2F1 or CMV-E2F-4). T[K1] and T[p] are pRb-binding-deficient and p53-binding-deficient T antigens, respectively.

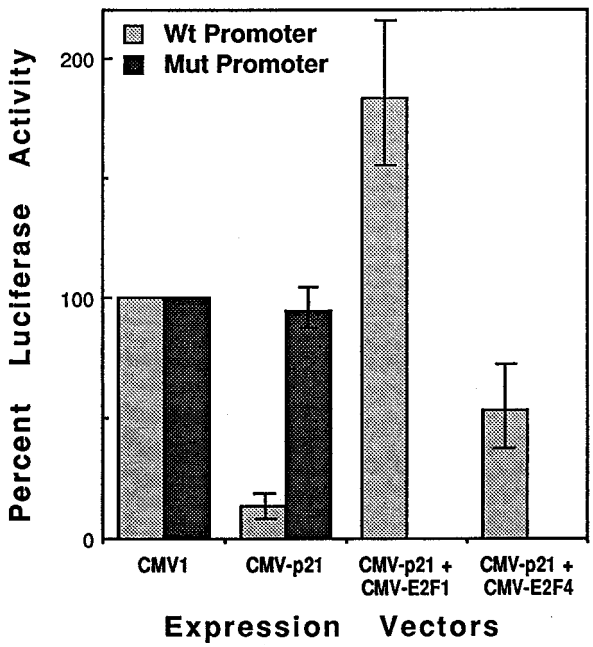

FIG. 4. E2F-1 abrogates p21 inhibition of E2F activity. Proliferating U2OS cells were transfected with 200 ng of CMV-β-gal; 2 μg of CMV1 (CMV1) or 1 μg (each) of CMV-p21 and CMV1 (CMV-p21), CMV-p21 and CMV-E2F1, or CMV-p21 and CMV-E2F4; and 2 μg of pGL2-AN (Wt) or pGL2-ANΔE2FA+B (Mut), which contain the wild-type or mutant E2F-1 promoter, respectively, driving luciferase expression (48). Transfected cells were assayed for luciferase and β-gal activities by using luminescent assays, as described in Materials and Methods. Luciferase activity was normalized for the β-Gal activity in each sample and is reported as the percent luciferase activity (in relative luminescent units) relative to the CMV1 control. The normalized values for the wild-type and mutant promoters were 0.08 and 0.1 relative luminescent units, respectively.

E2F-1 also reversed the p21-mediated inhibition of E2F activity. The E2F-1 expression vector actually stimulated E2F-1 promoter activity when transfected with CMV-p21 into U2OS cells (Fig. 4). By contrast, coexpression of E2F-4 with p21 only modestly countered the effect of p21 and certainly did not stimulate promoter activity.

Taken together, the results suggest that p21 inhibits DNA synthesis and E2F activity, at least in part, through its ability to inhibit pRb activity (phosphorylation). Additional experiments, however, suggest that p21 may inhibit both DNA synthesis and E2F activity through a pRb-independent mechanism as well (see below).

E2F-1 reverses p21-mediated inhibition of DNA synthesis in individual cells. Transfection data of the kind shown in Table 2 and Fig. 4 are often interpreted to suggest that E2F-1 abrogates the p21-mediated growth inhibition by acting within the same cell. Nonetheless, there is the possibility that antagonism of this sort may be due to indirect effects—for example, E2F-1 may stimulate production of a paracrine factor that allows cells to overcome the effects of p21. To confirm that E2F-1 acts in the same cells that overexpress p21, we transfected U2OS cells with p21 and E2F-1 expression vectors labeled with [^{3}H]thymidine and scored individual cells for p21 and E2F-1 expression by immunostaining and for DNA synthesis by autoradiography.

In untransfected proliferating cells, the endogenous p21 and E2F-1 proteins were undetectable by immunostaining (Fig. 5A, B, D, and E). As expected, most proliferating untransfected cells showed nuclear [^{3}H]thymidine labeling (96%) (Fig. 5C and F; see the legend for the values from which this and other percentages were calculated). In contrast to untransfected cells, transfected cells showed specific nuclear staining for p21 and E2F-1 (Fig. 5). Most transfected cells that expressed detectable levels of p21, but undetectable levels of E2F-1, were devoid of [^{3}H]thymidine labeling (87%), as expected (49) (data not shown). In contrast, and also as expected, most transfected cells expressing detectable levels of E2F-1, but undetectable levels of p21, were radiolabeled (91%) (data not shown). Cells expressing both p21 and E2F-1 (detected by different fluorochromes) were evident in cultures that were cotransfected with p21 and E2F-1 expression vectors. The majority (76%) of transfected cells expressing detectable levels of both p21 and E2F-1 were radiolabeled (Fig. 5).

We conclude that overexpression of p21 inhibits DNA synthesis and that overexpression of E2F-1 reverses this inhibition, within the same cell.

Effect of p21 on E2F DNA binding complexes in vitro. E2F binding sites are occupied by protein complexes, whose compositions—at least by in vitro analyses—depend on the cellular

TABLE 3. E2F-4 expression vector counteracts a partial p107-mediated inhibition of DNA synthesis[a]

Expression vector	% Labeled nuclei[b]
CMV1 (control)	100
CMV-p107	53 (49–59)
CMV-p107 + CMV-E2F4	82 (78–84)

[a] Proliferating C33a cells were transfected with 200 ng of CMV-β-gal and 1 μg (each) of expression vector or 2 μg of CMV1 (control). Where only CMV-p107 was added, 1 μg of CMV1 was included. Cells were radiolabeled, assayed for β-Gal activity, and processed for autoradiography, as described in Materials and Methods.

[b] Percentage of β-Gal-positive cells with labeled nuclei in plates receiving expression vectors, divided by the percentage of β-Gal-positive cells with labeled nuclei in control plates, × 100. Shown are the average, and range of, values from three independent experiments.

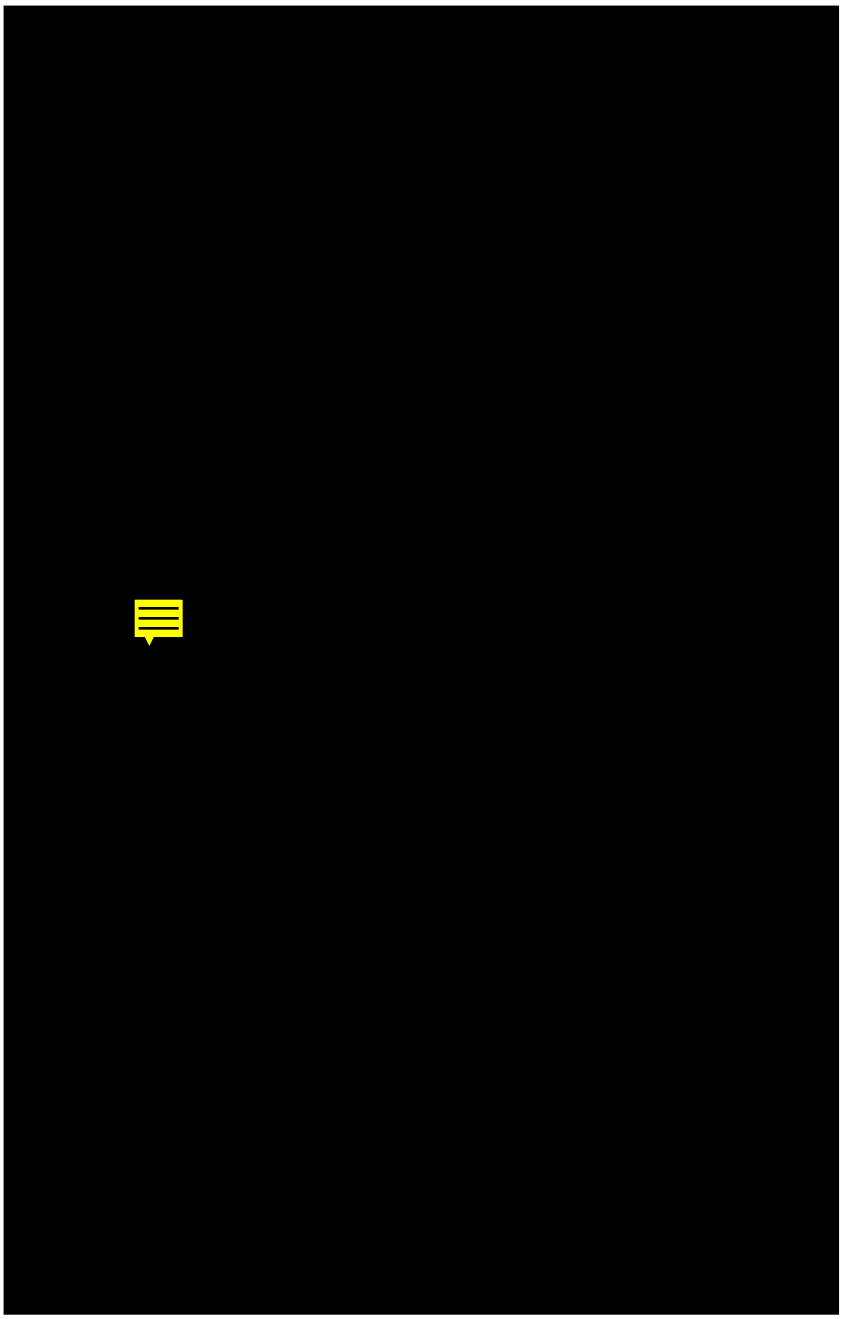

FIG. 5. E2F-1 and p21 expression and DNA synthesis in single cells. U2OS cells, proliferating on coverslips, were cotransfected with CMV-p21 and CMV-E2F1, labeled with [^{3}H]thymidine, immunostained for p21 (fluorescein isothiocyanate) and E2F-1 (Texas Red) protein, and processed for autoradiography, as described in Materials and Methods. (A and D) Cells were photographed through the fluorescein isothiocyanate filter in order to detect p21. Each panel shows a field containing one p21-positive cell. The fields also contain untransfected cells showing only background staining because endogenously expressed p21 is undetectable. (B and E) The same cells shown in panels A and D were photographed through the Texas Red filter to detect E2F-1. The p21-positive cells shown in A and D were also E2F-1 positive. Untransfected cells in the fields show only background staining because endogenously expressed E2F-1 is undetectable. (C and F) The same cells shown in panels A, B, D, and E photographed through phase-contrast optics to detect [^{3}H]thymidine-labeled nuclei (20% reduction). In both examples (A to C and D to F), the cell that coexpresses p21 and E2F1 is radiolabeled. Untransfected cells (no immunostaining) in the same field are also radiolabeled, as expected of a proliferating cell population. Overall, 96% (198 of 206) of untransfected cells were radiolabeled, as were 91% (99 of 109) of cells that stained for E2F-1 only. Of the cells that stained for p21 only, 87% (117 of 135) were unlabeled, as expected (49). Of the cells that stained for both p21 and E2F-1, 76% (58 of 76) were labeled.

growth state and stage of the cell cycle. The in vivo functions of these complexes are not yet clear, but some are believed to be stimulatory whereas others may be inhibitory. We used in vitro gel shift analyses to determine whether any of these complexes is a target for p21.

Nuclear extracts from quiescent cells contain complexes that associate with E2F binding sites. E2F family members other than E2F-1 constitute these complexes, since E2F-1 is not expressed by quiescent cells (16, 62, 66). Quiescent cell extracts have relatively low levels of free E2F or pRb-associated E2F but contain E2F components associated with p107 and p130 (2, 6, 24, 58, 70). By using the E2F binding sites in the DHFR

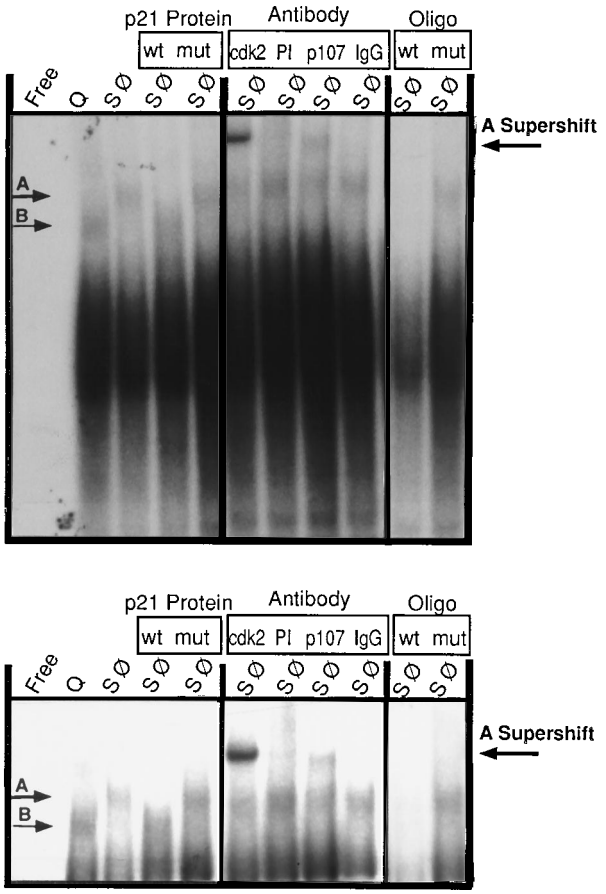

FIG. 6. p21 disrupts an E2F DNA binding complex. Nuclear extracts from A31 cells, quiescent (Q) or stimulated for 24 h (SØ), were analyzed for E2F DNA binding complexes, as described in Materials and Methods. (Free lanes) Extracts were omitted from the reaction mixture. (p21 Protein lanes) Extracts were incubated without or with recombinant glutathione *S*-transferase-fused p21 protein, either wild-type (wt) or mutant (mut). Arrows on the left indicate the E2F-p107-Cdk complex of stimulated cells (arrows A) and the E2F-p107 complex characteristic of quiescent cells (arrows B). (Antibody lanes) Extracts from stimulated cells were incubated with anti-Cdk2 antiserum (cdk2), preimmune serum (PI), anti-p107 IgG (p107), or nonspecific IgG (IgG); the Cdk2 and p107 antibodies supershifted complex A, as indicated by the arrows on the right. (Oligo lanes) Cold oligonucleotide containing either a wild-type (wt) or mutant (mut) E2F binding site was added to the binding reaction mixture to confirm binding specificity. The gel was run for 5 h to better separate the DNA-protein complexes. The lower panel shows a darker, high-contrast exposure of the upper portion of the same gel.

promoter, we detected a quiescent-cell-specific and late G_1-early S phase-specific E2F complex in A31 cell extracts (see the legend to Fig. 6). The quiescent-cell-specific E2F complex was not affected by the addition of recombinant p21 protein (Fig. 6), and none of the E2F complexes in quiescent cells were disrupted or supershifted by a p21 antibody (data not shown).

Nuclear extracts from cells in late G_1 contain more free E2F than quiescent cell extracts; E2F in association with pRb; and E2F-4 in association with p107, Cdk2 and cyclin E (mid to late G_1), or cyclin A (late G_1 to early S) (2, 6, 61, 70). The functions of E2F-p107-Cdk-cyclin complexes are not yet well understood; both stimulatory and inhibitory functions have been proposed, depending on the components (18, 40, 71). The E2F complex in A31 cell extracts from late-G_1-S-phase cells (Fig. 6) contained Cdk2 and p107. By using a long-running gel to better separate E2F complexes that differ between quiescent and proliferating cells, we found that specific Cdk2 antiserum or p107 IgG, but not preimmune serum or nonspecific IgG, disrupted and supershifted a portion of it (Fig. 6, bottom gel). In A31 cells, this complex may also contain p130 because p107 IgG was only partially effective at supershifting it, and preliminary data suggest that p130 IgG also supershifts it (not shown). This is in contrast to the Rb-deficient C33a and HTB-9 cells, in which the majority of the late-G_1-S E2F complex is supershifted by p107 IgG (see Fig. 8).

The late-G_1-S E2F complex was specifically disrupted by recombinant p21 (Fig. 6). The Δ17-52 mutant p21 protein failed to disrupt this E2F-p107-Cdk complex (Fig. 6). This mutant also failed to inhibit DNA synthesis and E2F-1 and DHFR promoter activities (Fig. 2 and 3) and does not bind Cdks (49). Thus, overexpression of p21 can disrupt existing E2F DNA binding complexes that contain Cdks and p107.

p21 inhibits E2F activity and DNA synthesis in Rb-deficient cells. Because p21 inhibits protein kinases that phosphorylate pRb (19, 26, 28, 29, 52, 75) and because one model of cell cycle control holds that unphosphorylated pRb negatively regulates E2F (51), pRb is presumed to be a central mediator of p21's effects. Our results thus far support this idea. However, the gel shift experiments (Fig. 6) suggested that p21 may inhibit E2F activity through additional pRb-independent pathways. To explore this idea, we tested the ability of p21 to inhibit DNA synthesis and E2F activity in C33a and HTB-9 cells, human cervical and bladder carcinoma cells that, unlike A31 and U2OS cells, do not express a functional pRb (see, e.g., references 6, 25, 69, and 70).

C33a (Fig. 7A) and HTB-9 (Fig. 7B) cells were clearly sensitive to both the growth and transcriptional inhibitory properties of p21 (Fig. 7). p21 inhibited DNA synthesis in HTB-9 cells to about the same extent that it did in A31 and U2OS cells. C33a cells were somewhat less sensitive, but p21 still had a substantial antiproliferative effect in these cells. C33a and HTB-9 cells were also about as sensitive to p21 inhibition of E2F activity (Fig. 7) as A31 and U2OS cells. In all cases, p21 overexpression reduced reporter gene activity driven by the wild-type, but not the mutant (E2F sites deleted), E2F-1 promoter.

The results clearly indicate that p21 can inhibit DNA synthesis and E2F activity through cellular targets other than pRb.

We next asked whether E2F-1 or E2F-4 could reverse the p21-mediated growth inhibition in pRb-deficient cells. E2F-1 was somewhat less effective at counteracting p21 in C33a cells (Table 4) relative to A31 and U2OS cells (Table 2). Moreover, E2F-4 was as potent as E2F-1 (Table 4). In HTB-9 cells, however, E2F-1 was as effective at counteracting p21 (Table 4) as it was in A31 and U2OS cells (Table 2), and E2F-1 was more effective than E2F-4 (Table 3). In these respects, the pRb-deficient HTB-9 and pRb-functional U2OS cells behaved very similarly (compare Tables 2 and 4).

The results support the idea that pRb is not an obligate intermediary in the growth arrest induced by p21 or in the ability of E2F-1 to overcome p21-mediated growth inhibition. The different responses to E2F-1 and E2F-4 among cell lines may well be due to differences in the levels of p107, p130, Cdks, and/or cyclins.

Effect of p21 on E2F DNA binding complexes in pRb-defective cells. Nuclear extracts from proliferating C33a and HTB-9 cells contained an E2F-Cdk2-p107 complex, as evidenced by its sensitivity to Cdk2 and p107 antibodies (Fig. 8), similar to the case for A31 cells. This complex—like that present in proliferating A31 cells—was disrupted by the addition of wild-type, but not mutant (Δ17-52), p21 protein (Fig. 8). A very fast migrating complex was similarly disrupted (see the legend to

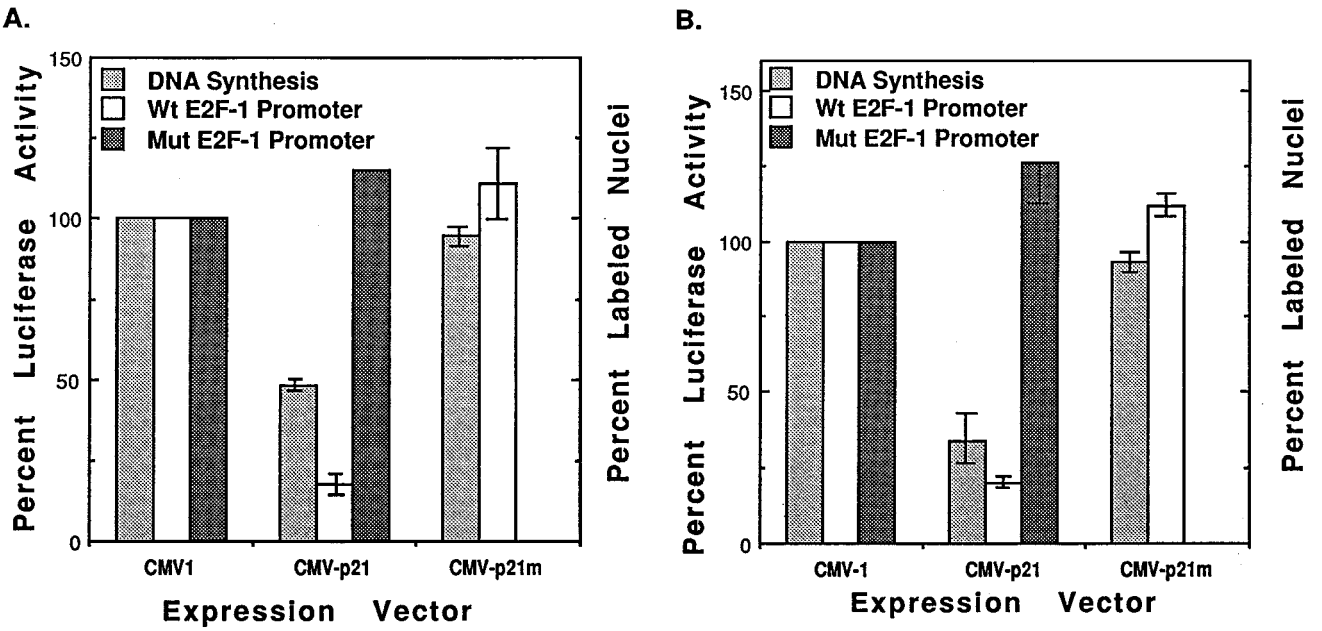

FIG. 7. p21 inhibits growth and E2F activity in pRb-deficient cells. Proliferating C33a (A) and HTB-9 (B) cells were transfected with 2 μg of CMV1, CMV-p21, or CMV-p21m (p21 Δ17-52 mutant) and 200 ng of CMV-β-gal. For transactivation, 2 μg of either pGL2-AN-luc (Wt E2F-1 Promoter) or pGL2-ANΔE2FA+B-luc (Mut E2F-1 Promoter) was included, as described in the legend to Fig. 4; cells were lysed and assayed for luciferase and β-Gal activities, as described in Materials and Methods. Reported is the normalized luciferase activity relative to that of the control (CMV1) cells. For DNA synthesis, cells were labeled with [^{3}H]thymidine, stained for β-Gal, and processed for autoradiography, as described in Materials and Methods. Reported is the percentage of β-Gal-positive cells with labeled nuclei relative to that of the control (CMV1-transfected) cells.

Fig. 8). This complex was insensitive to Cdk2 antibody and partially depleted p107 antibody; whether it is an as-yet-undescribed E2F complex or a partially degraded or dissociated complex remains to be seen. Taken together, these and the results described above suggest that p21 can inhibit DNA synthesis and E2F activity through a pRb-independent mechanism and that this may involve the disruption of an E2F complex containing the pRb-related protein p107.

DISCUSSION

p21 is a potent inhibitor of Cdk activity and cell proliferation (19, 26, 28, 29, 48, 52, 75). Current models of the events required for the G_0-to-S transition suggest that pRb is among the critical proteins whose phosphorylation is inhibited by p21. This scenario, in turn, predicts that E2F activity—particularly the pRb-sensitive E2F activity that is induced late in G_1—should be an important downstream target and effector of the growth inhibition caused by p21. In this investigation, we tested this idea, and we provide several lines of evidence to support it. However, our results also indicate that there is, in addition, a pRb-independent mechanism by which p21 inhibits cell proliferation, as well as E2F activity.

TABLE 4. Effects of E2F-1 and E2F-4 on p21-mediated growth inhibition in pRb-deficient cells[a]

Cell line	Expression vector	% Labeled nuclei[b]
C33a	CMV1 (control)	100
	CMV-p21	48 (47–48)
	CMV-p21 + CMV-E2F1	66 (64–68)
	CMV-p21 + CMV-E2F4	60 (57–63)
HTB-9	CMV1 (control)	100
	CMV-p21	34 (24–44)
	CMV-p21 + CMV-E2F1	83 (79–87)
	CMV-p21 + CMV-E2F4	64 (60–67)

[a] Proliferating cells were transfected, radiolabeled, assayed for β-Gal activity, and processed for autoradiography, as described in footnote *a* of Table 2 and Materials and Methods.

[b] Percentage of β-Gal-positive cells with labeled nuclei in plates receiving expression vectors, divided by the percentage of β-Gal-positive cells with labeled nuclei in control plates, × 100. In control plates, the percentage of β-Gal-positive cells with labeled nuclei ranged from 70 to 85%. Shown are the average, and range of, values from two independent experiments.

p21 inhibited E2F-dependent transcription from several (DHFR, cdc2, SV/E2F, and E2F-1) promoters. In the case of SV/E2F and E2F-1, mutations in the E2F binding sites abolished the inhibition. By contrast, p21 had little effect on the c-*fos* and SV promoters, which are not E2F regulated. Mutations that interfered with the ability of p21 to bind Cdks (49) also abolished its ability to inhibit DNA synthesis and abolished its ability to suppress E2F activity. A wild-type, but not a pRb-p107-p130-binding-deficient, T antigen reversed the p21-induced growth arrest and inhibition of E2F activity. In addition, both the growth arrest and inhibition of E2F activity were reversed strongly by E2F-1 but only weakly by E2F-4. E2F-1 binds pRb strongly but binds p107 and p130 only weakly, whereas E2F-4 binds p107 and p130, but not pRb. Thus, our results support the idea that E2F activity is a downstream effector of the growth arrest induced by p21 and the prediction that pRb is an intermediate in this growth arrest at least in some cells and under some circumstances.

Insertion of E2F binding sites into the SV promoter stimulated its activity in proliferating cells severalfold (see the legend to Fig. 1). E2F sites can be stimulatory or inhibitory, depending on the cellular pRb activity (74). They are expected to be stimulatory in proliferating cells, in which the level of active (unphosphorylated) pRb is low. Thus, the elevated activity of the SV/E2F promoter was not surprising. Unexpectedly, the SV/E2Fm promoter was as, or slightly more, active

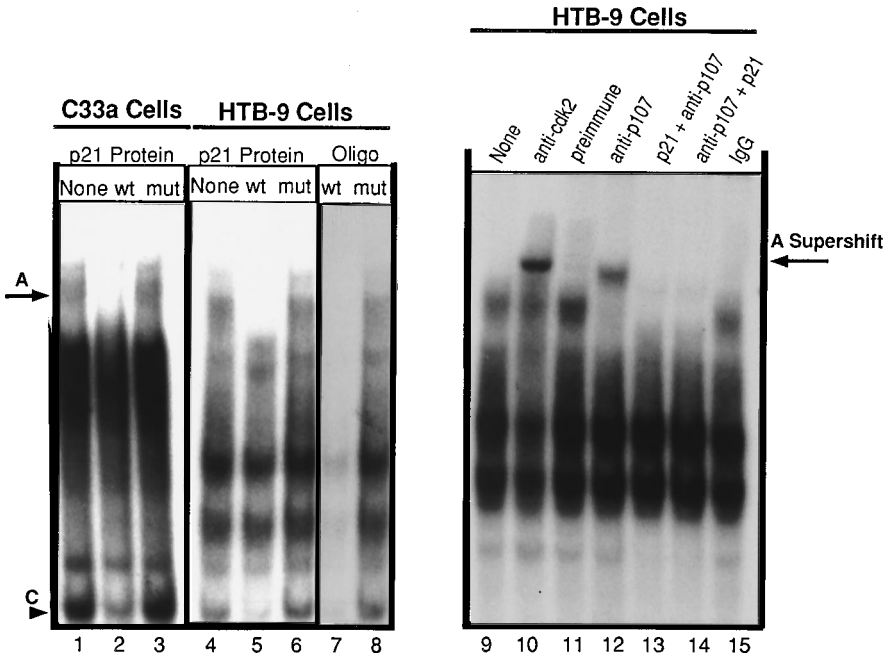

FIG. 8. p21 disrupts an E2F DNA binding complex in pRb-deficient cells. Nuclear extracts from proliferating C33a (lanes 1 to 3) and HTB-9 (lanes 4 to 15) cells were analyzed for E2F DNA binding complexes and incubated with recombinant p21 proteins, antibodies, and cold oligonucleotides, as described in the legend to Fig. 6 and Materials and Methods. The upper arrow on the left (A) indicates the E2F-p107-Cdk complex of stimulated cells, which is a major target for disruption by p21. The arrowhead (C; lower left) indicates the complex of unknown identity that is also disrupted by wild-type p21 protein. Extracts from proliferating HTB-9 cells were incubated with anti-Cdk2 antiserum, preimmune serum, anti-p107 IgG, or nonspecific IgG (lanes 10, 11, 12, and 15, respectively); the Cdk2 and p107 antibodies supershifted complex A, as indicated by the arrow on the right. In lanes 13 and 14, p21 protein and anti-p107 antibody were sequentially incubated with the extract in the indicated order. Because the gel was run for 5 h, the free probe migrated out of the gel and is not visible.

than the SV/E2F promoter (see the legend to Fig. 1). We speculate that inserting E2F sites—and adjacent sequences (see Materials and Methods)—into the SV promoter increased SV promoter activity by a mechanism that was independent of the E2F sites. Because the SV/E2Fm promoter was 20 to 40% more active than the SV/E2F promoter, we further speculate, on the basis of published data (74), that the SV/E2F promoter was partly inhibited because of the unphosphorylated pRb present in the early-G_1 cells in an exponentially growing culture. A preliminary experiment in the Rb-negative C33a cell line—in which SV/E2Fm was not more active than SV/E2F—supports this speculation.

Despite evidence that pRb mediates the inhibition of E2F activity in A31 and U2OS cells, p21 disrupted a p107-containing E2F-Cdk complex in nuclear extracts. This finding is consistent with results obtained with purified proteins (76). Moreover, p21 inhibited growth, as well as E2F-dependent transcription, and disrupted this complex, whether or not cells expressed a functional Rb gene. Thus, p21 can apparently inhibit growth and E2F activity by both pRb-dependent and pRb-independent mechanisms. One possibility is that p21 also inhibits the phosphorylation of p107. It was recently shown that the Cdk4-cyclin D kinase phosphorylates p107, which in turn results in the release of E2F-4 (1). p21 has been shown to inhibit Cdk4-cyclin D kinase activity (29, 75), and depending on the gene and cell, E2F-4 is capable of stimulating E2F-dependent transcription (2).

How might p21 act on E2F activity in both pRb-dependent and pRb-independent manners? In the case of pRb-dependent p21 action, inhibition of Cdk kinase activity very likely leads to an inhibition of pRb phosphorylation. This, in turn, maintains pRb in an unphosphorylated (active) form, which most likely inhibits E2F activity and, ultimately, growth by sequestering E2F-1. On the other hand, when p21 is overexpressed in proliferating cells—for example, by DNA damage (or experimentally by an overexpressing vector)—p21 may immediately disrupt the E2F-p107-Cdk-cyclin complex(es), which may be needed to transcribe genes important for the initiation of DNA synthesis. This action of p21 would, of course, be pRb independent. We speculate that overexpression of E2F-1 might overcome the effects of disrupting this complex, either by driving its reassembly by as-yet-unknown mechanisms or by stimulating transcription independent of the E2F-p107-Cdk-cyclin complex(es).

Our results also suggest that E2F-1 is an important downstream target of p21, but other E2F family members may also be targets of p21 action. p21 suppressed E2F-1-sensitive promoters, and this inhibition, as well as the p21-induced growth arrest, was reversed by E2F-1. However, the E2F-1 promoter was itself inhibited by p21. The E2F-1 promoter depends on two E2F binding sites and is repressed by unphosphorylated pRb, but its initial activity may not be controlled by E2F-1 during normal cell cycle progression. E2F-1 is not expressed in quiescent cells (16, 62, 66) and thus is unlikely to participate in the initial induction of its own transcription in mid-G_1 (E2F-1 could, however, act to positively maintain its own transcription once initiated). Thus, another E2F protein (e.g., E2F-4 or E2F-5), or a factor(s) that modulates the function of the E2F binding sites within the E2F-1 promoter, may also mediate the growth arrest caused by p21.

It is also possible that E2F-1 may be necessary, but not sufficient, to overcome the p21-induced growth arrest. Our measurements of cell growth, in fact, scored the ability of cells to initiate DNA synthesis. Recent findings suggest that, at least in some cells, E2F-1 overexpression can induce the initiation of DNA synthesis but not its completion and that this abortive S

phase is followed by massive apoptotic death (40). Although we have not observed massive apoptosis in our cultures, at least over the time course of our experiments, it is conceivable that E2F-1 overexpression did not induce a normal cell cycle in p21-inhibited cultures. This possibility suggests that p21 may inhibit cell proliferation through mechanisms in addition to the inhibition of E2F activity—mechanisms that might not be overcome by E2F-1 expression and might be manifest only after the onset of S phase. For example, p21 can also inhibit DNA replication through its interaction with PCNA (23, 72), and p21 inhibition of Cdk activity may also directly interfere with DNA replication (34). These activities of p21 may not affect the initiation of DNA synthesis, but rather, S phase progression or termination.

Our data provide solid evidence for speculations raised by several studies in which the effects of p21 and E2F on cell proliferation have been noted. For example, the findings that transforming growth factor β induces p21 (8) and that E2F-1 expression can overcome transforming growth factor β-induced growth arrest (60) suggest that transforming growth factor β may inhibit growth by suppressing E2F activity through p21. Recent data on the effects of γ-irradiation (11) similarly suggest that radiation may inhibit growth by suppressing E2F activity through p21. Likewise, the overexpression of p21 by senescent human fibroblasts (52) may be the immediate cause for the constitutive underphosphorylation of pRb in these cells (68), which in turn may be responsible for the failure of the cells to express E2F-1 and E2F DNA binding complexes (16).

In summary, the results show that E2F is an important downstream target of p21 action and, as such, may be a critical target for the growth inhibitory effects of p21. In addition, they suggest that p21 can inhibit E2F activity by both pRb-dependent and pRb-independent mechanisms.

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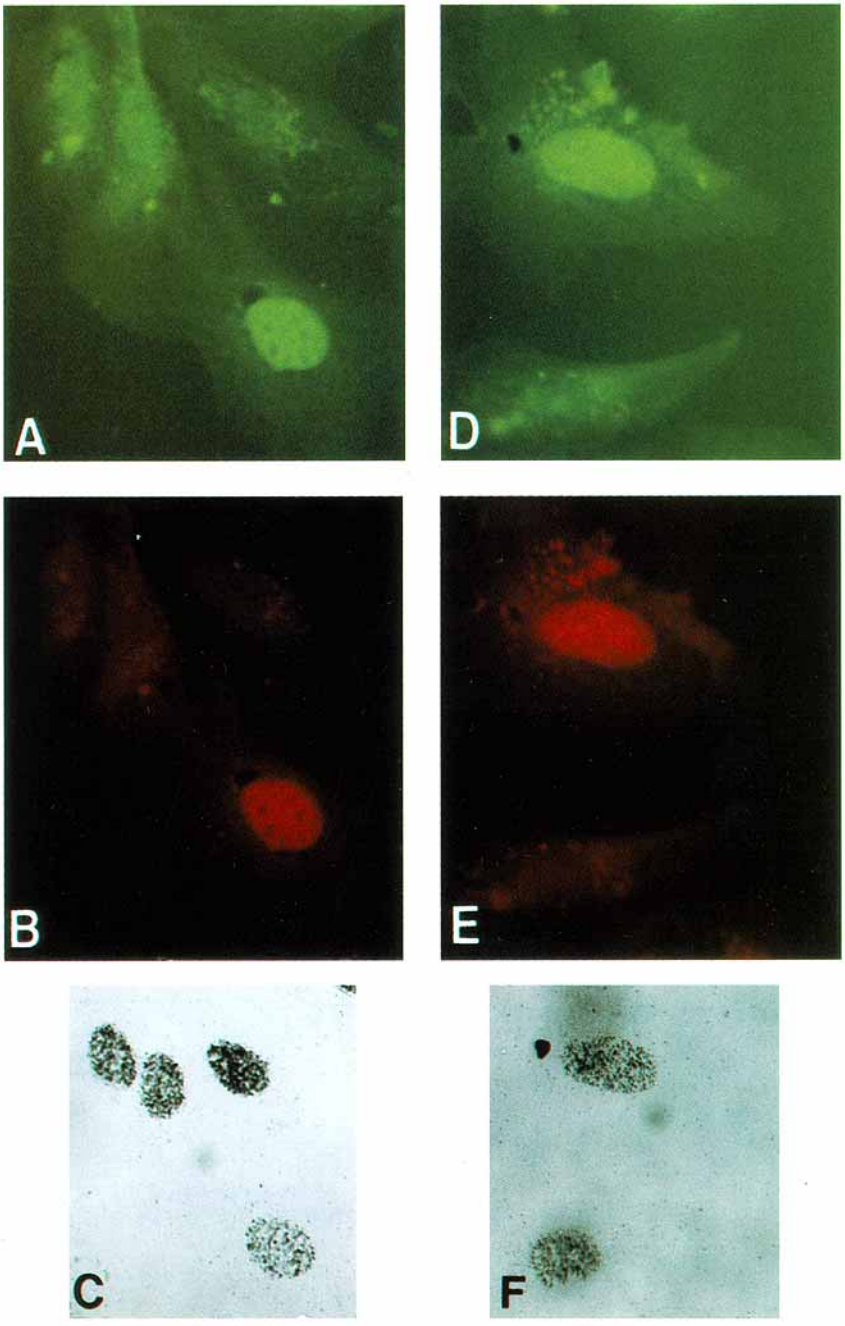
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