Induction of DNA Synthesis and Apoptosis in Cardiac Myocytes by E1A Oncoprotein

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Abstract. Beginning during the second half of gestation, increasing numbers of cardiac myocytes withdraw from the cell cycle such that DNA synthesis is no longer detectable in these cells by neonatal day 17 in vivo. The mechanisms that exclude these and other terminally differentiated cells from the cell division cycle are poorly understood. To begin to explore the molecular basis of the barrier to G1/S progression in cardiac myocytes, we used adenoviruses to express wild-type and mutant E1A proteins in primary cultures from embryonic day 20 rats. While most of these cardiac myocytes are ordinarily refractory to DNA synthesis, even in the presence of serum growth factors, expression of wild-type E1A stimulates DNA synthesis in up to 94% or almost all successfully transduced cells. Rather than complete the cell cycle, however, these cells undergo apoptosis. Apoptosis is limited to those cells that engage in DNA synthesis, and the kinetics of the two processes suggest that DNA synthesis precedes apoptopsis. Mutations in E1A that disable it from binding Rb and related pocket proteins have little effect on its ability to stimulate DNA synthesis in cardiac myocytes. In contrast, mutants that are defective in binding the cellular protein p300 stimulate DNA synthesis 2.4–4.1-fold less efficiently, even in the context of retained E1A pocket protein binding. In the absence of E1A pocket protein binding, the usual situation in the cell, loss of p300 binding severely decreases the ability of E1A to stimulate DNA synthesis. These results suggest that the barrier to G1/S progression in cardiac myocytes is mediated, at least in part, by the same molecules that gate the G1/S transition in actively cycling cells, and that p300 or related family members play an important role in this process.

Most adult mammalian cells exist in a quiescent state (G0), characterized by the absence of net DNA synthesis and cell division. While some G0 cells retain the ability to resume proliferative growth in response to certain signals, others that have undergone terminal differentiation are permanently excluded from the cell cycle. The mechanisms that prevent cell cycle reentry in terminally differentiated cells are unclear. Striated muscle cells, including skeletal and cardiac myocytes, exemplify the terminally differentiated state. In skeletal muscle, terminal differentiation involves three distinct processes: commitment, in which proliferating myoblasts withdraw irreversibly from the cell cycle; fusion of commited myoblasts into multinucleated myocytes; and induction of muscle specific genes (Nguyen et al., 1983). MyoD, myogenin, myf-5, and MRF 4 comprise a family of skeletal myogenic determination factors (MDFs),[1] whose members are sufficient to accomplish all three of these steps, as evidenced by their ability to convert a variety of nonmuscle cells to skeletal myocytes (reviewed in Weintraub, 1993). MDFs are basic helix-loop-helix transcription factors that transactivate the expression of multiple muscle-specific genes. In addition, these proteins exhibit intrinsic growth suppressive properties in both myogenic and nonmyogenic cells (Sorrentino et al., 1990; Crescenzi et al., 1990). MDF-induced growth inhibition requires member(s) of the retinoblastoma (Rb) family of pocket proteins (Gu et al., 1993), which also include p107 and p130. Although MDFs bind directly to pocket proteins in vivo (Gu et al., 1993; Schneider et al., 1994), the precise mechanism by which they suppress growth remains unclear, and recent work suggests that these effects may be indirect (Halevy et al., 1995; Skapek et al., 1995; Parker et al., 1995).

While both skeletal and cardiac myocytes are specialized contractile cells, they differ fundamentally from a developmental perspective. Skeletal myoblasts arise from the somites around midgestation (Holtzer et al., 1957); in contrast, cardiac myoblasts originate earlier and from the lateral plate mesoderm (Rawles, 1943; reviewed in Litvin et al., 1992). Furthermore, in contrast to skeletal myogenesis, where cell cycle withdrawal precedes fusion and the expression of muscle-specific genes (Nadal-Ginard, 1978), fully functioning cardiac myocytes develop from their pre-

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1. *Abbreviations used in this paper*: BrdU, 5-bromo-2′-deoxy-uridine; CR, conserved region; MDF, myogenic determination factor; MLC2v, ventricular myosin light chain 2; TUNEL, terminal deoxynucleotidyl transferase nick end labeling.

cursors in the absence of cell cycle withdrawal or cell fusion (Goldstein et al., 1974). As ontogeny progresses, however, an increasing proportion of cardiac myocytes withdraw from the cell cycle so that, by neonatal days 15–17, DNA synthesis is not detectable in cardiac myocytes in vivo (Claycomb, 1975; Rumyantsev, 1977; Kim et al., 1994). Forced expression of c-*myc* (Jackson et al., 1990) and SV-40 T antigen (Field, 1988; Behringer et al., 1988; Katz et al., 1992) in transgenic hearts beginning early in fetal life can induce cardiac myocyte hyperplasia and/or tumorigenesis; however, these genetic perturbations by themselves are insufficient to allow the direct establishment of permanent lines of differentiated cardiac myocytes (Jackson et al., 1990; Steinhelper et al., 1990). The molecules, which induce and maintain terminally differentiation in cardiac myocytes, have not been identified; specifically, the skeletal MDFs are absent (reviewed in Olson, 1993).

To begin to understand the molecular basis of terminal differentiation in cardiac myocytes, we have exploited two properties of adenoviruses. First, these viruses are able to infect primary cultures of cardiac myocytes with high efficiency (Kirshenbaum et al., 1993; Kass-Eisler et al., 1993). Second, adenoviruses express an endogenous oncogene, E1A, which facilitates G1 to S progression in nonterminally differentiated cells (Howe et al., 1990) and whose structure–function relationships have been particularly well delineated (reviewed in Bayley and Mymryk, 1994). We reasoned that if E1A were able to surmount the G1/S block in cardiac myocytes, it might be possible to explore the molecular nature of this barrier by assaying the ability of various E1A mutants to stimulate DNA synthesis in cardiac myocytes. Although the products of the *E1A* gene stimulate DNA synthesis in nonterminally differentiated cells, their ability to transform these cells is quite limited because they also induce apoptosis or programmed cell death through a p53-dependent mechanism (Debbas and White, 1993; Lowe and Ruley, 1993). E1A-induced apoptosis can be inhibited by the products of the adenovirus *E1B* gene and the cellular *bcl-2* gene (Rao et al., 1992; reviewed in White, 1993). Both the ability of E1A to induce cellular DNA synthesis and apoptosis map to residues at the extreme amino terminus and in conserved regions (CR) 1 (amino acids 40–80) and 2 (amino acids 120–138). These E1A functions are thought to be mediated through its interactions with several host proteins. These include p300, a transcriptional adaptor molecule (reviewed in Moran, 1993; Eckner, et al., 1994), which requires residues at the extreme amino terminus of E1A and the carboxy end of CR1 for full binding, as well as pocket proteins whose binding maps to residues in CR2 and the amino portion of CR1 (Wang et al., 1993). E1A mutants defective for p300 binding alone are modestly impaired in their abilities to stimulate cellular DNA synthesis in cycling cells while those unable to bind pocket proteins alone perform this function normally. Mutants defective for binding both of these classes of proteins, however, are completely crippled in this regard (Howe et al., 1990). Expression of E1A in skeletal myoblasts inhibits the activation of muscle-specific genes and differentiation of these cells (Webster et al., 1988; Enkemann et al., 1990; Braun et al., 1992; Mymryk et al., 1992; Caruso et al., 1993; Taylor et al., 1993). The effect of E1A expression in striated muscle cells that have already undergone terminal differentiation is not known, however.

In this study, we demonstrate that expression of E1A is sufficient to stimulate DNA synthesis in embryonic day 20 cardiac myocytes, most of which are ordinarily refractory to this process, even in serum-containing media. Rather than undergoing mitosis, however, these same cells undergo apoptosis. Finally, mutations disabling E1A from binding p300—both in the presence and absence of pocket protein binding—decrease the ability of this oncoprotein to stimulate DNA synthesis in these cells. These results suggest that p300 and/or related family members contribute to the regulation of DNA synthesis in cardiac myocytes.

Materials and Methods

Primary Rat Cardiac Myocyte Cultures

Cultures of cardiac myocytes were prepared from the hearts of embryonic day 20 Sprague-Dawley fetuses (Taconic Farms, Germantown, NY) as described (Kass-Eisler et al., 1993). After enzymatic dissociation, cells were pre-plated for 1 h to enrich for myocytes (90–95% of cells after this step). Cells were then plated at a density of 700/mm^2 onto 35- or 60-mm tissue culture dishes (Primaria, Falcon; Becton Dickinson & Co., Lincoln Park, NJ) and cultured in media consisting of Hanks' salts plus MEM vitamin stock, MEM amino acids, MEM nonessential amino acids, 2 mM L-glutamine, 0.67 mM glycine, 0.92 mM hypoxanthine (all from GIBCO BRL, Gaithersburg, MD), 19.6 mM $NaHCO_3$ (pH 7.1–7.2), penicillin, streptomycin, and 10% (vol/vol) FBS (Hyclone Laboratories, Logan, UT) at 37°C, 5% CO_2. 12 h after plating, cells were washed twice with media before the addition of fresh media.

Adenoviruses

Mutant human type 5 adenoviruses, derived from *dl*309 (Jones and Schenk, 1979), which lacks a functional E3 gene, but is phenotypically normal in cultured cells, were used throughout these experiments. *dl*309/E1B$^-$ (kindly provided by Dr. Eileen White, Center for Advanced Biotechnology and Medicine, Piscataway, NJ) contains the wild-type E1A gene, which is transcribed into alternatively spliced 12S and 13S mRNAs encoding 243- and 289-residue proteins, respectively, but lacks a functional E1B gene. *dl*520/E1B$^-$ and its derivatives (generous gifts from Dr. Stanley T. Bayley, McMaster University, Hamilton, Ontario, Canada) contain an E1A gene in which the 5′ splice site needed to generate the 13S transcript has been mutated, resulting in the production of only the 12S transcript and the 243-amino acid protein; these viruses also lack a functional E1B gene. Derivatives of *dl*520E1B$^-$ containing additional mutations in the E1A gene include *dl*1101/520E1B$^-$ (deletion of residues 14-25), *dl*1108/520E1B$^-$ (deletion of residues 124-127), and *dl*01/08/520E1B$^-$ (with both of these deletions) (Howe et al., 1990). *dl*312 lacks functional E1 and E3 genes (Jones and Shenk, 1979). Viruses were grown up on 293 cells, purified on CsCl gradients, and then titered by plaque assay on 293 cells (Jones and Shenk, 1979).

Infection

Cells were infected 36 h after plating at a multiplicity of infection (MOI) of 10 or 20 plaque-forming units (pfu) per cell as specified in a volume of 1 ml for 60-mm plates and 0.5 ml for 35-mm plates of serum-free media and incubated at 37°C, 5% CO_2. Plates were gently swirled every 15 min. After 1.5 h, serum-containing media was added to plates so that the final serum concentration was 10%. Infection efficiency of myocyte cultures was determined by double immunofluorescence for E1A (Ab-1 from Oncogene Science [Uniondale, NY]; used at 1:20 dilution) and ventricular myosin light chain 2 (MLC2v; Iwaki et al., 1990), which was generously provided by Dr. Ken Chien (University of California at San Diego) and used at 1:20 dilution or desmin (Sigma Chemical Co., St. Louis, MO; used at 1:10 dilution). Alternatively, staining was performed with a polyclonal adenoviral antibody (gift from Dr. Erik Falck-Pedersen, Cornell University Medical College, New York; used at 1:1,000 dilution). Steady-state E1A levels

were assessed by fractionating cellular lysates on 10% SDS-polyacrylamide gels, transferring to nitrocellulose membranes, and immunoblotting using the E1A antibody at a 1:200 dilution. Bands were detected by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL) and quantified by laser densitometry of autoradiograms exposed in the linear range (Molecular Dynamics, Sunnyvale, CA).

5-Bromo-2'-Deoxy-Uridine Incorporation

Nuclear 5-bromo-2'-deoxy-uridine (BrdU) incorporation and cytoplasmic muscle-specific antigens were simultaneously visualized using double indirect immunofluorescence. Cultures were pulsed with BrdU (Amersham) at a final concentration of 20 μM for the time period indicated. At the end of the pulse, cells on coverslips were fixed in 3.7% (vol/vol) formaldehyde for 10 min at room temperature, permeabilized with 70% ethanol for 30 min at −20°C, and incubated with 10% normal goat serum followed by a mouse monoclonal anti-BrdU antibody (undiluted; Amersham) and the rabbit polyclonal antibody against either MLC2v or desmin as noted above. Primary antibodies were then detected with rhodamine-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG (both used at 1:40; The Jackson Laboratory, Bar Harbor, ME), mounted with Flu-mount (Fisher Scientific, Pittsburgh, PA), and examined using an MRC 600 confocal microscope (Bio Rad, Hercules, CA) with a Kr/Ar laser.

Nucleosomal Ladder Assay

At the times indicated after infection, cells were harvested by scraping into the media. After centrifugation at 500 *g* for 5 min at 4°C, cells were lysed in 10 mM Tris, pH 8.0, 5 mM EDTA, 100 mM NaCl, 0.5% (vol/vol) SDS, and 1 μg/ml proteinase K (Sigma Chemical Co.) at 37°C for 5 h on an orbital rocker followed by the dropwise addition of 4 M NaCl to a final concentration of 1 M and incubation at 4°C overnight (Mymryk et al., 1994). After centrifugation at 12,000 *g* for 30 min at 4°C, the supernatants were recovered, extracted with an equal volume of 25:24:1 phenol (Tris-saturated, pH 8)/chloroform/isoamyl alcohol, and the combination of high molecular weight and fragmented DNAs were isopropanol precipitated, washed in 70% ethanol, resuspended in water, digested with 100 μg/ml RNase A for 30 min at 37°C, size fractionated on 1.2% agarose gels, and stained with ethidium bromide.

In situ Apoptosis Assay

48 h after infection, apoptotic cells were identified in situ using the terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL) assay (Gavrieli et al., 1992). After fixation in 10% neutral buffered formalin for 10 min at room temperature and permeabilization in 70% ethanol for 30 min at −20°C, the TUNEL assay was performed on cells on coverslips using the commercially available Fluorescein Apop Tag kit (Oncor, Gaithersburg, MD) according to the manufacturer's recommendations. In some experiments, indirect immunofluorescence was simultaneously used to identify striated myocytes by staining them with F 59 (Miller et al., 1989), a mouse monoclonal antisarcomeric myosin antibody (1:5 dilution; generous gift from Dr. Frank Stockdale, Stanford University, Palo Alto, CA), followed by rhodamine conjugated goat anti-mouse IgG (1:40 dilution; The Jackson Laboratory). In other experiments, cells were simultaneously scored for apoptosis and de novo DNA synthesis by pulsing with BrdU for the time period indicated, fixing the cells as for the TUNEL protocol, followed by sequentially performing TUNEL and BrdU staining.

Results

E1A Stimulates DNA Synthesis in Cardiac Myocytes

By embryonic day 20, most cardiac myocytes in the intact heart have withdrawn from the cell cycle (Rumyantsev, 1977; Kim et al., 1994). When placed into primary culture, only a low percentage of these cells exhibit evidence of ongoing DNA synthesis even in growth factor–supplemented or serum-containing media. To determine whether DNA synthesis could be stimulated in this population of cells, cultures were infected with *dl*309/E1B$^-$, a human type 5 adenovirus containing a wild-type E1A allele but lacking a functional E1B gene. We chose this particular virus to permit the effects of E1A to be assessed independently of the products of the E1B gene, which also interact with host proteins involved in cell cycle control. In preliminary studies, we confirmed by immunoblot that this virus does in fact produce E1A as early as 8 h after infection of cardiac myocytes (data not shown). In addition, the efficiency with which *dl*309/E1B$^-$ infects cardiac myocytes at MOI 10 was 70–80%, as determined by double immunofluorescence for E1A and MLV2v, a striated myocyte specific protein, 12 h after infection (data not shown). Control plates were infected with *dl*312, which lacks a functional E1A gene, or mock infected. Cells were pulsed with BrdU between 24 and 26 h after infection, after which they were fixed and analyzed by indirect immunofluorescence. Fig. 1 shows double staining for BrdU (*orange,* rhodamine) and MLC2v (*green,* FITC). Mock-infected myocytes (*a*) and those infected with *dl*312 (*b*) exhibited no evidence of BrdU incorporation by immunofluorescence. In contrast, 70–80% of the myocytes in plates infected with *dl*309/E1B$^-$ (*c*) showed marked nuclear staining for BrdU. Thus, E1A is sufficient to stimulate DNA synthesis in a large proportion of cardiac myocytes that do not ordinarily synthesize DNA.

E1A Induces Apoptosis in Cardiac Myocytes

To determine whether E1A-stimulated cardiac myocytes go on to mitosis, cells infected with *dl*309/E1B$^-$ or *dl*312 or mock infected, as above, were observed for longer periods of time. Beginning 36 h after infection, increasing numbers of cells were observed to round up and detach from the surface of the plates infected with *dl*309/E1B$^-$, but not those infected with *dl*312 or mock infected. By 72 h after infection, >70% of the cells on E1A infected plates were found floating in the media. Since E1A has been shown to induce apoptosis in cycling cells, we wished to determine whether cells in the *dl*309/E1B$^-$-infected plates were undergoing apoptotic death. Therefore, at various times after infection, DNA was assayed for nucleosomal laddering, a hallmark of apoptosis (Fig. 2). Only high molecular weight, unfragmented DNA was recovered from mock- and *dl*312-infected plates at all time points. In contrast, cells infected with *dl*309/E1B$^-$ exhibited nucleosomal ladders 48 and 72 h after infection. This experiment shows that cell death in E1A-infected plates occurred by apoptosis and that E1A by itself is sufficient to induce this process.

In rodent hearts, myocytes are outnumbered ~4:1 by nonmyocytes (predominantly fibroblasts). For this reason, various strategies to enrich for myocytes are used when preparing primary myocyte cultures from dissociated hearts. Although at the time of plating our cultures typically contain 90–95% myocytes, as judged by staining for sarcomeric myosin (data not shown), there is always a small degree of nonmyocyte contamination. Therefore, we wished to ascertain whether the apoptosis induced by E1A in these cultures actually occured in myocytes. Cultures that had been mock-infected or infected with *dl*312 or *dl*309/E1B$^-$ 48 h earlier were fixed and assayed on a cell-by-cell basis for the presence of sarcomeric myosin and apoptosis. The TUNEL in situ apoptosis assay is based on the increased number of DNA termini in nuclei of apoptotic cells, as compared with normal or necrotic cells. These

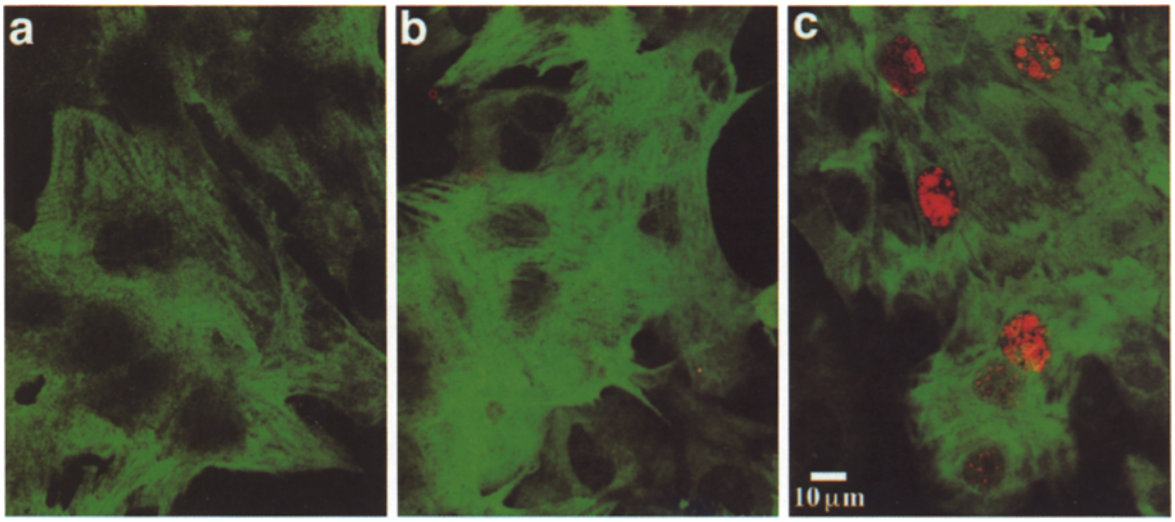

Figure 1. E1A-induced DNA synthesis in cardiac myocytes. Primary cultures of embryonic day 20 cardiac myocytes were plated on coverslips in 6-well dishes. Cells were mock-infected (*a*) or infected with adenoviruses *dl*312 (*b*) or *dl*309/E1B$^-$ (*c*) 36 h after plating, pulsed with 20 μM BrdU between 24 and 26 h after infection, fixed, and stained for BrdU (*orange*, rhodamine) and MLC2v (*green*, FITC). Duplicate coverslips were examined by confocal microscopy. In plates infected with *dl*309/E1B$^-$, 70–80% of myocytes were BrdU positive. In the field shown in *c*, six myocyte nuclei were scored as positive. In contrast, in plates infected with *dl*312 or mock infected, no BrdU-positive myocytes were found out of >300 myocytes scored. This experiment was performed using four independent preparations of cells with similar results.

were identified by covalently attaching digoxigenin-tagged dUTP to 3′ ends with terminal deoxytransferase and subsequent immunostaining for digoxigenin. As shown in Fig. 3 *c*, myosin-positive cells (*red,* rhodamine) from plates infected with *dl*309/E1B$^-$ show clear evidence of nuclear staining (*green,* FITC) indicative of ongoing apoptosis. In contrast, no nuclear staining was seen in mock- (Fig. 3 *a*) or *dl*312-infected (Fig. 3 *b*) plates. In addition, only occasional myosin negative cells were found to exhibit nuclear staining, suggesting that nonmyocytes in these cultures were not as efficiently infected by the virus and/or more resistant to E1A-induced apoptosis, as compared with

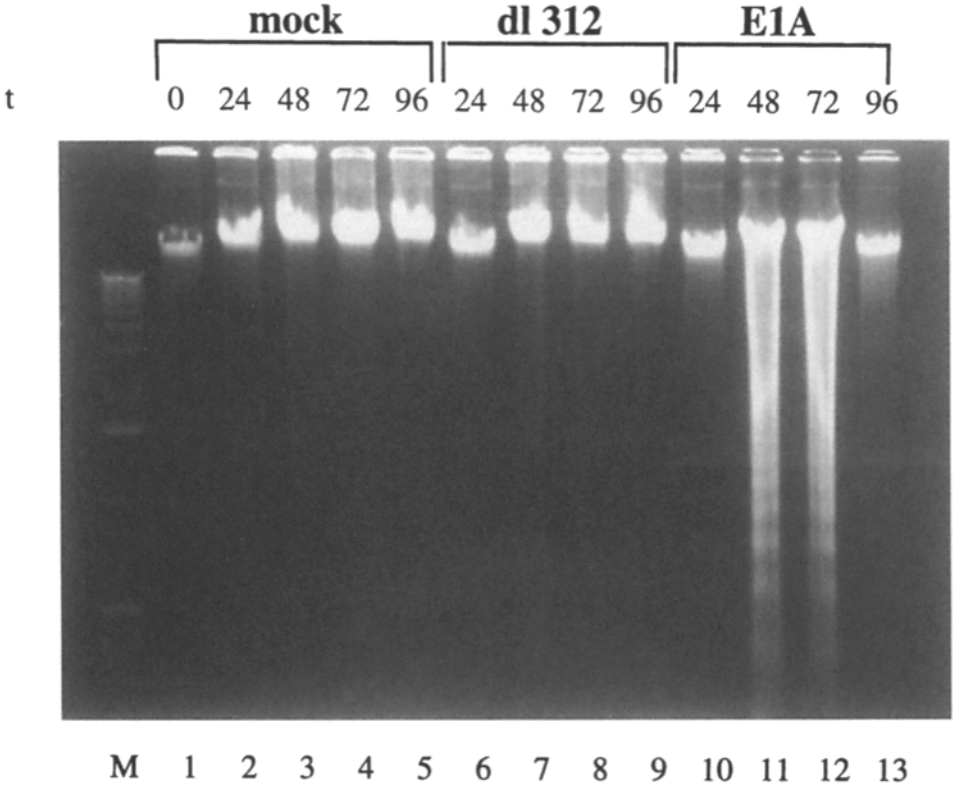

Figure 2. Time course of E1A-induced apoptosis in primary cultures of cardiac myocytes. Primary cultures of embryonic day 20 cardiac myocytes were plated on 60-mm dishes and mock infected (lanes *1–5*) or infected with adenoviruses *dl*312 (lanes *6–9*) or *dl*309/E1B$^-$ (lanes *10–13*) 36 h after plating. Cells were harvested 0–96 h after infection as indicated. DNA was isolated, size fractionated on a 1.2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. *M*, 1-kb mol wt standard. This experiment was performed using three independent preparations of cells with similar results.

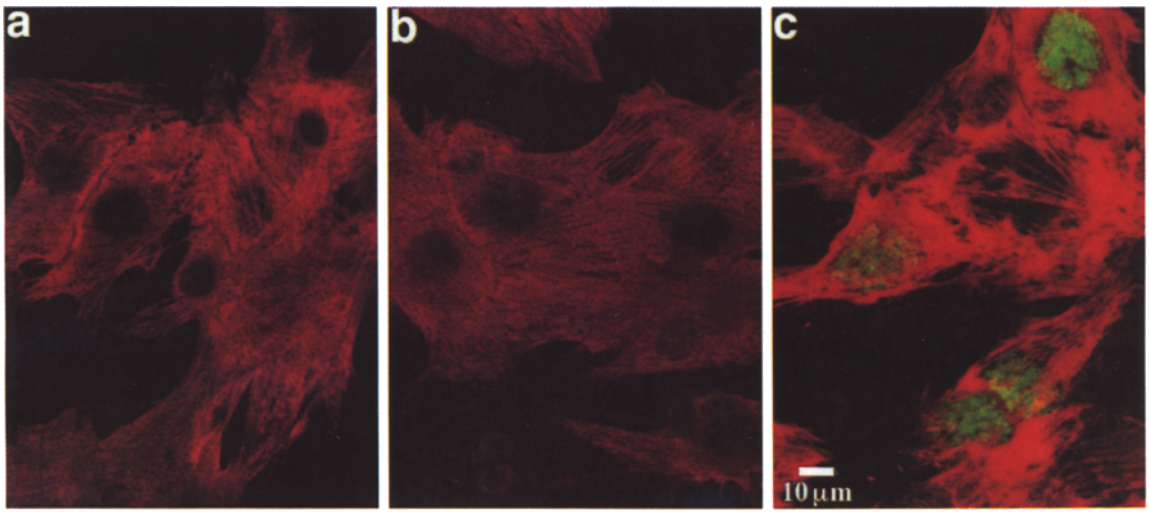

Figure 3. E1A-induced apoptosis occurs in cardiac myocytes. Primary cultures of embryonic day 20 cardiac myocytes were plated on coverslips in 6-well dishes and mock-infected (*a*) or infected with adenoviruses *dl*312 (*b*) or *dl*309/E1B (*c*) 36 h after plating. Cells were then fixed 48 h after infection, processed for double immunofluorescence for the TUNEL in situ apoptosis assay (*green*, FITC) and for sarcomeric myosin (*red*, rhodamine), and duplicate coverslips were examined by confocal microscopy. This experiment was performed using three independent preparations of cells with similar results.

myocytes. Thus, E1A clearly induces apoptosis in cardiac myocytes.

E1A-induced DNA Synthesis and Apoptosis Occur in the Same Cell

The experiments reported above show that E1A stimulates both DNA synthesis and apoptosis. These studies do not, however, address whether the same cells undergo both processes and, if so, which process occurs first. To address this issue, cells were mock infected or infected with *dl*309/E1B$^-$, pulsed with BrdU starting at 24 h after infection, harvested at 28 or 36 h after infection, and assayed for BrdU incorporation using immunostaining and apoptosis using the TUNEL assay. As demonstrated previously, no BrdU-positive or TUNEL-positive nuclei were observed in mock-infected cells at either time point. Although ~70% of nuclei were BrdU positive 28 h after infection with *dl*309/E1B$^-$, no TUNEL-positive nuclei were observed at this time (data not shown). The percentages of BrdU-positive and TUNEL-positive nuclei in cells harvested at 36 h after infection are shown in Table I. Although the total percentage of BrdU-positive nuclei (71%) was similar to that observed at the earlier time point, now 21% of total nuclei were TUNEL positive. Of the BrdU-positive nuclei, ~30% were also TUNEL positive, examples of which are shown in Fig. 4. Notably, however, BrdU-negative/TUNEL-positive nuclei were not observed. This experiment demonstrates that DNA synthesis and apoptosis can occur in the same myocyte and that apoptosis occurs only in those cells that are or have recently engaged in DNA synthesis. Taken together, the progressive increase in the number of TUNEL-positive nuclei from 0% at 28 h to 21% at 36 h after infection (with ~70% of nuclei BrdU positive at both time points) and the absence of BrdU-negative/TUNEL-positive nuclei at either time point suggest that apoptosis is preceded by DNA synthesis.

Ability of E1A Mutants to Stimulate DNA Synthesis and Apoptosis in Cardiac Myocytes

The effects of E1A on DNA synthesis and apoptosis in cycling cells are mediated by its interactions with cellular proteins including Rb (and related pocket proteins p107 and p130) and p300 (Howe et al., 1990; Mymryk et al., 1994). To gain insight into whether these same cellular proteins play a role in the G1/S barrier to cell cycle progression in cardiac myocytes, we expressed wild-type and mutant E1A proteins in these cells using E1B$^-$ deficient adenoviruses. *dl*520E1B$^-$ was used as the wild-type control in these experiments because each of the mutants tested was derived from it. In contrast to *dl*309/E1B$^-$ used in the previous experiments, which encodes both the 243– and 289– amino acid alternative splice products of the E1A gene, *dl*520E1B$^-$ gives rise to only the shorter of the proteins. In cycling cells, however, this gene product possesses all of the host cell DNA synthetic and apoptosis functions of the longer protein (reviewed in Bayley and Mymryk, 1994). In addition, it binds p300, pRb, p107, and p130 normally. Mutant E1A proteins were encoded by derivatives of *dl*520/E1B$^-$ including: (*a*) *dl*1108/520E1B$^-$, whose E1A lacks residues 124–127. Quantitative immunoprecipitation studies have demonstrated that this E1A mutant is completely unable to bind Rb and p130, and that it binds only 3% as much p107 as wild-type (Barbeau et al., 1992). In contrast, p300 binding is near normal. (*b*) *dl*1101/520E1B$^-$, encoding an E1A protein lacking residues 14–25, rendering it completely defective with respect to p300 binding. In contrast, it retains the ability to bind Rb, p107, and p130, although slightly less well than wild-type (Barbeau et al., 1992). (*c*) *dl*01/08/520E1B$^-$, which contains both of the above mutations. All four *dl*520E1B$^-$ viruses were titered on 293 cells to establish the concentrations of infective particles in each stock. Cultured cardiac myocytes were infected with these viruses, pulsed with BrdU

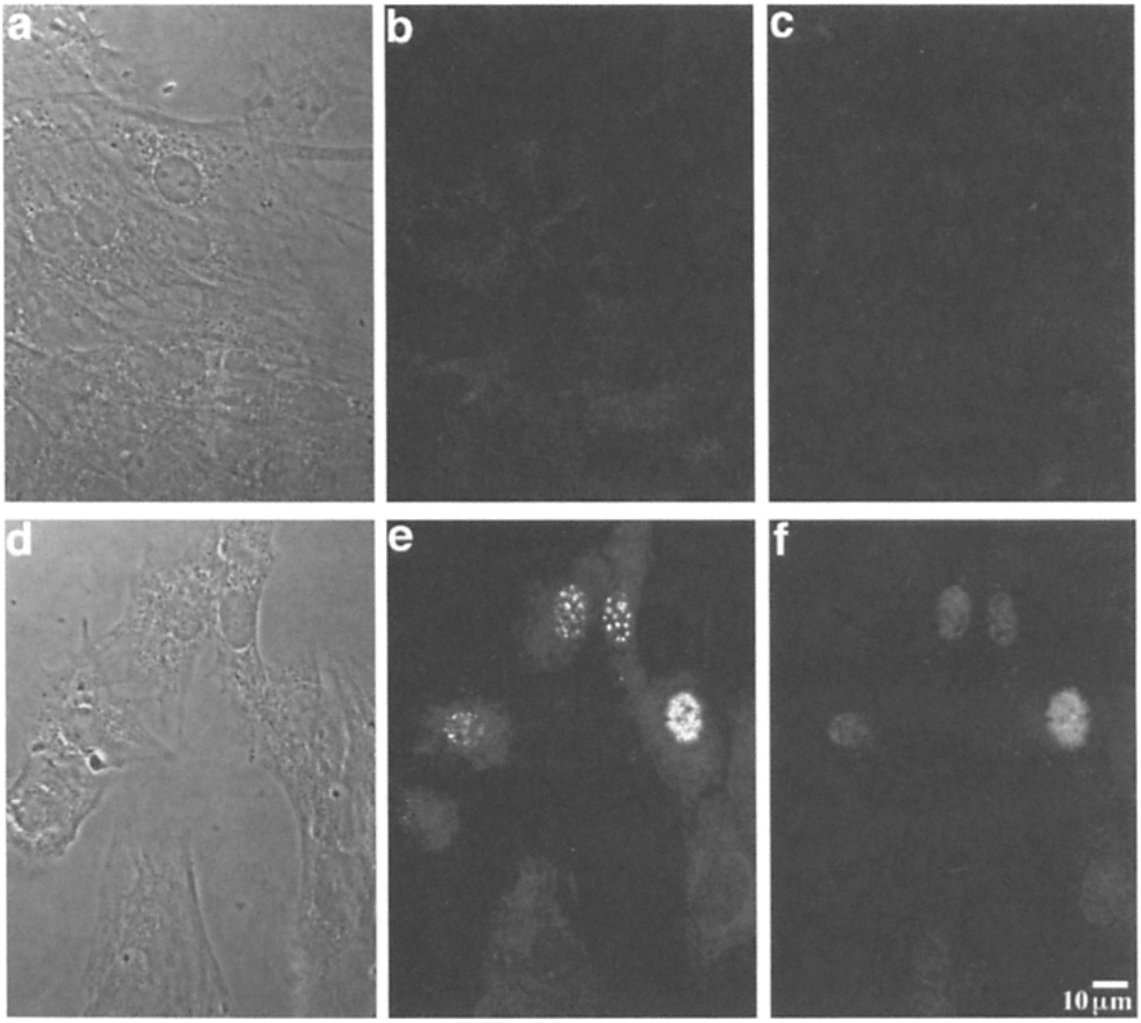

Figure 4. E1A-induced DNA synthesis and apoptosis occurs in the same cells. Primary cultures of embryonic day 20 cardiac myocytes were plated on coverslips in 6-well dishes and mock infected (*a–c*) or infected with *dl*309/E1B$^-$ (*d–f*) 36 h later. Cells were then pulsed with 20 μM BrdU 24–36 h after infection, fixed, and processed for double immunofluorescence for BrdU (*b* and *e*, rhodamine) and the TUNEL in situ apoptosis assay (*c* and *f*, FITC). Duplicate coverslips were examined by confocal microscopy. *a* and *d* are phase contrast views of the corresponding immunofluorescence images. Note the BrdU-positive/TUNEL-positive nuclei in the field shown. In other fields, significant numbers of BrdU-positive/TUNEL-negative nuclei were seen, but BrdU-negative/TUNEL-positive nuclei were not observed (see Table I).

between 24 and 36 h after infection, and analyzed for BrdU incorporation and MLC2v expression by double immunofluorescence.

Fig. 5 *A* shows the percentage of myocyte nuclei containing BrdU. As observed previously, BrdU was not detectable by immunofluorescence in the nuclei of mock-infected myocytes. On the other hand, 86% (MOI 10) and 94% (MOI 20) of myocyte nuclei were BrdU positive after infection with *dl*520E1B$^-$, confirming that the 243-residue E1A protein is sufficient to stimulate DNA synthesis in cardiac myocytes. At both MOI 10 and 20, the E1A mutant defective for pocket protein binding was almost as effective as the wild-type protein at stimulating DNA synthesis. In contrast, the mutant unable to bind p300 was 4.1- (MOI 10, $P < 0.001$) to 2.4-fold (MOI 20, $P < 0.005$) less efficient. The percentage of myocytes positive for E1A protein at 24 h after infection was similar for each of the viruses, as was the abundance of E1A protein by immunoblot (data not shown). Thus, the decreased ability of the p300 binding mutant to stimulate DNA synthesis in cardiac myocytes is attributable to the mutation itself rather than to a decrease in the amount of E1A protein per myocyte.

Infection with *dl*01/08/520E1B$^-$, encoding the mutant defective for binding both pocket proteins and p300, resulted in a very low percentage of BrdU-positive nuclei. Although its infection efficiency, as determined by immu-

Table I. Quantitation of BrdU and TUNEL-positive Nuclei 36 h after Infection

	Mock infection	dl309/E1B$^-$
BrdU$^-$/TUNEL$^-$	155	39
BrdU$^-$/TUNEL$^+$	0	0
BrdU$^+$/TUNEL$^-$	0	69
BrdU$^+$/TUNEL$^+$	0	28
Total	155	136

Methods are as described in Fig. 4.

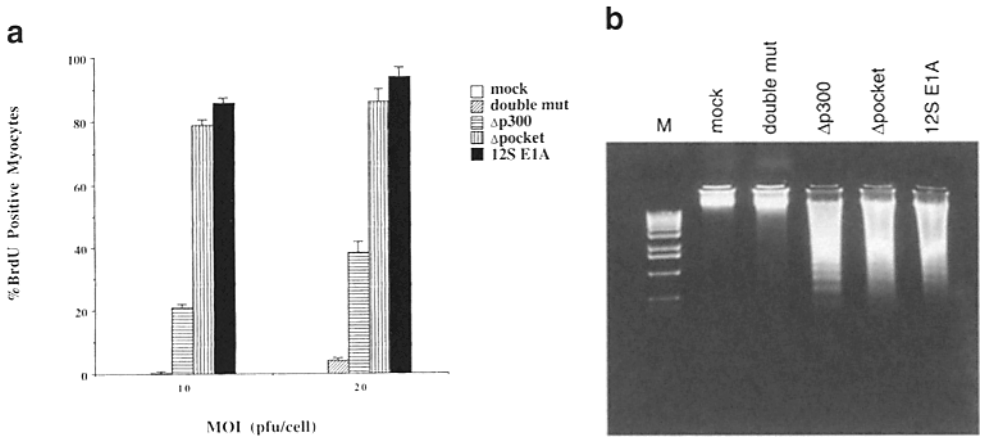

Figure 5. Ability of E1A mutants to stimulate DNA synthesis and apoptosis in cardiac myocytes. Primary cultures of embryonic day 20 cardiac myocytes were mock infected (*mock*) or infected with adenoviruses encoding various E1A proteins: *double mut*, defective for binding p300 and pocket proteins; *Δp300*, defective for p300 binding; *Δpocket*, defective for pocket protein binding; and *12SE1A*, wild-type 12S E1A. (*a*) After infection at MOI 10 or 20 as indicated, cells were pulsed with 20 μM BrdU 24–28 h after infection, fixed, stained for BrdU and MLC2v, and examined by confocal microscopy. In each experiment, >200 cells were scored on each of two coverslips for the percentage of MLC2v-positive cells with BrdU-positive nuclei. The results from a representative experiment are presented as the means ± SE of these counts. This experiment was performed on three independent preparations of cells with similar results. Statistical comparisons: MOI 10, $P < 0.001$ for 12S E1A vs mock, double mut, and Δp300, and for Δpocket vs double mut; $P < 0.005$ for Δp300 vs double mut. MOI 20, $P < 0.001$ for 12S E1A vs mock and double mut; $P < 0.005$ for 12SE1A vs Δp300; and for Δpocket vs double mut; $P < 0.05$ for Δp300 vs double mut. (*b*) Cells were harvested 48 h postinfection at MOI 10 and assayed for nucleosomal laddering. *M*, 1-kb mol wt standard. This experiment was performed on three independent preparations of cells with similar results.

nostaining with a polyclonal adenoviral antibody, was similar to that of *dl*520E1B⁻, the double-mutant consistently resulted in lower E1A levels by immunoblot (data not shown). In an attempt to increase the abundance of the doubly mutated E1A, we infected *dl*01/08/520E1B⁻ at MOI 20 and compared the resulting E1A levels and percentage of BrdU-positive myocyte nuclei with those resulting from infection of *dl*520E1B⁻ at MOI 10. Infection with *dl*01/08/520E1B⁻ at MOI 20 resulted in E1A levels that were 55% of those resulting from infection with *dl*520E1B⁻ at MOI 10. In contrast, infection with *dl*01/08/520E1B⁻ at MOI 20 resulted in >20-fold fewer BrdU-positive myocyte nuclei, as compared with infection with *dl*520/E1B⁻ at MOI 10. Although the possibility of a threshold effect cannot be excluded, this result suggests that simultaneous mutations in residues required for pocket protein and p300 binding severely limit the ability of E1A to stimulate DNA synthesis in cardiac myocytes.

We also assessed the effect of the various mutants on the ability of E1A to induce apoptosis in cardiac cultures by assaying lysates for DNA fragmentation 48 h after infection at MOI 10 (Fig. 5 *B*) and 20 (results similar, data not shown). The DNA of cells infected with *dl*520E1B⁻ (12S E1A) exhibits marked nucleosomal laddering confirming that the 243-residue E1A protein is sufficient to induce apoptosis. In contrast, no detectable apoptosis was seen in mock-infected cells (*mock*) or in those infected with the *dl*01/08/520E1B⁻ (*double mut*). Definite nucleosomal laddering was present, however, in lanes containing DNA from cells infected with *dl*1108/520E1B⁻ (*Δpocket*) and *dl*1101/520E1B⁻ (*Δp300*). Thus, mutants that retain either pocket protein or p300 binding function are sufficient to stimulate apoptosis in these cells.

Discussion

Most cultured cardiac myocytes do not engage in net DNA synthesis, even in the presence of serum growth factors. E1A stimulates this process in almost all successfully transduced myocytes. This observation suggests that the block to DNA synthesis normally present in these cells is mediated by the same or similar molecules that gate the G1/S transition in cycling cells. Since E1A is thought to induce DNA synthesis in cycling cells through its interactions with cellular proteins, it follows that at least some of the proteins involved in blocking DNA synthesis in cardiac myocytes may be targets for E1A binding.

To gain insight into which cellular proteins may be involved, we assessed the abilities of various E1A mutants to stimulate DNA synthesis in cardiac myocytes. Mutations disrupting p300 binding decrease E1A-stimulated DNA synthesis in cardiac myocytes. This effect is severalfold in the context of an E1A molecule that retains the ability to bind, and presumably inactivate, pocket proteins. In the absence of E1A pocket protein binding, the usual situation in the cell, loss of p300 binding decreases the ability of E1A to stimulate DNA synthesis markedly, although we are unable to quantitate this effect precisely because of the slightly lower levels in the cell of the E1A double mutant. While this paper was in review, a study was reported evaluating the effects of an independent set of E1A mutants in neonatal cardiac myocytes (Kirshenbaum and Schneider, 1995). While the results of the two studies are in general agreement, they differ in that the effect of isolated loss of p300 binding on DNA synthesis reported herein was not observed in the other study. This difference may reflect subtle effects of the precise mutations used in the two studies or differences between fetal and neonatal cardiac myocytes. Regardless, both studies demonstrate that residues required for p300 binding play an important role in the ability of E1A to stimulate DNA synthesis in cardiac myocytes.

The most straightforward interpretation of this data is that p300 or a p300-like protein exists in cardiac myocytes

and, in the absence of being sequestered by E1A, contributes to the inhibition of cellular DNA synthesis. We hasten to emphasize, however, that since the identities and functions of proteins in cardiac myocytes that interact with E1A residues required for p300 binding have not yet been determined, this interpretation must be regarded as conditional. In addition, this model assumes that the absence of DNA synthesis in cardiac myocytes results from active repression rather than simply being the default mode. This view is supported by skeletal myoblast/myocyte heterokaryon experiments demonstrating dominance of the phenotype characterized by the absence of net DNA synthesis (Clegg and Hauschka, 1987). It remains to be determined whether this is also the case in cardiac myocytes in light of experiments demonstrating the lack of dominance of cardiac gene expression in fusions between cardiac myocytes and fibroblasts (Evans et al., 1994).

p300 is the prototype member of a family of bromodomain-containing transcriptional adaptor molecules that are thought to transactivate the expression of target genes by interacting simultaneously with specific enhancer binding proteins and components of the basal transcriptional apparatus (Eckner et al., 1994; discussed in Arany et al., 1994). Members of the p300 family appear to be expressed ubiquitously. How then can the presence of p300 family members in cycling as well as terminally differentiated cells be reconciled with the possibility that these proteins play an important role in excluding the latter from the cell cycle? One possibility is that different p300 family members exist in proliferating and postmitotic cells. Another is that the activity of the same p300 family member is differentially modulated in these cells by changes in abundance, posttranslational modifications, or interactions with other proteins. The answers to these questions will become clearer as the different p300 family members and their modes of regulation become better defined.

If the role postulated above for p300 in cardiac myocytes is correct, one pathway by which it might act to inhibit DNA synthesis is suggested by recent work in terminally differentiating keratinocytes, demonstrating that it plays a role in activating the promoter of the gene encoding $p21^{CIP1/WAF1}$ (Missero et al., 1995). This protein, in turn, is a member of the family of universal cyclin-dependent kinase inhibitors and blocks DNA synthesis and cell cycle progression (reviewed in Sherr, 1994). Interestingly, $p21^{CIP1/WAF1}$ is also induced during differentiation of C2C12 myotubes (Halevy et al., 1995; Parker et al., 1995; Missero et al., 1995).

It is well established that Rb plays an important role in regulating the G1/S transition in mammalian cells, as well as in the terminal differentiation of skeletal myocytes (Gu et al., 1993; Schneider et al., 1994), neurons (Lee et al., 1994), and lens fiber cells (Pan and Griep, 1994; Morgenbesser et al., 1994; and Fromm et al., 1994). Although its presence in wild-type cardiac myocytes has not been formally documented, it is present in extracts of whole-heart tissue and in cardiac myocytes that express an SV-40 T antigen transgene (Kim et al., 1994). Therefore, it was somewhat surprising that the E1A mutant that is completely defective for Rb and p130 binding and binds p107 only 3% as well as the wild-type protein (Barbeau et al., 1992) retains the ability to stimulate DNA synthesis in cardiac myocytes almost as efficiently as the wild type. One possible explanation for this observation is that the expression of E1A leads to the inactivation of Rb through an alternative mechanism. In support of this, E1A has been shown in other cells to induce the phosphorylation of Rb through a mechanism independent of its ability to bind this pocket protein (Wang et al., 1991).

As in cycling cells containing functional p53, the end result of E1A overexpression in cardiac myocytes is apoptosis. Direct evidence that both processes occur in the same cells is shown in Fig. 4. In addition, the data demonstrate that: (*a*) while ~70% of nuclei are BrdU positive both 28 and 36 h after infection, the number of TUNEL-positive nuclei increases from 0 to 21% during the same period of time; and (*b*) while many BrdU-positive nuclei are TUNEL-negative 36 h after infection, all TUNEL-positive nuclei are BrdU positive (Table I). Taken together, these findings suggest that DNA synthesis precedes apoptosis.

Although tight associations between forced DNA synthesis and apoptosis have been observed in the present study and previously (Wu and Levine, 1994; Qin et al., 1994; Shan and Lee, 1994; Morgenbesser et al., 1994), a cause and effect relationship between these two processes has not been demonstrated. Alternatively, the temporal association between these two processes could reflect shared proximal signaling events (discussed in Evan et al., 1995). Conversely, it is clear that a block to DNA synthesis is not required for E1A-induced apoptosis because E1A elicits apoptosis in cycling cells as well as in those which do not ordinarily engage in DNA synthesis. Thus, while in some situations apoptosis may represent the resolution of conflicting positive and negative growth signals, this does not appear to be the case with E1A-induced apoptosis. Moreover, the outlines of a plausible scenario by which E1A could induce apoptosis have emerged from known epistatic relationships: E1A increases steady-state levels of p53 through transcriptional (Braithwaite et al., 1990) and posttranscriptional mechanisms (Lowe et al., 1993). p53, in turn, is known to activate the expression of the *bax* gene (Miyashita and Reed, 1995), whose product can promote apoptosis (Oltvai et al., 1993). Further work is needed to determine whether these events constitute the actual signaling pathway mediating E1A-induced apoptosis.

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