Adenovirus E1A proteins influence cell growth and phenotype through physical interactions with cellular proteins that regulate basic processes such as cell cycle progression, DNA synthesis, and differentiation. p120E4F is a low-abundance cellular transcription factor that represses the adenovirus E4 promoter and is regulated by E1A, through a phosphorylation-induced reduction of its DNA binding activity, to permit activation of the E4 promoter during early infection. To determine the normal biological role of p120E4F, we assessed its ability to influence fibroblast cell growth and transformation. p120E4F suppressed NIH 3T3 fibroblast colony formation but had little effect when coexpressed with E1A and/or activated ras. Cells that overexpressed p120E4F were inhibited in their ability to enter S phase, had elevated levels of the cdk inhibitor p21WAF1, and reduced cyclin D-cdk4/6 kinase activity. The increase of p21WAF1 levels occurred through a p53-independent posttranscriptional mechanism that included a three- to fourfold increase in the half-life of p21WAF1 protein. Coexpression of activated ras with p120E4F stimulated cyclin D1 expression, elevated cyclin D-cdk4/6 kinase activity, and accelerated cell growth. These data suggest an important role for p120E4F in normal cell division and demonstrate that p21WAF1 can be regulated by protein turnover.

Adenovirus E1A oncoproteins serve as the major regulators of the adenoviral life cycle by controlling two aspects of lytic infection: E1A coordinately activates high-level expression of viral genes during the early phase of infection and, in addition, induces the onset of S phase in the host cell to allow viral DNA replication (3, 41). Both capacities are due to the ability of E1A to influence the activity of cellular factors involved in the control of basic processes such as cell cycle progression, DNA synthesis, and differentiation (12, 25, 34, 35). When taken out of the context of a lytic infection, E1A can act as a transforming protein (24, 41); this is due, in part, to its ability to bind to products of the retinoblastoma tumor suppressor gene, Rb, and two Rb-related proteins, p107 and p130 (35, 47).

In G0 or early G1, Rb family proteins are hypophosphorylated and associate with E2Fs, a family of heterodimeric transcription factors that regulate the expression of genes whose products are required for S phase (35). The association of Rb family proteins with E2F factors inactivates E2F-mediated transcriptional stimulation or, on some promoters, can function to actively repress transcription (35). In response to mitogenic signals, cyclin D-cdk4/6 kinase activities, and subsequently cyclin E-cdk2 kinase activity, progressively phosphorylate Rb and cause the release of E2F complexes to enable S phase entry (42, 43). In the absence of mitogenic signals or the presence of growth inhibitory signals, Rb phosphorylation is prevented by decreased levels of cyclin proteins and/or elevated levels of the universal cyclin-dependent kinase inhibitors p21WAF1/Cip1, p27Kip1, and p57Kip2, or the D-type cyclin-dependent kinase inhibitors INK4a-d (23, 44). The binding of E1A to Rb family proteins circumvents this entire regulatory circuit by releasing E2Fs from Rb proteins and thus dysregulating E2F activity to promote cell cycle progression (35).

Cyclin-cdk’s and cdk inhibitors regulate a number of important processes in addition to the Rb-E2F pathway (5, 6, 11, 26, 45), and E1A has also evolved a number of mechanisms to directly control their function. Recent evidence indicates that E1A can bind to and inactivate p27, as well as repress p15INK4b transcription, to help overcome transforming growth factor β (TGF-β)-mediated G1 arrest in epithelial cells (9, 33). However, the complexity of cell growth regulation suggests that additional mechanisms must also be involved.

E4F is a ubiquitously expressed, low-abundance cellular transcription factor that exists in two forms, a predominant full-length 120-kDa protein (p120E4F) and a less-abundant proteolytically derived 50-kDa amino-terminal fragment (p50E4F) (17). Both forms bind to the same sites in the adenovirus E4 promoter, but they have opposite functional effects: p120E4F represses the E4 promoter in the absence of E1A, whereas p50E4F activates the E4 promoter only in the presence of E1A. E1A differentially regulates the DNA binding activities of both forms through induced phosphorylation, down-regulating the DNA binding activity of the p120E4F repressor while stimulating that of p50E4F, and thus controls a precise regulatory circuit. Analysis of the murine p120E4F repressor (also called φAP3) showed that p120E4F phosphorylation is reduced in serum-starved cells but is reactivated by the addition of serum or tetradecanoyl phorbol acetate, or by adenovirus infection, suggesting that p120E4F may regulate some aspect of cell proliferation (18).

Here we report that enforced expression of p120E4F in NIH 3T3 fibroblasts has profound negative effects on cell growth that are alleviated by coexpression of E1A, activated ras, or both. The growth effects of p120E4F are accompanied by an elevation of p21WAF1 levels, which occurs through a posttranscriptional mechanism that includes reduced turnover of the p21 protein.
TABLE 1. Effect of E4F cDNA overexpression on NIH 3T3 colony formation

| E4F | Amount (μg) | Coexpressed oncogene(s) | Fold change in colony no. (SD) |
|-----|-------------|-------------------------|-----------------------------|
| pCMV4 | 1.00 | | |
| E4F | 0.15 | 0.63 (0.18) | |
| | 0.3 | 0.61 (0.17) | |
| | 0.5 | 0.70 (0.15) | |
| E4F | 0.15 | + E1A(13S) | 1.19 (0.16) |
| | 0.3 | + E1A(13S) | 0.89 (0.03) |
| | 0.5 | + E1A(13S) | 1.01 (0.30) |
| E4F | 0.15 | + E1A(12S) | 0.86 (0.11) |
| | 0.3 | + E1A(12S) | 0.68 (0.16) |
| | 0.5 | + E1A(12S) | 0.92 (0.54) |
| E4F | 0.15 | + T24-ras | 1.79 (0.31) |
| | 0.3 | + T24-ras | 2.02 |
| | 0.5 | + T24-ras | 0.76 |
| E4F | 0.15 | + E1A(13S) + T24-ras | 1.75 (0.36) |
| | 0.3 | + E1A(13S) + T24-ras | 1.47 (0.21) |
| | 0.5 | + E1A(13S) + T24-ras | 1.26 (0.13) |
| E4F | 0.15 | + E1A(12S) + T24-ras | 2.35 (1.44) |
| | 0.3 | + E1A(12S) + T24-ras | 2.10 |
| | 0.5 | + E1A(12S) + T24-ras | 1.47 |
| E4F262 | 1.0 | | 1.16 (0.06) |
| E4F | 0.5 | + p53-281 | 0.17 (0.02) |
| | 0.5 | + p53-22,23,281 | 0.30 (0.07) |

*Calculated as the ratio of the number of G418-resistant colonies from pCMV-E4F2.5K (E4F) or pCMV-E4F2.62 (E4F262)-containing transfections to the number of G418-resistant colonies from pCMV4 control transfections. SD, standard deviation. For each set of E4F transfections, parallel transfections containing pCMV4 (instead of an E4F plasmid) and the indicated coexpressed oncogenes were used as controls. Values are derived from three independent experiments unless otherwise noted; values with no standard deviations are from one experiment. pCMV4 control transfections typically produced 50 to 100 colonies.

*Values derived from five independent experiments.

*Each E4F262-containing transfection was performed with the calcium phosphate procedure and contained 5 × 10⁴ NIH 3T3 fibroblasts, 1.0 μg of pCMV4 (pCMV) or CMVs-E4F262 (E4F262), 1.0 μg of pβ21-neo plasmid, and 200 μg of total DNA; for E4F262, each independent experiment was performed in duplicate or triplicate. These conditions produced numbers of pCMV4 control colonies similar to those of the transfections with E4F.

*Values derived from 10 independent experiments.

*Each p53-containing transfection was performed with Lipofectamine reagent and contained 6 × 10⁴ NIH 3T3 fibroblasts, 150 ng of the indicated mutant p53 expression plasmid or empty vector (pCMVneo), 500 ng of pCMV4 (pCMV) or pCMV-E4F2.5K (E4F), 0.5 μg of p53-281 (p120 E4F) plasmid, and 0.15 μg of total DNA.

**Clonal antibody (Santa Cruz Biotechnology) and detected by ECL. Cyclins and cyclin-dependent kinase inhibitor proteins were detected with antibodies against p21**

**WAF1/CIP1** (Santa Cruz Biotechnology), p27**KIP1** (Santa Cruz Biotechnology), p21**WAF1** (Santa Cruz Biotechnology), cyclin A (PharMingen), cyclin D1 (C. J. Serr, St. Jude Children’s Research Hospital), and cyclin E (Santa Cruz Biotechnology). Blots were visualized by ECL.

*In vitro kinase assays. Immunoprecipitation and analysis of kinase activities from whole-cell extracts of E4F, E4F4, and control cell lines were performed essentially as described previously (34, 48). Kinase activities were immunoprecipitated from 100 μg of extract with antisera to purified antibodies against cyclin D1 (C. J. Serr), cyclin E (Santa Cruz Biotechnology), cyclin A (PharMingen), cdk2, and cdk4 and cdk6 (C. J. Serr). All reactions were performed at 30°C in 20 μl of kinase buffer (50 mM HEPES [pH 7.5], 10 mM MgCl₂, 1 mM dithiothreitol) containing 20 μM ATP and 10 μCi of [γ-³²P]ATP (6,000 Ci/mmol; New England Nuclear) linked to horseradish peroxidase (Transduction Laboratories) or cdk1 and cdk2 immune complexes were incubated for 90 min and used 50 ng of glutathione S-transferase (GST)-Rh (379–928) protein (S. Hiebert, Vanderbilt University, Nashville, Tenn.) as a substrate. Reaction mixtures containing cyclin E, cyclin A, and cdk2 immune complexes were incubated for 15 min and used 1 μg of histone H1 (Sigma) as a substrate. Phosphorylation of substrate protein was determined by
Northern blot analysis. Total RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (7) and used to prepare polyadenylated RNA with the PolyATtract mRNA isolation system (Promega). Poly(A) RNA (10 μg) was electrophoresed through a 1.2% formaldehyde-agarose gel (37) in parallel with RNA size markers (Life Technologies) and transferred to nitrocellulose. Probes were gel-purified plasmid inserts that were labeled by the MegaPrime DNA labeling system (Amersham). Individual probes were excised from the following I.M.A.G.E. Consortium [LLNL] cDNA clones (29): mouse p21 (clone 533961), mouse cyclin A (clone 476912), mouse cyclin D1 (clone 402690), and mouse cyclin E (clone 315063). All clones were obtained through Genome Systems, Inc.

RESULTS

p120E4F can suppress fibroblast colony formation and growth. The effect of p120E4F on cell growth was assessed by colony formation assays using NIH 3T3 fibroblasts. A cytomegalovirus (CMV) promoter-driven expression construct encod-
ing the full-length E4F cDNA (pCMVs-E4F2.5K) or empty vector (pCMV4) was cotransfected into NIH 3T3 fibroblasts with a neomycin resistance marker alone or in combination with constructs expressing E1A(13S), E1A(12S), or activated ras (Table 1). Increasing amounts of the E4F construct markedly suppressed G418-resistant colony formation relative to vector controls. In contrast, when cotransfected with E1A(13S), E1A(12S), or activated ras expression constructs, the E4F construct had little or no suppressive effect on colony formation and at lower levels had a slight stimulatory effect with activated ras. Coexpression of the E4F construct with combinations of E1A(13S) plus ras or E1A(12S) plus ras also had modest stimulatory effects (Table 1). Because coexpression of E1A has little or no effect on expression from pCMVs-E4F2.5K (17), alleviation of p120E4F-induced colony suppression by E1A was not due to effects on p120E4F expression.

Although the majority of protein expressed from the E4F cDNA exists in cells as full-length p120E4F, a minor fraction is proteolytically converted to p50E4F (17). As such, the growth effects seen with expression of full-length E4F cDNA could be a function of p120E4F, p50E4F, or both. Therefore, a construct expressing E4F-encoded amino acids 1 to 262 (E4F262) was also tested, as described above, in NIH 3T3 colony formation assays (Table 1); the E4F262-encoded protein displays all of the functional properties of endogenous p50E4F with regard to DNA binding, transcriptional activation, and E1A regulation (17). In contrast to full-length E4F cDNA, expression of E4F262 had no significant effect on colony formation. Therefore, the growth-suppressive effects of E4F cDNA in untransformed NIH 3T3 cells are due to the p120E4F protein.

To further analyze this effect, individual clones of NIH 3T3 fibroblasts were isolated from the colony formation assays described above and assayed for expression of p120E4F or E4F262 and for changes in their growth characteristics; representative clones of each type are illustrated (Fig. 1A). The growth rates of cell lines that overexpress p120E4F were markedly reduced in comparison to control cell lines, and the degree of growth rate reduction roughly correlated with the level of p120E4F expression (Fig. 1B). In contrast, the growth rates of E4F262 cell lines were no different than those of the control cell lines. Cell lines that coexpress p120E4F and activated ras had normal or accelerated growth rates, similar to those of ras-transformed NIH 3T3 fibroblasts (Fig. 2). Cell viability, as measured by trypan blue exclusion, was generally greater than 90% for all cell lines (not shown). Thus, the phenotypic effect of p120E4F expression in stable cell lines correlated with what was observed in colony formation assays.

**FIG. 2.** Coexpression of activated ras alleviates the suppression of cell growth by p120E4F. (A) NIH 3T3 cell lines that coexpress p120E4F and activated ras were plated at 10^5 cells per 35-cm-diameter well, and cell counts were determined at the indicated times. Cell lines include the parental line (NIH 3T3), two ras-expressing cell lines (3T3/ras 17-1 and 17-2), two p120E4F cell lines (E4F2.5K/3T3-4 and -7), and three lines that coexpress p120E4F and activated ras (E4F2.5K/ras-1, -3, and -7). Growth curves are from a single representative experiment, with each point being the average from duplicate wells; the standard error between duplicates was less than 6% at all points. Growth rates of all lines were determined in parallel in three independent experiments. (B) Expression levels of p120E4F and Ras proteins in E4F2.5K/ras and control cell lines. Ectopically expressed p120E4F protein was detected by precipitation with S-protein-agarose and Western blotting using o-E4F-Nterm antiserum after separation of proteins by SDS–10% PAGE. Ras proteins were detected by Western blotting using a pan anti-Ras monoclonal antibody after separation of protein extracts by SDS–14% PAGE, as described in Materials and Methods. The positions of p120E4F and Ras are indicated by arrows.

**FIG. 3.** p120E4F attenuates S-phase entry following serum stimulation. Cells were grown to 30% confluency and synchronized in the quiescent G0 state by incubation in media containing 0.1% serum for 48 h prior to stimulation with 10% serum. At the indicated times after serum addition, the distribution of cells with 2C (G0-G1), intermediate (S), and 4C (G2-M) DNA content were determined by FACS analysis of propidium iodide-stained nuclei. FACS profiles were determined for several serum stimulation experiments; values from a representative experiment are shown. (Top panel) Percentage of NIH 3T3 cells in S phase after serum stimulation. (Bottom panel) Percentage of E4F2.5K/3T3-7 cells in S phase after serum stimulation. Note that the time intervals are spaced differently on the two graphs (x axes).
Enforced p120E4F expression inhibits S-phase entry. Alleviation of p120 E4F’s negative growth effects by E1A and ras suggested that p120 E4F was affecting some aspect controlling G1-to-S-phase progression. To determine if E4F cell lines exhibited a delay in S-phase entry, a p120 E4F cell line (E4F2.5K/3T3-7) and a control cell line (NIH 3T3) were synchronized in G0 by serum starvation for 48 h and then analyzed by FACS for cell cycle progression following serum stimulation. For the control cell line (Fig. 3, top panel), S-phase entry occurred sharply after 10 h poststimulation, with the number of cells in S phase peaking at 14 to 16 h poststimulation. For the p120E4F cell line (Fig. 3, bottom panel), a slow accumulation of cells in S phase started approximately 10 h poststimulation and then sharply accelerated at 27 h, with the number of cells in S phase peaking at 33 h poststimulation. The period between maximal accumulation in S phase and accumulation in the next G1 phase was approximately 5 to 7 h for both cell lines, indicating a similar duration of G2-M phases for both lines (not shown). These results indicate a delay in the G1-to-S-phase transition in p120E4F-expressing cells.

To determine if the delay in S-phase entry was directly attributable to p120E4F function and not a result of clonal selection, we constructed fibroblast cell lines in which p120 E4F expression was under dexamethasone-inducible control from the mouse mammary tumor virus long terminal repeat (pMAM-E4F2.5K/3T3) (Fig. 4C). FACS analysis of pMAM-E4F2.5K/3T3 cell lines and the pMAM/3T3-1 control line following a 72-h treatment with 1 μM dexamethasone (+, −, −, untreated), p120E4F protein was detected by Western blotting using α-E4F-Nterm antiserum after S-protein–agarose precipitation from 100 μg of protein extract; the position of p120E4F is indicated.

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p120E4F cell lines contain elevated levels of p21WAF1. Inhibition of G1-to-S-phase progression in p120E4F cell lines suggested that p120E4F/ras expression was affecting the activity of one or more G1-specific cyclin-cdk complexes. Therefore, lysates of p120E4F cells, p120E4F/ras cells, and control cells were immunoprecipitated by antisera against cyclin D1, cyclin E, cyclin A, cdk2, and cdk4/6, and the immunoprecipitates were tested in vitro for associated kinase activity (Fig. 5A and B). Cyclin D1- and cdk4/6-associated kinase activities were reduced 10- to 20-fold in p120E4F cells, whereas cyclin E- and cdk2-associated kinase activities were reduced 4- to 5-fold and cyclin A-associated kinase activity was reduced 2-fold. In contrast, all cyclin-associated and cdk kinase activities were elevated 1.5- to 4-fold in p120E4F/ras cells, correlating with their increased growth rates.

The relative protein levels of cyclins D1, E, and A and universal cdk inhibitors p21 and p27 in lysates of p120E4F, p120E4F/ras, and control cell lines were determined by immunoblot analyses. p120E4F cells had significant increases in p21 levels and more modest increases in p27 levels (Fig. 5C), which could account for inhibition of S-phase entry. p120E4F cell lines also had higher levels of cyclin E, which may allow these cells to partially overcome the higher levels of p21 and p27 and grow, albeit at a low rate. p120E4F/ras cell lines still had elevated levels of p21, p27, and cyclin E, but they also had markedly higher levels of cyclin D1 (Fig. 5D), which might titrate...
out the increased levels of p21 and p27 and permit the observed accelerated growth rates.

*p21^E4F* protein half-life is stabilized in p120^E4F* cell lines independent of p53. Although p21 and cyclin E protein levels were higher in p120^E4F* cell lines, Northern blot analysis showed no accompanying increase in p21 mRNA levels and a significant decrease in cyclin E mRNA levels (Fig. 6A). Also, cyclin D1 and cyclin A mRNA levels did not appreciably change. These results, and the lack of E4F binding sites in the p21^E4F* and cyclin E promoters, indicate that the p21^E4F* transcription factor must be indirectly affecting the expression of these genes. In contrast, cyclin D1 mRNA levels were elevated in p120^E4F*/ras cell lines (Fig. 6B), accounting for the higher levels of cyclin D1 protein. To determine if the higher level of p21 protein in p120^E4F* cells was due to increased protein stability, the p21 half-life was measured in p120^E4F* and control cells by pulse labeling for 1 h with [35S]methionine and [35S]cysteine, followed by a chase with an excess of unlabeled methionine and cysteine. Quantitation of immunoprecipitated p21 protein showed a half-life of ~45 min in control cells and a half-life of ~165 min in p120^E4F* cells (Fig. 7), indicating that p120^E4F* overexpression leads to a decrease in p21 protein turnover.

Although the p21 promoter has been identified as a transcriptional target of wild-type p53 (13), the posttranscriptional up-regulation of p21 in p120^E4F* cells suggests it is not likely to depend upon wild-type p53 function. Also, p53 protein levels are not elevated in p120^E4F* cells (16). To confirm that p53 is not involved, the E4F cDNA was transfected into NIH 3T3 fibroblasts in combination with constructs expressing two forms of mutant p53, p53-281 and p53-22,23,281 (22, 31). Both forms can oligomerize with and inactivate wild-type p53, but
FIG. 6. p120E4F cell lines do not contain increased levels of p21WAF1 mRNA. (A) Northern blot analysis of poly(A) RNA isolated from three p120E4F cell lines (E4F2.5K/3T3-4, -5, and -7) and two control cell lines (NIH 3T3 and 3T3/neo 23-1). Blots (10 μg of RNA/lane) were hybridized with 32P-labeled cDNA probes for p21WAF1, cyclin D1, cyclin E, and cyclin A; a cDNA probe for gelsolin was used as a control for RNA concentration and loading. (B) Northern blot analysis of poly(A)+ RNA isolated from two control cell lines (NIH 3T3 and 3T3/neo 23-1), two p120E4F cell lines (E4F2.5K/3T3-4 and E4F2.5K/3T3-7), and two p120E4Fras cell lines (E4F2.5K/3T3-1 and E4F2.5K/3T3-3). Blots were hybridized with 32P-labeled probes for p21WAF1 and cyclin D1.

Mechanisms that control cyclin inhibitory expression or activity have been documented at multiple levels, including transcription, mRNA stability, translational control, posttranslational stabilization, and protein sequestration (10, 13, 21, 23, 30, 33, 36, 39, 46), but for p21 most studies have focused on the means of transcriptional induction. In this study, the effect of p120E4F expression on p21 turnover is a clear indication that protein stability also plays a part in p21 regulation. Whether or not protein stabilization is the only mechanism altering p21 levels in p120E4F-overexpressing cells remains to be determined.

The 22,23,281 mutant lacks a functional transcriptional activation domain. Neither p53 altered the effect of p120E4F expression on colony formation (Table 1), indicating that the effects elicited by p120E4F were p53 independent.

**DISCUSSION**

Genetic and functional analyses have linked many of the biological effects of the adenovirus E1A proteins to two E1A-mediated transcriptional regulatory mechanisms, the dysregulation of E2F factors through the interaction of E1A with members of the Rb protein family and the repression of enhancer-activated transcription through the interaction of E1A with p300 and CBP (12, 35, 47). We demonstrate in this study that another cellular transcription factor targeted by E1A, p50E4F, can also influence cellular growth and phenotype.

Ectopic expression of p120E4F in NIH 3T3 fibroblasts markedly suppressed colony formation and led to extremely low growth rates in stably expressing cell lines, effects that were alleviated by coexpression of activated ras (49) elevated cyclin D1-cdk4/6 activity by as much as 80-fold and alleviated the G1-to-S block. Furthermore, activated ras expression did not reduce the elevated levels of p21 that were induced by p120E4F, suggesting that p120E4F was still functional in these cells. We therefore presume that the elevated levels of p21 and p27 are responsible for the G1-to-S block but cannot discount the possibility that other p120E4F-induced effects may also be involved.

Mechanisms that control cyclin inhibitory expression or activity have been identified at multiple levels, including transcription, mRNA stability, translational control, posttranslational stabilization, and protein sequestration (10, 13, 21, 23, 30, 33, 36, 39, 46), but for p21 most studies have focused on the means of transcriptional induction. In this study, the effect of p120E4F expression on p21 turnover is a clear indication that protein stability also plays a part in p21 regulation. Whether or not protein stabilization is the only mechanism altering p21 levels in p120E4F-overexpressing cells remains to be determined.

**FIG. 7.** p21WAF1 protein half-life is greater in p120E4F cells. (A) Quantitation of p21 protein half-life. Subconfluent cultures of E4F2.5K/3T3-7 cells and 3T3/neo 23-1 control cells were labeled for 1 h with [35S]methionine and [35S]cysteine and chased with an excess of unlabeled methionine and cysteine for the times indicated. [35S]-labeled p21 protein was immunoprecipitated, separated by SDS–14% PAGE, and quantitated by PhosphorImager analysis. The data at individual time points represent the relative amounts of 35S-labeled p21 protein compared to the start of the chase (time zero) and are the average of three independent experiments; standard deviations are shown as thin bars, and the positions where p21 levels are reduced by 50% (t1/2) are indicated. 35S-labeled p21 protein was immunoprecipitated, separated by SDS–14% PAGE, and quantitated by PhosphorImager analysis. The data at individual time points represent the relative amounts of 35S-labeled p21 protein compared to the start of the chase (time zero) and are the average of three independent experiments; standard deviations are shown as thin bars, and the positions where p21 levels are reduced by 50% (t1/2) are indicated by dotted lines. (B) Immunoprecipitation of p21 protein from a representative pulse-chase experiment.
be determined, as it is unclear if the three- to fourfold increase in p21 half-life can account for the entire observed increase of p21 protein (10- to 30-fold by densitometry). Therefore, we cannot rule out additional posttranscriptional mechanisms, such as the increased translation that occurs during cell cycle regulation of p27, with p53 regulation of cdk4, or with overexpression of mdm2 in some human tumors (14, 21, 28).

How p120<sup>OE4F</sup> affects p21 turnover remains unclear. We observed that, in addition to p21, cyclin E and p27 protein levels were also elevated, albeit more modestly, in p120<sup>OE4F</sup>-overexpressing cells, suggesting a common link in their regulation. In the case of cyclin E, protein levels increased while mRNA levels significantly dropped, suggesting that cyclin E stabilization was on the same order as that of p21. Recent studies have demonstrated that p21 can be degraded through the ubiquitin-proteosome pathway (4, 32), the mechanism responsible for cyclin E and p27 degradation (8, 36, 50). Although there are no indications as yet that p21 turnover is cell cycle regulated, as occurs with cyclin E and p27 (1, 8, 36, 50), one interpretation of our results is that the specific pathways regulating the turnover of p21 and cyclin E may share a common component whose transcription is affected by p120<sup>OE4F</sup> (e.g., a specific E2, E3, or deubiquitinating enzyme). However, ubiquitination of cyclin E and p27 appears to require prior phosphorylation by cdk’s (and possibly mitogen-activated protein kinases for p27) (2, 8, 50), and thus their relative stabilization in p120<sup>OE4F</sup>-cells could also be a secondary reflection of the inhibition of cdk activities by p21.

**Biological relevance of p120<sup>OE4F</sup>.** Although the inhibitory growth effects reported here occurred with overexpression of p120<sup>OE4F</sup>, they do correlate with its regulation observed in serum-starved CREF cells (18); i.e., the stimulation of p120<sup>OE4F</sup> phosphorylation that occurs with serum, tetradecanoyl phorbol acetate, or adenosine infection (E1A) down-regulates p120<sup>OE4F</sup> DNA binding activity and would thereby lessen its negative effects on cell cycle progression and growth. Also, we recently observed similar negative effects on colony formation and cellular growth rates when p120<sup>OE4F</sup> was overexpressed in H35 hepatoma cells, indicating that the effects reported here are not peculiar to our NIH 3T3 clone or to fibroblast cell lines in general (16).

Another situation where p120<sup>OE4F</sup> may be physiologically relevant involves the effect of E1A on TGF-β growth inhibition. The ability of E1A to overcome TGF-β growth inhibition involves a number of different mechanisms that include the direct physical binding and inactivation of p27, repression of TGF-β-induced p15<sup>INK4B</sup> gene transcription, and a block to TGF-β induction of p21 (9, 33). Although the effect on p21 induction appears to involve the repression of TGF-β-induced activation of the p21 promoter, dependent on an E1A-p300/CBP interaction (5, 9, 30), p21 mRNA induction in TGF-β-treated HaCaT human keratinoocyte cells is reduced by only 50% with adenosine infection (9). This suggests that the marked reduction of p21 protein must also have a posttranscriptional component, and it is possible that this may be due to E1A-mediated down-regulation of p120<sup>OE4F</sup>. Examination of this mechanism and other physiological processes in which p21 levels increase (e.g., senescence or differentiation) may ultimately reveal a wider array of circumstances in which E4F proteins play important roles.

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**REFERENCES**

1. Agrawal, D., F. Dong, Y. Z. Wang, D. Kayda, and W. J. Pledger. 1995. Regulation of cyclin E and p27kip during mitosis in BALB/c 3T3 cells. Cell Growth Differ. 6:1199–1205.
2. Alessandri, A., D. S. Chiaur, and M. Pagano. 1997. Regulation of the cyclin-dependent kinase inhibitor p27 by phosphorylation and phosphorylation. Leukemia 11:342–345.
3. Berk, A. J. 1986. Functions of adenovirus E1A. Cancer Surv. 5:367–387.
4. Blaggesklover, M. V., G. S. Wu, S. Omura, and W. S. el-Deiry. 1996. Protease-dependent regulation of p21/WAF1/CIP1 expression. Biochem. Biophys. Res. Commun. 227:506–510.
5. Botchan, M. 1996. Coordinating DNA replication with cell division: current status of the licensing concept. Proc. Natl. Acad. Sci. USA 93:9997–10000.
6. Chevalier, S., and J. J. Blow. 1996. Cell cycle control of replication initiation in Cercopis erythrocephala.Curr. Opin. Gen. Dev. 6:815–821.
7. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156–159.
8. Clark, R. E., R. J. Sheafe, K. Thress, M. Grondine, and J. M. Roberts. 1996. Turnover of cyclin E by the ubiquitin-proteosome pathway is regulated by cdk2 binding and phosphorylation. Genes Dev. 10:1979–1990.
9. Datto, M. B., P. P.-C. Hu, T. F. Kowalik, J. Yingling, and X.-F. Wang. 1997. The viral transactivator E1A blocks transforming growth factor β-mediated induction of p21/WAF1/Cip1 and p15/INK4B. Mol. Cell. Biol. 17:2030–2037.
10. Datto, M. B., Y. Li, J. F. Panus, D. J. Howe, Y. Xiong, and X. F. Wang. 1995. Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. Proc. Natl. Acad. Sci. USA 92:5545–5549.
11. Doree, M., and S. Galas. 1994. The cyclin-dependent protein kinases and the control of cell division. FASEB J. 8:1114–1121.
12. Eckner, R. 1996. p300 and CBP as transcriptional regulators and targets of oncogenic events, Biol. Chem. 377:685–688.
13. el-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein. 1996. ras downregulation of protein kinase C mRNA in C3H 10T1/2 fibroblasts. Mol. Carcinog. 17:23–34.
14. Fornace, E. R., and C. L. Ashendel. 1990. Transformation by the human adenoviruses. Semin. Cancer Biol. 1:225–234.
15. Fornace, E. R., and C. L. Ashendel. 1995. WAF1, a potential mediator of p53 tumor suppression. Cell 81:825–835.
16. Fornace, J. A., P. Leavitt, G. Muscat, S.-Y. Ng, and L. Kedes. 1987. A human beta-actin expression vector system directs high-level accumulation of antisense transcripts. Proc. Natl. Acad. Sci. USA 84:8381–8385.
17. Harlow, E., and D. Lane. 1988. Antibodies; a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
18. Hengst, L., and S. I. Reed. 1996. Translational control of p27kip accumulation during the cell cycle. Science 274:1681–1684.
19. Hinds, P. W., C. A. Finlay, R. S. Quartin, S. J. Baker, E. R. Fearon, B. Vogelstein, and A. J. Levine. 1990. Mutant p53 DNA clones from human colon carcinomas cooperate with ras in transforming primary rat cells: a comparison of the “hot spot” mutant phenotypes. Cell Growth Differ. 1:571–580.
20. Jacks, T., and R. A. Weinberg. 1996. Cell-cycle control and its watchman. Nature 381:643–644.
21. Jones, N. C. 1990. Transformation by the human adenoviruses. Semin. Cancer Biol. 1:432–435.
22. Jones, N. C. 1995. Transcriptional modulation by the adenovirus E1A gene. Curr. Top. Microbiol. Immunol. 199:59–80.
23. King, R. W., R. J. Deshaies, J. Peters, and M. W. Kirschner. 1996. How proteostasis drives the cell cycle.
24. Krishan, A. 1975. Rapid flow cytometric analysis of the mammalian cell cycle.
25. Downloaded from http://mcb.asm.org/ on May 2, 2019 by guest
by propidium iodide staining. J. Cell Biol. 66:188–193.
28. Landers, J. E., D. S. Haines, J. F. Strauss III, and D. L. George. 1994. Enhanced translation: a novel mechanism of mdm2 oncogene overexpression identified in human tumor cells. Oncogene 9:2745–2750.
29. Lentz, G., C. Aufray, M. Polymeropoulos, and M. B. Soares. 1996. The I.M.A.G.E. consortium: an integrated molecular analysis of genomes and their expression. Genomics 33:151–152.
30. Li, J.-M., M. A. Nichols, S. Chandrasekharan, Y. Xiong, and X.-F. Wang. 1995. Transforming growth factor B activates the promoter of cyclin-dependent kinase inhibitor p15INK4B through an Sp1 consensus site. J. Biol. Chem. 270:26750–26753.
31. Lin, J., A. K. Teresky, and A. J. Levine. 1995. Two critical hydrophobic amino acids in the N-terminal domain of the p53 protein are required for the gain of function phenotypes of human p53 mutants. Oncogene 10:2387–2390.
32. Maki, C. G., and P. M. Howley. 1997. Ubiquitination of p53 and p21 is differentially affected by ionizing and UV radiation. Mol. Cell. Biol. 17:355–363.
33. Mal, A., R. Y. Poon, P. H. Howe, H. Toyoshima, T. Hunter, and M. L. Harter. 1995. Inactivation of p27Kip1 by the viral E1A oncoprotein in TGFbeta-treated cells. Nature 380:262–265.
34. Matsushima, H., D. E. Quelle, S. A. Shurtleff, M. Shibuya, C. J. Sherr, and J. Kato. 1994. D-type cyclin-dependent kinase activity in mammalian cells. Mol. Cell. Biol. 14:2066–2076.
35. Nevins, J. R. 1995. Adenovirus E1A: transcription regulation and alteration of cell growth control. Curr. Top. Microbiol. Immunol. 199:25–32.
36. Pagano, M., S. W. Tam, A. M. Theodoras, P. Beer-Romero, G. Del Sal, V. Chau, P. R. Yew, G. F. Draetta, and M. Rolfe. 1995. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science 269:682–685.
37. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
38. Santos, E., S. R. Tronick, S. A. Aaronson, S. Pulcianni, and M. Barbacid. 1982. T24 human bladder carcinoma oncogene is an activated form of the normal human homologue of BALB and Harvey-MSV transforming genes. Nature 298:343–347.
39. Schwaller, J., H. P. Koeffler, G. Nikolaus, P. Loetscher, S. Nagel, M. F. Fey, and A. Tobler. 1995. Posttranscriptional stabilization underlies p53-independent induction of p21WAF1/CIP1/SDI1 in differentiating human leukemic cells. J. Clin. Invest. 95:973–979.
40. Sellers, W. R., and W. G. Kaelin. 1996. Rb as a modulator of transcription. Biochim. Biophys. Acta 1288:M1–M5.
41. Shenk, T., and J. Flint. 1991. Transcriptional and transforming activities of the adenovirus E1A proteins. Adv. Cancer Res. 57:47–85.
42. Sherr, C. J. 1995. D-type cyclins. Trends Biochem. Sci. 20:187–190.
43. Sherr, C. J. 1996. Cancer cell cycles. Science 274:1672–1677.
44. Sherr, C. J., and J. M. Roberts. 1995. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev. 9:1149–1163.
45. Stillman, B. 1996. Cell cycle control of DNA replication. Science 274:1659–1664.
46. Vlach, J., S. Hennecke, K. Alevizopoulos, D. Conti, and B. Amati. 1996. Growth arrest by the cyclin-dependent kinase inhibitor p27Kip1 is abrogated by c-Myc. EMBO J. 15:6595–6604.
47. Whyte, P. K. 1995. The retinoblastoma protein and its relatives. Semin. Cancer Biol. 6:83–90.
48. Winston, J. F., D. Dong, and W. J. Pledger. 1996. Differential modulation of G1 cyclins and the Cdk inhibitor p27Kip1 by platelet-derived growth factor and plasma factors in density-arrested fibroblasts. J. Biol. Chem. 271:11253–11260.
49. Winston, J. T., S. R. Coats, Y. Z. Wang, and W. J. Pledger. 1996. Regulation of the cell cycle machinery by oncogenic ras. Oncogene 12:127–134.
50. Won, K. A., and S. I. Reed. 1996. Activation of cyclin E/CDK2 is coupled to site-specific autophosphorylation and ubiquitin-dependent degradation of cyclin E. EMBO J. 15:4182–4193.