JOURNAL OF VIROLOGY, Apr. 1995, p. 2491–2500 Vol. 69, No. 4
0022-538X/95/$04.00+0

E2F1 Overexpression in Quiescent Fibroblasts Leads to Induction of Cellular DNA Synthesis and Apoptosis

TIMOTHY F. KOWALIK, JAMES DEGREGORI, JAMES K. SCHWARZ,† AND JOSEPH R. NEVINS*

Department of Genetics, Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina 27710

Received 25 October 1994/Accepted 12 January 1995

Various experiments have demonstrated a role for the E2F transcription factor in the regulation of cell growth during the G_0/G_1/S phase transition. Indeed, overexpression of the E2F1 product, a component of the cellular E2F activity, induces DNA synthesis in quiescent fibroblasts. To provide an approach to a more detailed biochemical analysis of these events, we have made use of a recombinant adenovirus containing the E2F1 cDNA in order to efficiently express the E2F1 product in an entire population of cells. We demonstrate an induction of DNA synthesis when quiescent cells are infected with the E2F1 recombinant virus. However, we also find that the induction does not lead to a complete replication of the cellular genome, as revealed by flow cytometry. The incomplete nature of cellular DNA replication is due, at least in part, to the fact that E2F1 overexpression leads to massive cell death that is characteristic of apoptosis. This E2F1-mediated induction of apoptosis is largely dependent on endogenous wild-type p53 activity and can be subverted by introducing mutant forms of p53 into these cells or by overexpressing E2F1 in fibroblasts derived from p53-null mouse embryos. We conclude that E2F1 can induce events leading to S phase but that the process is not normal and appears to result from the activation of a cell death pathway.

The events that control the orderly passage of cells through a cell cycle are under tight regulatory control. Through the combined use of genetic and biochemical approaches, the identification of regulatory activities controlling the cell cycle has progressed at a rapid pace. Although yeast systems have provided many of the initial advances (13), more recent contributions have been made from the study of mammalian cell growth control and in particular the events that are disrupted during oncogenic transformation. These studies have led to the realization that the so-called tumor suppressor genes, including *RB1* (retinoblastoma tumor suppressor gene) and the p53 gene, encode proteins that control the progression of cells through the cell cycle and are often targets for the action of viral oncoproteins. Since the initial studies of *RB1* function, most attention has focused on the role of the *RB1*-encoded protein (Rb) in controlling cell growth during the G_1-to-S phase transition. Indeed, direct assays demonstrate an ability of Rb to bring about a G_1 growth arrest in Rb-negative cells (1, 19).

The *RB1* gene is now recognized as one member of a family of mammalian genes that encode proteins that bind to the E2F transcription factor. At least one other member of this family, the p107 gene product, has also been shown to have growth-suppressing activity that, as in the case of Rb, correlates with its ability to interact with and inhibit E2F (45). The product of the most recently cloned member of the family, the p130 gene (12, 25, 30), also interacts with E2F, specifically during the G_0 and early G_1 phases of the cell cycle (5), and cytogenetic studies suggest that p130 may be altered in several different human cancers (12). Moreover, as cells begin to grow out of quiescence, there is a dramatic change in the phosphorylation state of the Rb protein as cells proceed through the G_1/S transition (2, 4, 8, 9, 31). Since other experiments suggest that it is the unphosphorylated form of Rb that is active in growth suppression (11, 29), it appears that the activity of Rb is regulated as cells pass through G_1 and into S phase.

Additional experiments have provided convincing evidence that the cellular transcription factor E2F is a relevant target of the action of Rb as a growth suppressor. The interaction of Rb with E2F results in an inhibition of E2F transcriptional activity, apparently by blocking the transcriptional activation domain of E2F (6, 14, 15, 23, 44). E2F appears to normally function to control the transcription of a group of genes that encode proteins important for S-phase events, including dihydrofolate reductase, thymidine kinase, and DNA polymerase α (32). These genes are coordinately regulated during the cell growth cycle, being induced in late G_1 and S phases at a time when active E2F accumulates. Thus, the action of Rb or Rb family members can be seen as the control of expression of these S-phase genes. The ability of Rb to interact with E2F directly correlates with the ability of Rb to arrest cell growth in G_1, as seen by the analysis of Rb mutants isolated from human tumors as well as in vitro-constructed mutants that have been assayed for growth suppression activity. In each case, mutants that lack the ability to cause a growth arrest also lack the ability to bind E2F and inhibit E2F-dependent transcription (16, 17, 32, 34).

A series of recent studies using the E2F1 gene, which encodes a component of cellular E2F activity, have directly demonstrated a role for E2F in cell proliferation control. These experiments have shown that expression of the E2F1 product can block cells from entering quiescence when serum is removed from the culture and that overexpression of the E2F1 product can induce quiescent cells to enter S phase, both events dependent on the ability of E2F1 to bind DNA and activate transcription (22). Other recent experiments have shown that a G_1 arrest resulting from treatment of cells with transforming growth factor β, likely a consequence of the inhibition of G_1 cyclin/kinase activity, can be overcome by the

* Corresponding author. Phone: (919) 684-2746. Fax: (919) 681-8973.

† Present address: Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115.

expression of the E2F1 product (37). Finally, very recent data demonstrate that E2F1 overexpression can lead to transformation of an immortalized cell line (39) or, in conjunction with activated *ras*, transformation of primary rat embryo cells (21).

A common aspect of the transformation process is the loss of regulated DNA replication resulting in genomic instability. Since E2F1 expression and function appear to be tightly linked to a cell proliferation pathway that when deregulated can lead to transformation, we wished to examine in more detail the DNA replication process associated with deregulated E2F1 expression. Using a recombinant adenovirus which overexpresses E2F1, we found that the pattern of DNA synthesis was incomplete and that this was largely due to the induction of apoptosis. Further examination revealed that apoptosis could be induced by E2F1 overexpression under a number of conditions, including serum deprivation, γ-irradiation, and even normal culture conditions in fibroblasts. Finally, introducing dominant p53 mutations into these cells or infecting fibroblasts derived from p53-null ($p53^{-/-}$) mice blocked most of the apoptotic events induced by overexpressed E2F1, thereby implicating endogenous p53 in signalling the apoptotic program.

MATERIALS AND METHODS

Virus construction and infections. Recombinant adenoviruses in which E1A and E1B sequences have been replaced with the cytomegalovirus promoter (AdCMV) or the E2F1 cDNA under the control of the cytomegalovirus promoter (AdE2F1) (Fig. 1A) have been described previously (37). An adenovirus deleted for both the E1B (*dl*118 mutation) and 13S E1A genes, LL2-140, was graciously provided by L. Babiss (Glaxo). All viruses were grown and their titers were determined on 293 cells. Virus titers were determined by immunofluorescent staining of the adenovirus 72-kDa E2 gene product with an anti-72-kDa protein antiserum.

REF-52 cells expressing endogenous wild-type (wt) p53 (27) were a gift from E. Ruley (Vanderbilt University) and were grown in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS). To make cells quiesce, REF-52 cells were plated at a density of 3,500 cells per cm^2 and incubated overnight. The next day, the culture medium was removed, the cells were washed once in phosphate-buffered saline (PBS), and the culture medium was replaced with DMEM containing 0.1% FCS. Cells were further incubated for 48 h prior to virus infection or serum stimulation by replacement with culture medium containing 10% FCS. Infections of REF-52 cells were done at a multiplicity of infection of 500.

REF-52 cells expressing mutant p53 were created by transfecting one of two plasmids, pIE.p53.217.neo and pIE.p53.238.neo, which express forms of p53 with mutations at codon positions 217 and 238, respectively, as well as neomycin resistance. Both constructs were kindly provided by J. Marks (Duke University). After transfection of the DNA by $CaPO_4$ coprecipitation (22), drug-resistant colonies were identified by selection in culture medium containing 400 μg of geneticin per ml. Several colonies from each transfection were expanded and examined for functional inactivation of endogenous p53 activity by bypassing a G_1 block induced by γ irradiation (data not shown). Those isolates which did not arrest in G_1 but proceeded to S phase and DNA synthesis (217.4 and 238.3) as well as an isolate containing vector alone (neo.3) were used for subsequent experimentation. Mouse embryo fibroblasts containing wt ($p53^{+/+}$) or germ line-inactivated p53 ($p53^{-/-}$) were a gift from T. Jacks (Massachusetts Institute of Technology).

Cell irradiation. Cycling REF-52 cells were γ-irradiated with 12.5 Gy or sham treated (control) on 60-mm-diameter culture dishes. The culture media was then removed, and the cells were infected with either AdCMV or AdE2F1 as described above. Cells were harvested at the indicated times postinfection and analyzed by flow cytometry.

Western immunoblotting. Quiescent REF-52 cells were infected with the appropriate adenovirus construct, and samples were harvested by scraping at the indicated times to create whole cell extracts. Pelleted cells (5×10^5) were washed twice with cold PBS and lysed in 100 μl of whole cell extract buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 250 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid [EGTA], 0.1% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.3 mM sodium orthovanadate, 2 mM sodium fluoride, 2 μg of apoprotinin per ml, 1 μg of pepstatin per ml, 2 μg of leupeptin per ml) by incubation for 30 min on ice. Soluble proteins were collected by centrifugation at 15,000 rpm in a microcentrifuge, and the supernatant was stored at −70°C. Aliquots (30 μg) were analyzed by electrophoresis through 10% polyacrylamide gels, and the resolved proteins were transferred to nitrocellulose membranes by electroblotting. E2F1 protein expression was detected with an anti-E2F1 monoclonal antibody (Santa Cruz Biotechnology) by using enhanced chemiluminescence (Amersham) according to the manufacturers' recommendations.

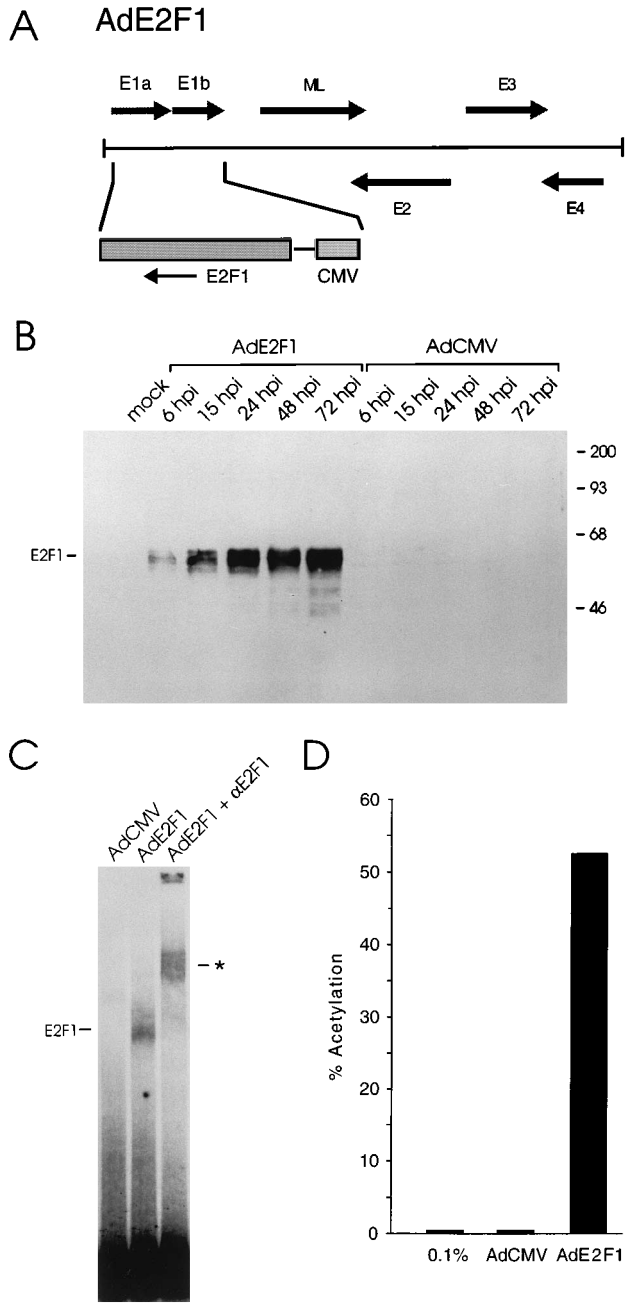

FIG. 1. (A) Schematic depiction of AdE2F1. ML, major late promoter; CMV, cytomegalovirus promoter. AdCMV is identical but lacks the E2F1 cDNA. (B) Western blot analysis of E2F1 expression in recombinant adenovirus infections of serum-starved REF-52 cells. E2F1 expression was detected on Western blots by an anti-E2F1 monoclonal antibody and enhanced chemiluminescence. Sizes are indicated in kilodaltons. (C) Comparison of E2F1 expression by electrophoretic mobility shift assays 48 h after recombinant virus infection. Asterisk, supershifted complex. (D) Transactivation of 4X-E2-CAT by E2F1 in AdE2F1-infected cells.

Gel retardation assays. DNA binding activity by E2F1 was measured as described previously (24). Essentially, 0.5 μg of whole cell extract from cells harvested 48 h postinfection (hpi) was incubated with a binding solution containing 20 mM HEPES (pH 7.5), 40 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM EGTA, 0.02% sodium azide, 0.1% Nonidet P-40, 10% glycerol, 1 μg of

sonicated salmon sperm DNA, and 0.1 ng of ^{32}P-labeled E2 DNA probe in a final volume of 20 μl. The mixture was incubated for 30 min at room temperature prior to resolution by electrophoresis through a nondenaturing 4% polyacrylamide gel at 4°C. Where indicated, 1 μl of the anti-E2F1 monoclonal antibody used in Western blotting was included in the binding reaction.

[^{3}H]thymidine incorporation. Immediately after serum-starved cells were infected with a recombinant adenovirus or serum stimulated, 10 μCi of [^{3}H]thymidine (2.00 Ci/mmol) per ml was added to the culture medium. At 30 or 72 hpi, cells were washed twice with PBS and lysed on the plates with 0.5 ml of lysis buffer (10 mM Tris [pH 8.0], 100 mM NaCl, 5 mM EDTA, 0.5% sodium dodecyl sulfate, 1 mg of proteinase K per ml). Any floating cells were pelleted, washed with PBS, and included with the attached cells. After incubation for 2 to 4 h at 37°C, the lysates were sheared by pipetting to reduce viscosity, transferred to microcentrifuge tubes, and stored at −20°C. To determine the degree of acid-precipitable labeling, 50 μl of each lysate was spotted onto a glass filter, air dried, and then washed for 10 min in cold 95% ethanol–5% trichloroacetic acid. Filters were dried in vacuo, and ^{3}H incorporation was monitored by liquid scintillation counting.

Flow cytometry. Serum-starved REF-52 cells on 100-mm-diameter culture plates were serum stimulated or infected with the adenovirus constructs as described above. At the indicated times postinfection, cells were then trypsinized, combined with any floating cells, pelleted, washed with PBS, repelleted, and resuspended in 400 μl of PBS. All centrifugations were at 500 × *g* for 2 min at 4°C. Subsequently, the cells were fixed in cold ethanol (70%, final concentration) and stored for at least 30 min at 4°C. The fixed cells were centrifuged, washed twice with PBS, and resuspended in 0.5 ml of PBS containing propidium iodide (50 μg/ml) and RNase A (0.5 mg/ml). Samples were incubated for 30 min to overnight at 4°C prior to analysis by flow cytometry with a Becton Dickinson FACScan.

Cell viability assays. Viability of infected cells was monitored by trypan blue exclusion. Floating cells from mock- and virus-infected samples were collected, and attached cells were trypsinized, resuspended in DMEM–10% FCS, and pooled with the floating cells. These cells were centrifuged at 500 × *g* for 2 min, and the pellets were resuspended in DMEM without serum. An aliquot of the suspension was mixed with trypan blue (0.1%, final concentration) and incubated for 5 min at room temperature. Cells were then counted with a hemacytometer. Viable cells were defined as those excluding the trypan blue dye. More than 300 cells were counted for each variable.

Analysis for DNA degradation. Preparation of DNA for agarose gel electrophoresis was based on the method of Hirt as modified by Debbas and White (7). Floating cells from mock- and virus-infected cells were collected, pelleted, and washed once with PBS. Cells were then repelleted and resuspended in 40 μl of PBS. Lysis buffer was added to 400 μl (final volume), and the cells were incubated at 37°C for 2 h. NaCl was then added to a final concentration of 1 M, and the lysates were incubated at 4°C overnight. The lysates were centrifuged at 15,000 rpm for 15 min, and the supernatants were collected and recentrifuged. Two volumes of ethanol was added to the supernatants, which were then incubated overnight at −20°C. After centrifugation and a 70% ethanol wash, pellets were resuspended and analyzed by electrophoresis through 1.25% agarose gels.

CAT assays. Promoter transactivation by E2F1 was measured by using chloramphenicol acetyltransferase (CAT) activity as expressed from transfection of 4X-E2-CAT and virus infection (22). The reporter plasmid (5 μg), a Rous sarcoma virus–β-galactosidase standard (2 μg), and 13 μg of salmon sperm carrier DNA were transfected into growing REF-52 cells by calcium phosphate coprecipitation for 12 h in culture medium lacking serum. Cells were subsequently cultured for an additional 24 h in culture medium containing 0.1% FCS prior to infection with the appropriate adenovirus construct. Cells were harvested 48 h after transfection and processed for CAT activity as described previously (22). Results were adjusted to values for the β-galactosidase standard.

RESULTS

Expression of E2F1 upon infection with a recombinant adenovirus. Previous experiments have shown that E2F1 overexpression in quiescent fibroblast cultures leads to an induction of cellular DNA synthesis (22). However, since these experiments have used either microinjection or transfection to introduce the E2F1 cDNA into target cells, it has not been possible to analyze the nature of this replication in detail since only a small fraction of the cells receive and express the exogenous E2F1 construct. To alleviate this limitation, we have generated a recombinant adenovirus in which the E2F1 cDNA has replaced the E1A and E1B genes (Fig. 1A). Two aspects of the biology of adenovirus are particularly important for these studies. First, high-titer stocks that allow for quantitative, high-multiplicity infection of the entire cell population, thus giving high-level expression of the recombinant gene product, can easily be generated. Second, and most critical for these experiments, is the fact that adenovirus can efficiently infect quiescent cells.

TABLE 1. Induction of DNA synthesis in AdE2F1-infected cells[a]

Sample	cpm in 1/10 of sample	
	30 h	72 h
Control	1,504	5,984
AdCMV	2,817	7,803
AdE2F1	10,456	22,658
10% serum	47,058	278,632

[a] Serum-starved REF-52 cells were mock infected, virus infected, or serum stimulated and then monitored for [^{3}H]thymidine incorporation at the indicated times.

As shown in Fig. 1B, expression of the E2F1 product was readily detected following infection of REF-52 cells with AdE2F1 but not the recombinant virus lacking the cDNA insert (AdCMV). E2F1 protein was detected as early as 6 hpi, increased through 15 hpi, and then remained relatively constant from 24 through 72 hpi. Expression of functional E2F1 in infections with AdE2F1 was also demonstrated by a gel retardation assay, in which increased levels of E2F binding activity were seen to accumulate, as well as CAT assays, in which the AdE2F1 infection resulted in activation of a transfected E2-CAT reporter plasmid (Fig. 1C and D).

Induction of cellular DNA synthesis by AdE2F1 infection. To extend previous observations concerning the ability of E2F1 to induce S phase and allow a biochemical approach to the events associated with this induction, we examined the ability of the AdE2F1 to induce DNA synthesis following infection of quiescent REF-52 cells. As shown in Table 1, AdE2F1 infection of serum-starved REF-52 cells resulted in a four- to sixfold increase in [^{3}H]thymidine incorporation over that observed in mock-infected cells or cells infected with an empty adenovirus vector. The extent of DNA synthesis induced by AdE2F1 was approximately one-fifth that of serum-stimulated cells. In addition, analysis of the labeled DNA from these experiments by isopycnic banding through CsCl gradients confirmed that the newly synthesized DNA was of cellular, not viral, origin (data not shown). Therefore, these results verify that E2F1 can induce quiescent cells to enter S phase, as measured by the induction of DNA synthesis (22). However, it is also clear from this assay that the induction of DNA replication is quantitatively not equal to that obtained by serum stimulation.

To provide additional information regarding the nature of the cellular DNA replication process following E2F1 overexpression, we used flow cytometry to measure DNA content in quiescent REF-52 cells infected with AdE2F1 (Fig. 2). Serum-starved cells infected with the AdCMV control segregated into two peaks of DNA contents representing G_1 and G_2/M cells, respectively, with little or no cells exhibiting intermediate amounts of DNA. Serum-stimulated cells had similar profiles until 15 h poststimulation, when approximately 8% of the cells were in S phase, as indicated by an increase in cells with a greater than G_1 DNA content. By 24 h after stimulation, the majority of serum-stimulated cells were in S and G_2/M phases (16 and 54%, respectively), and numerous mitotic figures were observed at this time. At later times poststimulation, the majority of serum-stimulated cells returned to G_1 (62 to 71%), with fewer in S or G_2/M phase (24 and 16% in S phase at 48 and 72 h after stimulation, respectively, versus 12% in G_2/M phase at both of these time points). Infection of serum-starved cells with AdE2F1 resulted in no detectable increase in the DNA content through 24 hpi. By 48 hpi, however, approxi-

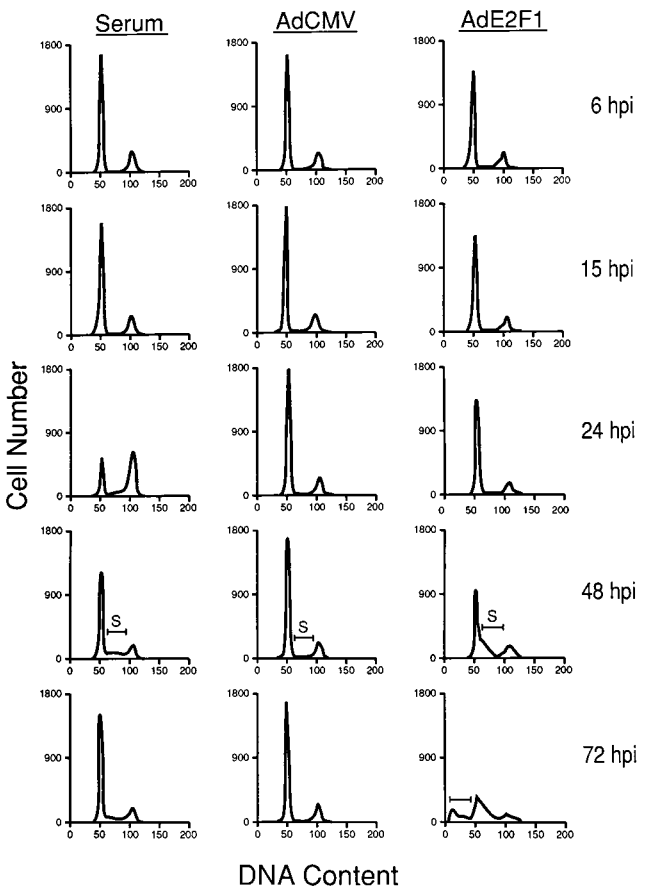

FIG. 2. Induction of DNA synthesis by AdE2F1 infection. Serum-starved REF-52 cells were infected with AdE2F1 or AdCMV vector or stimulated with serum, harvested at the indicated times, and examined for DNA content by propidium iodide staining and flow cytometry. Each plot represents the analysis of 10,000 gated events. Bars labeled S mark cells in S phase, and the bar in the panel for 72-hpi AdE2F1 infection marks a population of cells with a DNA content less than that of the G_1 population, which is apparent as the adjacent peak.

mately 45% of the AdE2F1-infected cells had DNA contents in excess of G_1 but less than the G_2/M peak. The distribution of these cells having increased DNA levels was grouped near the cell population containing a G_1 DNA content, a difference from serum-stimulated cells, the S-phase population of which is spread relatively evenly between the G_1 and G_2/M peaks. By 72 hpi, the proportion of cells having a greater than G_1 DNA content had further increased, causing the G1 peak to broaden and skew toward the G_2/M peak.

Two additional observations could be made from these analyses. First, there was little or no evidence for an increase in cells possessing a G_2 DNA content. Thus, despite the fact that there was induction of cellular DNA synthesis, there was little evidence of completed replication events. Second, it was apparent that by 72 hpi, the distribution of AdE2F1-infected cells had changed to define a new population with DNA contents less than that of G_1 cells (bar in panel for 72-hpi AdE2F1 infection in Fig. 2). This population represented approximately 20% of the gated events observed with the flow cytometer. Populations with less than G_1 DNA contents have been observed in other systems in conjunction with apoptosis (3, 10, 43).

Induction of cell death and DNA fragmentation by E2F1 overexpression. To extend the results obtained by flow cytometric analysis of AdE2F1-infected cells, we examined these cells for morphological changes associated with apoptosis. As shown in Fig. 3A, mock-infected cells had a flat shape typical of serum-starved REF-52 cells. In contrast, 24 to 48 h following infection by AdE2F1, many of the cells appeared refractile, exhibiting a thin and elongated appearance. By 72 hpi, most of the cells had detached from the culture plate and many of the remaining cells had rounded up, with some displaying cytoplasmic blebs indicative of apoptosis. No mitotic cells were observed in either the mock-infected or the AdE2F1-infected serum-starved cultures, although atypical mitotic figures would be difficult to discern from the rounding phenotype observed in the AdE2F1-infected cells at later times postinfection. For comparison, cycling cells were also examined for morphological changes after infection (Fig. 3B). Mock-infected cells displayed a normal fibroblast morphology with many mitotic figures. In contrast to AdE2F1 infections of serum-starved cells, more dramatic changes had occurred by 24 hpi in cycling REF-52 cells infected with AdE2F1. Morphological changes in infected cycling cells included a significant number of detached cells by 24 hpi, with the remaining adherent cells displaying elongated or rounded phenotypes. These changes continued through 48 hpi, and by 72 hpi the majority (>80%) of the cells had detached from the plate, with the few remaining cells having primarily a very thin, elongated appearance.

The morphological changes in AdE2F1 infections suggested the occurrence of cell death. Indeed, as shown in Fig. 4A and B, AdE2F1-infected cells exhibited a significant loss in viability, whereas both starved and cycling mock-infected cells remained viable during the course of this assay. Interestingly, the kinetics of viability loss were nearly identical in the infections of starved and cycling cells, while morphological changes in the infected cycling cells were more immediate and severe. This discrepancy suggests that many of the rounded and floating cells observed in infected cycling cells were still viable at the earlier times postinfection.

Genomic DNA was examined for the characteristic DNA banding pattern associated with apoptosis. Floating cells from a duplicate set of plates used for Fig. 4A were processed for enrichment of small DNAs by a modified Hirt procedure (7). In a time course of AdE2F1 infection of starved cells, only the 72-hpi sample exhibited a significant degree of DNA fragmentation (Fig. 4C). Infection of cells under both stressed (serum-starved) and normal growing conditions resulted in the ladder pattern characteristic of apoptosis (Fig. 4D). The higher background observed in the infected cycling cells was likely due to a greater number of viable floating cells which had not yet undergone significant DNA degradation. Neither of the uninfected controls (serum-starved or cycling cells) showed any significant degree of DNA degradation. Induction of apoptosis in cells that were not stressed was somewhat unexpected, since previous experiments have shown that induction of apoptosis by Myc and E1A requires some form of growth restriction, such as the removal of growth factors. Nevertheless, we find that infection of cycling cells with an adenovirus expressing the $E1A_{12S}$ product does result in apoptosis and is likely due to the high levels of E1A expression generated from the multiplicities used in these infections (data not shown). Furthermore, E2F1-associated apoptosis in the presence of growth factors has recently been observed by others (35, 38, 42).

Enhanced induction of apoptosis in γ-irradiated, arrested cells by E2F1 overexpression. Our recent work has demonstrated that E2F1 overexpression can bypass a G_1 arrest induced by γ irradiation (9a). To determine if this bypass also results in apoptosis, genomic DNAs from floating cells were examined by agarose gel electrophoresis (Fig. 5A). Significant

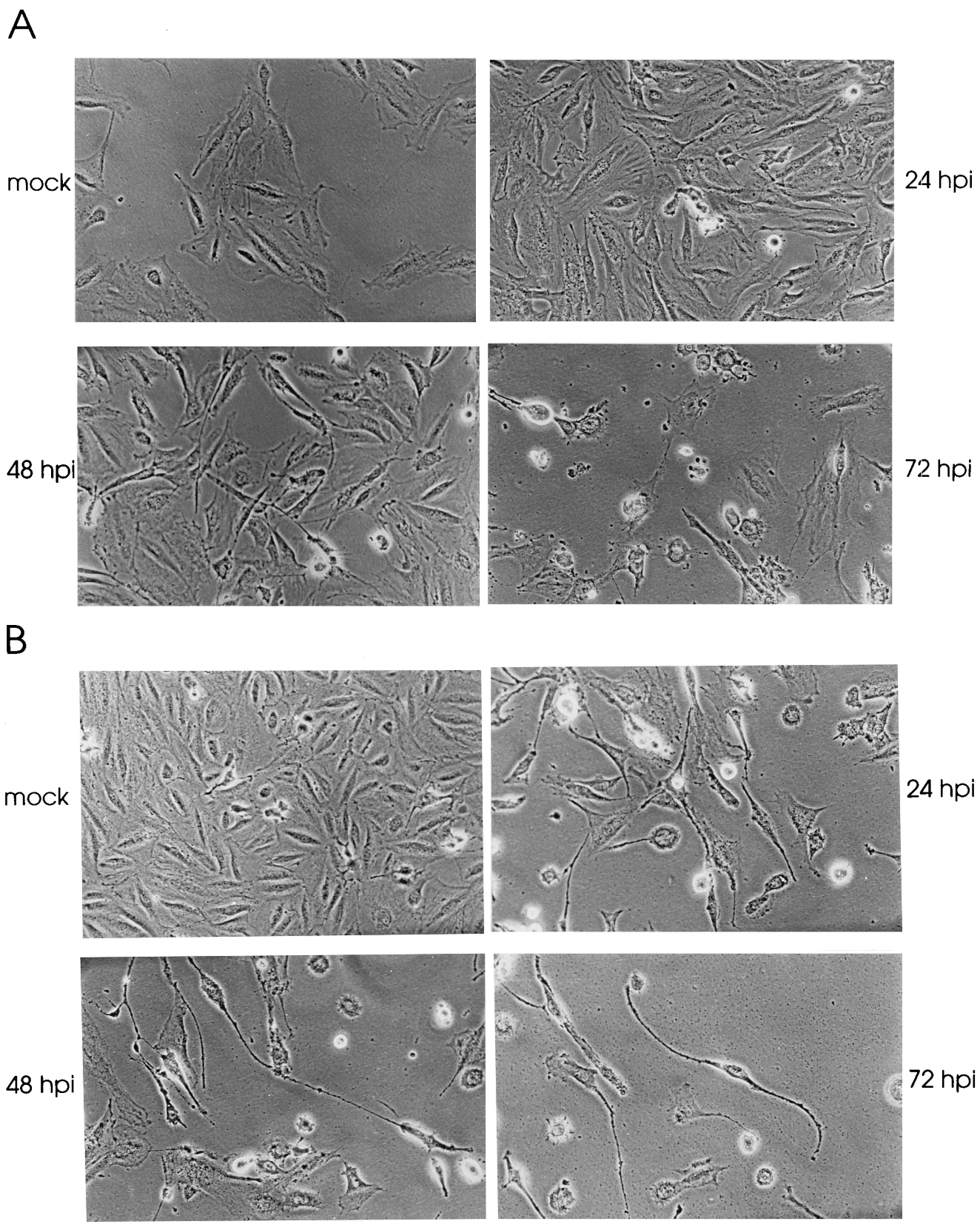

FIG. 3. Morphological changes in AdE2F1-infected REF-52 cells. (A) Serum-starved cells were infected with AdE2F1 and examined for morphological changes at the indicated times. (B) Same as panel A except that cycling cells were infected with AdE2F1.

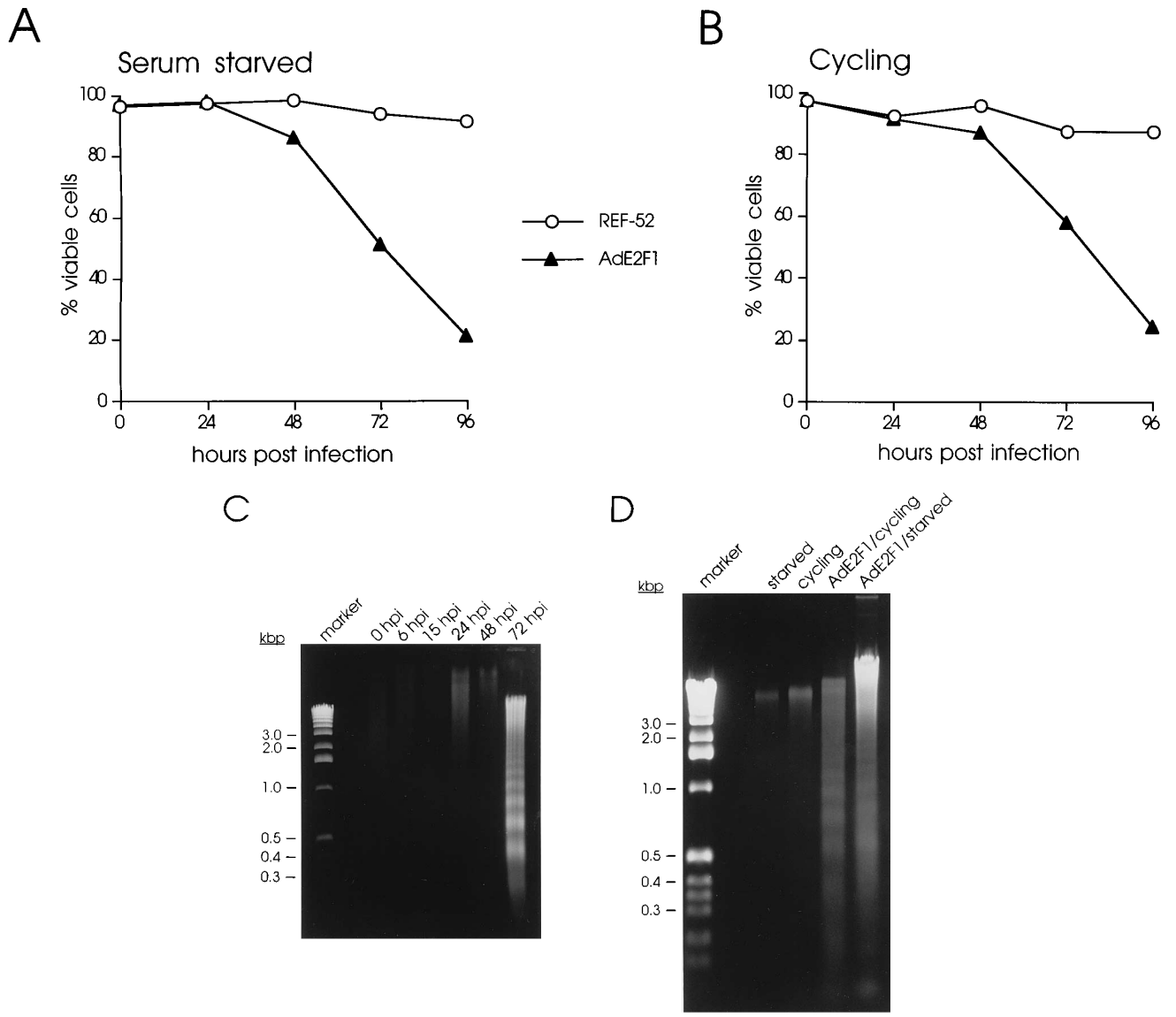

FIG. 4. Cell viability and DNA fragmentation upon AdE2F1 infection. (A) Serum-starved REF-52 cells were infected with AdE2F1 and examined for viability by trypan blue exclusion at the indicated times. (B) Cycling cells were examined for viability after AdE2F1 infection. (C) Floating cells from AdE2F1 infections of serum-starved cells were harvested at the indicated times, and their DNA was analyzed for fragmentation by electrophoresis through 1.25% agarose gels. (D) DNA fragmentation analysis comparing serum-starved and cycling cells in the presence or absence of AdE2F1 infection. Floating cells from each sample were harvested at 72 hpi.

fragmentation was observed in both control and γ-irradiated cells infected with AdE2F1, whereas infections with AdCMV under these conditions did not result in detectable DNA fragmentation. The amount of degraded DNA was significantly higher in the cells infected with AdE2F1 and irradiated versus infection alone. We then used flow cytometry to determine if the differences observed in Fig. 5A would correlate with differences in the cell populations containing less than normal DNA contents. Figure 5B summarizes these results by plotting the percentage of cells with a less than G_1 DNA content (defined as apoptotic index). In fact, the differences observed by electrophoresis correlate well with the differences in the apoptotic peaks between AdE2F1 infections of unirradiated and γ-irradiated cells.

Effect of p53 activity on E2F1-induced cell death and S-phase abnormalities. A number of recent experiments dem-

TABLE 2. Loss of γ-irradiation-induced G_1 arrest in REF-52 cell lines expressing mutant p53 proteins

Cell line	Treatment[a]	cpm[b] (10% total sample)	Fold repression
neo.3	Control	127,870	
	γ-irradiation	7,102	18
217.4	Control	241,406	
	γ-irradiation	189,040	1.2
283.3	Control	238,732	
	γ-irradiation	136,296	1.75

[a] See Materials and Methods for processing of control and γ-irradiated (12.5 Gy) samples.

[b] Acid-precipitable [^{3}H]thymidine incorporation after labeling from 24 to 32 h postirradiation.

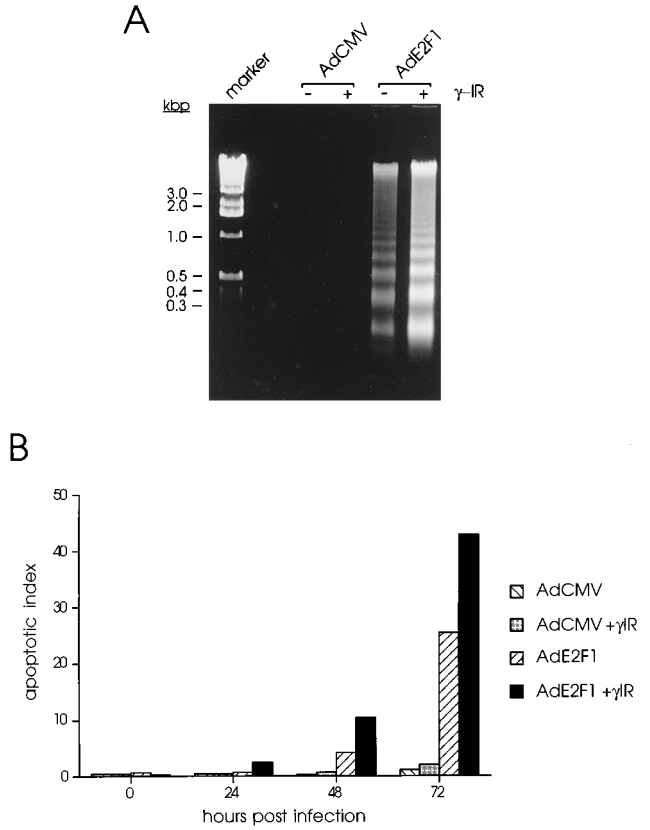

FIG. 5. Apoptosis upon AdE2F1 infection of γ-irradiated cells. (A) Cells were γ-irradiated (γ-IR; 12.5 Gy) and infected with either AdCMV or AdE2F1. Floating cells were harvested at 72 hpi and examined for small genomic DNAs as described for Fig. 4C. (B) Floating and adherent cells from plates treated as for panel A were pooled, stained with propidium iodide, and subjected to flow cytometry to quantitate the percentage of cells displaying an apoptotic phenotype (i.e., having a less than G_1 DNA content). Values are plotted as an apoptotic index for each variable.

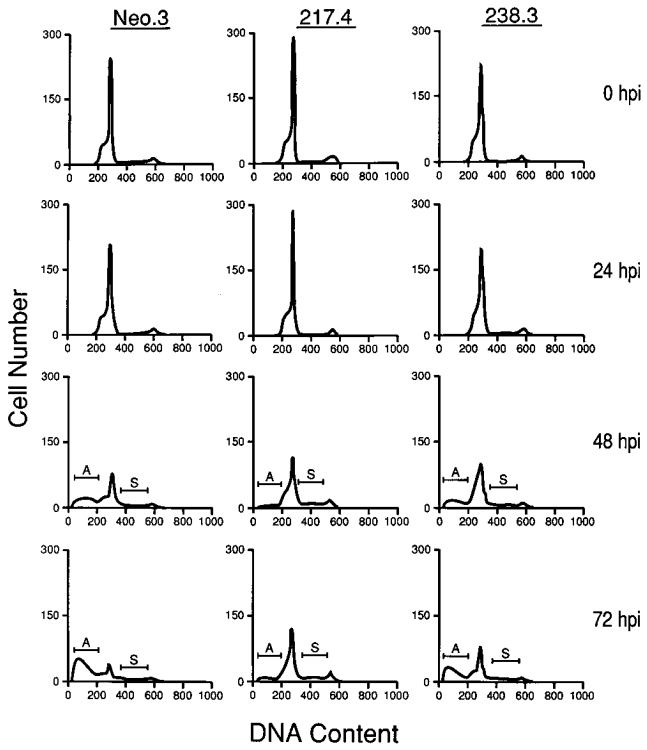

FIG. 6. Flow cytometric analysis of p53 mutant REF-52 cell lines upon AdE2F1 infection. Serum-starved cell lines containing the control vector or plasmids expressing mutant p53 forms were infected with AdE2F1 and examined for DNA content by propidium iodide staining and flow cytometry. Bars labeled A mark positions of apoptotic cells, and bars labeled S show locations of cells in S phase.

onstrate a role for p53 in activating apoptosis in response to a variety of conditions, including the expression of E2F1 (7, 26, 28, 42). Furthermore, we show here that γ-irradiation, a treatment that causes a strong induction of p53, enhances E2F1-dependent apoptosis. To examine if wt p53 activity in REF-52 cells contributes to both apoptosis and the aberrant S phase associated with E2F1 overexpression, we created REF-52 cell lines constitutively expressing dominant mutant forms of p53. We chose to use two naturally occurring mutant forms of p53 identified from primary breast tumors whose mutations occur in the DNA binding domain of p53 (codons 217 and 238). Mutation 217 is a deletion of this codon, and mutation 238 contains a one-base change resulting in a cysteine being replaced with an arginine (29a). Table 2 summarizes the functional inactivation of wt p53 in mutant cell lines by their ability to bypass a p53-dependent G_1 arrest induced by γ-irradiation. The neo.3 control cell line was blocked from entering S phase, as measured by [^{3}H]thymidine incorporation after 12.5 Gy of γ irradiation, resulting in an 18-fold decrease in thymidine incorporation. In contrast, the 217.4 and 238.3 cell lines were still able to incorporate [^{3}H]thymidine under these conditions, although with slightly different efficiencies. Therefore, stable transfection of REF-52 cells with plasmids expressing either of these mutant p53 products resulted in dominant suppression of wt p53 activity.

To better define the extent of apoptosis and S-phase induction in these cell lines, we subjected serum-starved, AdE2F1-infected cells to flow cytometry (Fig. 6). By 48 hpi, all of the cell lines exhibited an increase in the population of cells in S phase (12% for neo.3, 22% for 217.3, and 14% for 238.3). The percentage of cells in S phase had significantly decreased in the neo.3 cells by 72 hpi, whereas only minimal differences were apparent in the p53 mutant lines. These differences seemed to coincide with the appearance and size of populations representing cells undergoing apoptosis in each cell type at 48 and 72 hpi. Five times as many neo.3 cells had undergone apopto-

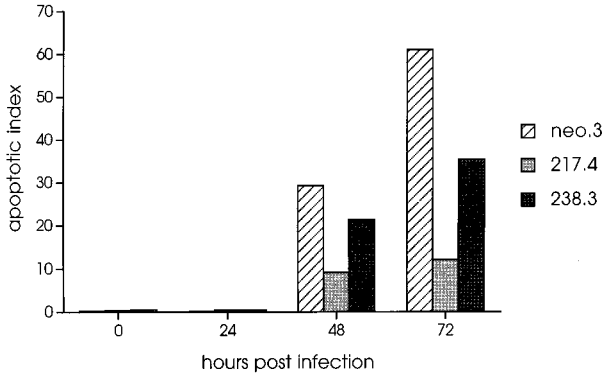

FIG. 7. Apoptotic index of AdE2F1-infected, p53 mutant cell lines. Percentages of cells displaying a DNA content less than that of G_1 cells from Fig. 6 are plotted as an apoptotic index.

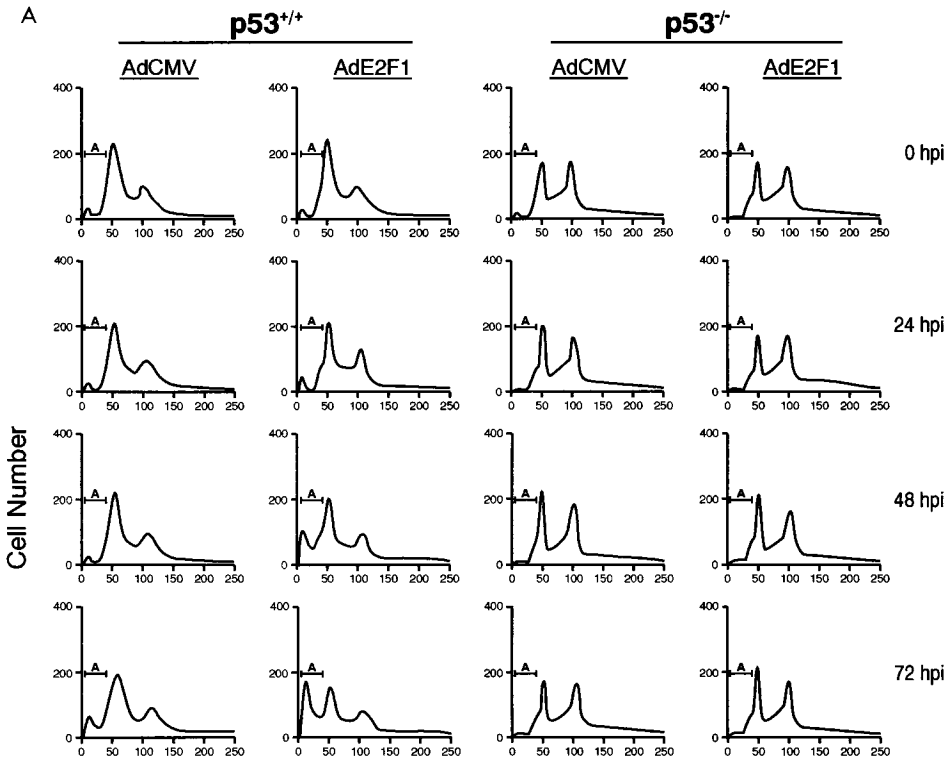

FIG. 8. Flow cytometric analysis of $p53^{+/+}$ and $p53^{-/-}$ mouse embryo fibroblasts upon AdCMV or AdE2F1 infection. (A) Proliferating $p53^{+/+}$ and $p53^{-/-}$ mouse embryo fibroblasts were infected with either AdCMV or ADE2F1, harvested at the indicated times postinfection, and examined for DNA content by propidium iodide staining and flow cytometry. (B) Percentages of cells displaying a DNA content less than that of G_1 cells in the analysis of panel A are plotted as an apoptotic index.

sis, as indicated by the appearance of the $<G_1$ peak, compared with the 217.4 cell line (61% versus 12%); the 238.3 cell line had an intermediate apoptotic phenotype (Fig. 7). Parallel infections with AdCMV resulted in no change in S phase or induction of apoptosis (data not shown).

The results described above and by others (35, 42) suggest that apoptosis induced by E2F1 is dependent on wt p53 function. However, the possibility exists that we introduced a clonal bias by choosing cell lines which did not respond to γ-irradiation and G_1 arrest. Likewise, bias could have been introduced in previous studies by clonally selecting for viability of p53 mutant cells in the presence of E2F1 overexpression (42). To further examine the dependency for p53 in E2F1-mediated apoptosis, we infected fibroblasts derived from control ($p53^{+/+}$) and $p53^{-/-}$ mouse embryos. As shown in Fig. 8, infections of $p53^{+/+}$ mouse fibroblasts with AdE2F1 resulted in the appearance of a population of cells with a less than G_1 DNA content by 48 hpi. This is in contrast to AdE2F1 infection of $p53^{-/-}$ fibroblasts, in which case no clearly distinguishable population with less than G_1 DNA content was apparent through 72 hpi. We therefore conclude that p53 activity does indeed contribute to E2F1-mediated apoptosis.

DISCUSSION

Previous experiments have shown that E2F1 overexpression can induce DNA synthesis in otherwise quiescent cells (22). Nevertheless, because of their design, these experiments did not provide detail as to the nature of the replication events. The ability to analyze this induction in biochemical detail, through the use of an adenovirus expression system that can deliver the E2F1 gene to the entire population of quiescent cells, has now permitted this more detailed analysis. These experiments again demonstrate an ability of E2F1 to induce cellular DNA synthesis and now reveal that the extent of DNA synthesis under these conditions is not complete, with cells apparently never reaching a G_2 DNA content. In large part, this appears to be due to the concomitant activation of an apoptosis pathway that leads to the degradation of cellular DNA before replication can be completed. Surprisingly, even in experiments in which apoptosis was minimized (Fig. 6 and 8), there still does not appear to be an increase in the G_2 population, suggesting that cells overexpressing E2F1 do not complete the DNA replication process. The significance of this observation awaits further study, but among the possibilities is the potential for some degree of E2F1-mediated apoptosis that is independent of p53.

Recent work by several groups has shown that expression of the E1A and E7 viral oncoproteins in cultured cells, as well as in transgenic mice, leads to apoptotic cell death. The cytocidal effects of E1A are well known and have been shown to be due in large part to induction of apoptosis mediated by p53 (7, 28, 41). Recently E7 has been shown to be cytocidal in culture (40), and it appears to enhance a normal process of regulated apoptosis in the lens of the developing eye (18, 33). Mutational analyses have shown a correlation between the stimulation of cell growth by these oncoproteins and their ability to induce apoptosis. This activity is dependent on the capacity of the viral proteins to disrupt the interaction between Rb family members and E2F (20, 36), consistent with the experiments presented here and elsewhere that suggest that E2F1-mediated apoptosis coincides with the ability of E2F1 to stimulate growth (35, 38, 42).

When viewed from the perspective of the viruses that encode activities that can activate E2F, an induction of cellular DNA synthetic capacity in an otherwise quiescent cell is a rational event. These viruses encode many of the activities necessary to replicate viral DNA but cannot do so in a quiescent cell that lacks the components that are critical for DNA replication such as DNA polymerase α and deoxyribonucleotides. Apparently, the DNA tumor viruses have evolved a capacity to induce the necessary cellular functions that create the S-phase environment. In this respect, the action of the viral proteins would not be to induce a normal mitotic event but rather merely to create the environment for viral DNA replication to take place efficiently. Thus, the induction of cellular DNA synthesis might be viewed as a consequence of these events, mediated by the immediate-early viral gene products such as E1A, rather than as the primary target. The experiments presented here, as well as previous experiments, demonstrate that the action of viral proteins such as E1A to create this environment involves the activation of the E2F transcription factor, a view entirely consistent with the role of E2F in activating transcription of the S-phase genes.

Given these considerations, it is not unreasonable to suggest that the creation of this S-phase environment resulting in the induction of cellular DNA synthesis is not a normal process. That is, one might view it as only a part of the normal proliferation signal, sufficient to allow DNA replication but not involving other aspects leading to a mitotic event. Results of a large number of experiments now suggest that one consequence of such an abnormal proliferative signal is the activation of apoptosis and that this effect is dependent on the action of the p53 gene product.

The role of p53 in activating apoptosis upon E2F1 overexpression has been demonstrated by inducing a wt p53 phenotype in cells overexpressing a temperature-sensitive p53 mutant (42). We show that the endogenous p53 is sufficient to activate apoptosis upon E2F1 overexpression and that the overexpression of dominant p53 mutants relieves most of the observed apoptosis, resulting in a greater number of cells synthesizing DNA. However, under our conditions there is still a background of apoptosis taking place. This observation suggests that other, p53-independent events can activate apoptosis after E2F1 overexpression. Alternately, the mutants used may not be fully dominant by nature of their mutation, or there may be a background of wt p53 activity still present. We addressed these issues by repeating the assays in a genetically clean background, using fibroblasts from $p53^{-/-}$ and wt mouse embryos. These results again support the conclusion that wt p53 contributes to E2F1-mediated apoptosis. However, there may not be a total dependency on normal p53 function for this response, as our preliminary results suggest that some E2F1-mediated apoptosis can still occur in the $p53^{-/-}$ background (unpublished observations). Clearly, further work will be required to determine the nature of the events involved in activating an apoptotic program in the absence of p53.

Although the role of p53 in responding to these abnormal proliferative signals such as E2F1 overexpression is now clear, it is not apparent how p53 recognizes these events. In short, what is the link between E2F1-mediated S-phase induction and p53-mediated apoptosis? Clearly, the DNA tumor viruses solve this problem by encoding proteins that inactivate p53 function. It is tempting to speculate that a similar process may operate during a normal proliferative response. In this case, p53 would recognize the E2F-mediated activation process but would be counteracted by a cellular activity similar in nature to the viral proteins that inactivate p53 function. In this view, normal proliferation would involve both the activation of E2F as well as a p53 antagonist. Abnormal proliferation induced by E2F1 alone would lack this p53 antagonist function. Determination of whether this simple scheme, modeled after the DNA tumor virus strategies, is accurate must await further experiments, although it does provide a framework for such analysis.

ACKNOWLEDGMENTS

We thank Mike Cook and Alan Fisher of the Duke Comprehensive Cancer Center Flow Cytometry Facility for the flow cytometric analyses. We also thank Laszlo Jakoi for assistance with the gel retardation assay and David Johnson and Douglas Cress for technical advice during the course of this study. We thank Kaye Culler for assistance in preparing the manuscript.

T.F.K. is a Leukemia Society of America Special Fellow, J.K.S. was a Pfizer postdoctoral fellow, and J.D. is supported by an Individual National Research Service Award CA59259 from the NIH.

REFERENCES

1. **Bookstein, R., J.-Y. Shew, P.-L. Chen, P. Scully, and W.-H. Lee.** 1990. Suppression of tumorigenicity of human prostate carcinoma cells by replacing a mutated RB gene. Science **247:**712–715.
2. **Buchkovich, K., L. A. Duffy, and E. Harlow.** 1989. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell **58:**1097–1105.
3. **Carson, W. E., S. Haldar, R. A. Baiocchi, C. M. Croce, and M. A. Caligiuri.** 1994. The c-kit ligand suppresses apoptosis of human natural killer cells through the upregulation of bcl-2. Proc. Natl. Acad. Sci. USA **91:**7553–7557.
4. **Chen, P.-L., P. Scully, J.-Y. Shew, J. Y. J. Wang, and W.-H. Lee.** 1989. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell **58:**1192–1198.
5. **Cobrinik, D., P. Whyte, D. S. Peeper, T. Jacks, and R. A. Weinberg.** 1993. Cell cycle specific association of E2F with the p130 E1A-binding protein. Genes Dev. **7:**2392–2404.
6. **Cress, W., D., D. G. Johnson, and J. R. Nevins.** 1993. A genetic analysis of the E2F1 gene distinguishes regulation by Rb, p107, and adenovirus E4. Mol. Cell. Biol. **13:**6314–6325.
7. **Debbas, M., and E. White.** 1993. Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev. **7:**546–554.
8. **DeCaprio, J. A., Y. Furukawa, F. Ajchenbaum, J. D. Griffin, and D. M. Livingston.** 1992. The retinoblastoma-susceptibility gene product becomes phosphorylated in multiple stages during cell cycle entry and progression. Proc. Natl. Acad. Sci. USA **89:**1795–1798.
9. **DeCaprio, J. A., J. W. Ludlow, D. Lynch, Y. Furukawa, J. Griffin, H. Piwnica Worms, C. M. Huang, and D. M. Livingston.** 1989. The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. Cell **58:**1085–1095.

9a. **DeGregori, J., T. F. Kowalik, and J. R. Nevins.** Submitted for publication.

10. **Gottschalk, A. R., L. H. Boise, G. B. Thompson, and J. Quintans.** 1994. Identification of immunosuppresant-induced apoptosis in a murine B-cell line and its prevention by bcl-x but not bcl-2. Proc. Natl. Acad. Sci. USA **91:**7350–7354.
11. **Hamel, P. A., R. M. Gill, R. A. Phillips, and B. L. Gallie.** 1992. Transcriptional repression of the E2-containing promoters EIIaE, c-*myc*, and RB1 by the product of the RB1 gene. Mol. Cell. Biol. **12:**3431–3438.
12. **Hannon, G. J., D. Demetrick, and D. Beach.** 1993. Isolation of the Rb-related p130 through its interaction with cdk2 and cyclins. Genes Dev. **7:**2378–2391.
13. **Hartwell, L. H., and T. A. Weinert.** 1989. Checkpoints: controls that ensure the order of cell cycle events. Science **246:**629–633.

14. **Helin, K., E. Harlow, and A. Fattaey.** 1993. Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. Mol. Cell. Biol. **13:** 6501–6508.
15. **Helin, K., J. A. Lees, M. Vidal, N. Dyson, E. Harlow, and A. Fattaey.** 1992. A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F. Cell **70:**337–350.
16. **Hiebert, S. W.** 1993. Regions of the retinoblastoma gene product required for its interaction with the E2F transcription factor are necessary for E2 promoter repression and pRb-mediated growth suppression. Mol. Cell. Biol. **13:**3384–3391.
17. **Hiebert, S. W., S. P. Chellappan, J. M. Horowitz, and J. R. Nevins.** 1992. The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. Genes Dev. **6:**177–185.
18. **Howes, K. A., N. Ransom, D. S. Papermaster, J. G. H. Lasudry, D. M. Albert, and J. J. Windle.** 1994. Apoptosis or retinoblastoma: alternative fates of photoreceptors expressing the HPV-16 E7 gene in the presence or absence of p53. Genes Dev. **8:**1300–1310.
19. **Huang, H.-J. S., N.-P. Wang, B. Y. Tseng, W.-H. Lee, and E. Y.-H. P. Lee.** 1988. Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. Science **242:**1563–1566.
20. **Ikeda, M.-A., and J. R. Nevins.** 1993. Identification of distinct roles for separate E1A domains in the disruption of E2F complexes. Mol. Cell. Biol. **13:**7029–7035.
21. **Johnson, D. G., W. D. Cress, L. Jakoi, and J. R. Nevins.** 1994. Oncogenic capacity of the E2F1 gene. Proc. Natl. Acad. Sci. USA **91:**12823–12827.
22. **Johnson, D. G., J. K. Schwarz, W. D. Cress, and J. R. Nevins.** 1993. Expression of transcription factor E2F1 induces quiescent cells to enter S phase. Nature (London) **365:**349–352.
23. **Kaelin, W. G., W. Krek, W. R. Sellers, J. A. DeCaprio, F. Ajchenbaum, C. S. Fuchs, T. Chittenden, Y. Li, P. J. Farnham, M. A. Blanar, et al.** 1992. Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. Cell **70:**351–364.
24. **Kovesdi, I., R. Reichel, and J. R. Nevins.** 1986. Identification of a cellular transcription factor involved in E1A trans-activation. Cell **45:**219–228.
25. **Li, Y., C. Graham, S. Lacy, A. M. V. Duncan, and P. Whyte.** 1993. The adenovirus E1A-associated 130-kd protein is encoded by a member of the retinoblastoma gene family and physically interacts with cyclins A and E. Genes Dev. **7:**2366–2377.
26. **Lowe, S. W., T. Jacks, D. E. Housman, and H. E. Ruley.** 1994. Abrogation of oncogene-associated apoptosis allows transformation of p53-deficient cells. Proc. Natl. Acad. Sci. USA **91:**2026–2030.
27. **Lowe, S. W., and H. E. Ruley.** 1993. Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. Genes Dev. **7:**535–545.
28. **Lowe, S. W., H. E. Ruley, T. Jacks, and D. E. Housman.** 1993. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell **74:**957–967.
29. **Ludlow, J. W., J. Shon, J. M. Pipas, D. M. Livingston, and J. A. DeCaprio.** 1990. The retinoblastoma susceptibility gene product undergoes cell cycle-dependent dephosphorylation and binding to and release from SV40 large T. Cell **60:**387–396.
29a. **Marks, J.** Unpublished data.
30. **Mayol, X., X. Grana, A. Baldi, N. Sang, Q. Hu, and A. Giordano.** 1993. Cloning of a new member of the retinoblastoma gene family (pRb2) which binds to the E1A transforming domain. Oncogene **8:**2561–2566.
31. **Mihara, K., X.-R. Cao, A. Yen, S. Chandler, B. Driscoll, A. L. Murphree, A. T'Ang, and Y.-K. T. Fung.** 1989. Cell cycle-dependent regulation of phosphorylation of the human retinoblastoma gene product. Science **246:**1300–1303.
32. **Nevins, J. R.** 1992. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. Science **258:**424–429.
33. **Pan, H., and A. E. Griep.** 1994. Altered cell cycle regulation in the lens of HPV-16 E6 or E7 transgenic mice: implications for tumor suppressor gene function in development. Genes Dev. **8:**1285–1299.
34. **Qian, Y., C. Luckey, L. Horton, M. Esser, and D. J. Templeton.** 1992. Biological function of the retinoblastoma protein requires distinct domains for hyperphosphorylation and transcription factor binding. Mol. Cell. Biol. **12:**5363–5372.
35. **Qin, X.-Q., D. M. Livingston, W. G. Kaelin, and P. D. Adams.** 1994. Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. Proc. Natl. Acad. Sci. USA **91:**10918–10922.
36. **Raychaudhuri, P., S. Bagchi, S. H. Devoto, V. B. Kraus, E. Moran, and J. R. Nevins.** 1991. Domains of the adenovirus E1A protein required for oncogenic activity are also required for dissociation of E2F transcription factor complexes. Genes Dev. **5:**1200–1211.
37. **Schwarz, J. K., C. H. Bassing, I. Kovesdi, M. B. Datto, M. Blazing, S. George, X. Wang, and J. R. Nevins.** 1995. Expression of the E2F1 transcription factor overcomes TGF-β mediated growth suppression. Proc. Natl. Acad. Sci. USA **92:**483–487.
38. **Shan, B., and W. Lee.** 1994. Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. Mol. Cell. Biol. **14:**8166–8173.
39. **Singh, P., S. W. Wong, and W. Hong.** 1994. Overexpression of E2F-1 in rat embryo fibroblasts leads to neoplastic transformation. EMBO J. **13:**3329–3338.
40. **White, A. E., E. M. Livanos, and T. D. Tlsty.** 1994. Differential disruption of genomic integrity and cell cycle regulation in normal human fibroblasts by the HPV oncoproteins. Genes Dev. **8:**666–677.
41. **White, E., R. Cipriani, P. Sabbatini, and A. Denton.** 1991. Adenovirus E1B 19-kilodalton protein overcomes the cytotoxicity of E1A proteins. J. Virol. **65:**2968–2978.
42. **Wu, X., and A. J. Levine.** 1994. p53 and E2F-1 cooperate to mediate apoptosis. Proc. Natl. Acad. Sci. USA **91:**3602–3606.
43. **Yamada, T., S. Yamaoka, T. Goto, M. Nakai, Y. Tsujimoto, and M. Hatanaka.** 1994. The human T-cell leukemia virus type I Tax protein induces apoptosis which is blocked by the Bcl-2 protein. J. Virol. **68:**3374–3379.
44. **Zamanian, M., and N. B. La Thangue.** 1993. Transcriptional repression by the Rb-related protein p107. Mol. Biol. Cell **4:**389–396.
45. **Zhu, L., S. van den Heuvel, K. Helin, A. Fattaey, M. Ewen, D. Livingston, N. Dyson, and E. Harlow.** 1993. Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. Genes Dev. **7:**1111–1125.