Adenovirus E1A Inhibits Cardiac Myocyte-specific Gene Expression through Its Amino Terminus*

(Received for publication, January 27, 1997, and in revised form, April 25, 1997)

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Adenovirus E1A oncoproteins inhibit muscle-specific gene expression and myogenic differentiation by suppressing the transcriptional activating functions of basic helix-loop-helix proteins. As one approach to identifying cardiac-specific gene regulatory proteins, we analyzed the functional regions of E1A proteins that are required for muscle gene repression in cardiac cells. Myocyte-specific promoters, including the α-actins and α-myosin heavy chain, were selectively and potently inhibited (>90%) by E1A, while the ubiquitously expressed β-actin promoter was only partially (~30%) repressed; endogenous gene expression was also affected. Distinct E1A protein binding sites mediated repression of muscle-specific and ubiquitous actin promoters. E1A-mediated inhibition of β-actin required both an intact binding site for the tumor repressor proteins pRb and p107 and a second E1A domain (residues 15–35). In contrast, cardiac-specific promoter repression required the E1A amino-terminal residues 2–36. The proximal skeletal actin promoter (3’ to base pair −153) was a target for repression by E1A. Although E1A binding to p300 was not required for inhibition of either promoter, co-expression of p300 partially reversed E1A-mediated transcriptional repression. We conclude that cardiac-specific and general promoter inhibition by E1A occurs by distinct mechanisms and that cardiac-specific gene expression is modulated by cellular factors interacting with the E1A p300/CBP-binding domain.

Differentiated mycardium appears very early during embryonic development, and contracting cardiac myocytes continue to proliferate until shortly after birth. Cells committed to the cardiac lineage express a unique set of differentiation-dependent genes. A hallmark of the muscle phenotype, including cardiac, smooth, and skeletal muscle cells, is expression of genes encoding contractile proteins such as α-actin and myosin heavy chain. Although certain myofilament protein isoforms are only expressed in one cell type (e.g. cardiac α-myosin heavy chain), many sarcomeric genes are expressed in both skeletal and cardiac muscle.

In contrast to skeletal muscle, the mechanisms regulating tissue-specific gene expression in the heart are poorly understood. A family of transcription-activating proteins characterized by a basic helix-loop-helix (bHLH) motif, including the factors myogenin, MyoD, myf-5, and MRF4/herculin/myf-6, are now known to govern skeletal myogenesis and muscle gene expression (1–8). These myogenic proteins share the ability to induce skeletal muscle gene expression in a broad range of cell types and are required for the genesis of muscle tissue in the limbs and somites during development (5, 9–12). MyoD and its homologues also interact with components of the cell cycle regulatory apparatus to induce differentiation in myoblasts and maintain skeletal myotubes in a state of growth arrest (13, 14). Despite intense scrutiny, functional homologues of skeletal bHLH proteins have not been identified in the heart (9, 15, 16).

Several years ago, we demonstrated that expression of the Ad2/5 E1A gene inhibited tissue-specific gene expression and differentiation of skeletal myocytes (17). E1A is a virus-encoded nuclear phosphoprotein that primes the host cell for viral replication by repression of differentiated cell functions and re-activating cell machinery involved in DNA synthesis. Like the related polyoma virus T antigens (18, 19), E1A exerts these effects by interacting with host cell proteins involved in growth regulation, transcription, nuclear DNA synthesis, and apoptosis (reviewed in Refs. 20–22; see also Refs. 23–28). Proteins known to bind E1A include the transcriptional co-activator proteins p300 and CBP, retinoblastoma (Rb) protein p105, and Rb-related proteins p107 and p130, as well as cell cycle regulatory proteins, including cyclin D and p27Kip1/Waf1. Binding sites for many of these proteins have been mapped by mutagenesis and functional analysis (29–33). Inhibition of muscle-specific gene expression appears to correlate with the binding of E1A amino-terminal sequences to cellular proteins p300 (34) and the bHLH protein myogenin (35). Interestingly, other bHLH-regulated tissue-specific promoters, including the immunoglobulin and insulin enhancers (36–38), are susceptible to repression by E1A (39, 40). Since E1A proteins can bind both cell cycle-regulating and bHLH-proteins, there are a number of ways in which E1A could disrupt the myogenic program in skeletal and cardiac cells.

In this study, we report that E1A preferentially disrupts transcriptional activation of cardiac sarcomeric genes via an amino-terminal domain implicated in transformation and tissue-specific gene repression in other cell types (41, 42). Disruption of binding to p300, pRb, or both did not eliminate this effect. In contrast, E1A-mediated repression of the non-tissue-specific myosin heavy chain is mediated through an amino-terminal domain.
specific β-actin promoter required an intact binding site for p105Rb/p107. E1A-mediated repression mapped to a proximal element of the muscle-specific skeletal β-actin promoter (hsA) and was partly reversed by co-expression of p300. Our studies delineate a short region of amino-terminal E1A residues that regulate cardiac-specific transcription, probably through binding or modulation of tissue-specific factors, and implicate a proximal tissue-specific element in the human skeletal β-actin promoter as a target for E1A repression.

EXPERIMENTAL PROCEDURES

Cell Culture—Cardiac myocytes were prepared from 1-3-day-old neonatal Harlan Sprague Dawley rats as described previously (43). After preplating to reduce the number of non-myocytes, cultures were plated at a density of 4 × 10^6/80-mm dish and allowed to attach overnight in EM, 5% FCS. Cells were re-fed with and maintained in this medium for the duration of the experiments. Under these conditions, cardiac myocytes are plated at near-confluent density, and contact inhibition limits the number of non-myocytes to ~5% of the total as determined by specific immunofluorescent staining (not shown). Non-myocytes were studied in parallel as a control for contamination by these cells in the myocyte cultures; selectively preplated non-myocytes were allowed to grow to 80% confluence in the same MEM, 5% FCS medium and then passage twice before use.

Materials—Truncated E1A genes expressing wild-type 12 S and mutant forms were kindly provided as mammalian expression vectors and as recombinant adenoviruses by E. Moran (Fels Institute, Philadelphia). The expression vectors comprise genomic fragments corresponding to E1Awt and mutations therein cloned into pUC18 or pUC18(41, 42). Viruses encoding these E1A proteins also express E1B. The antibody M73 was the gift of Ed Harlow (44) and is directed against an epitope common to the above wild-type (wt) and mutant E1A proteins. The human skeletal β-actin, cardiac β-actin, and β-actin constructs, and the vector containing full-length E1A, have been described previously (17, 43, 45). The α-myosin heavy chain promoter/CAT (α-MHC-CAT) containing 3000 bp of upstream sequence was provided by T. Gustafson, the proenkephalin-CAT reporter chimera (46) by M. Tom Curran (St. Jude Children’s Research Hospital, Memphis, TN). The p300 expression plasmid, pCMVβ-p300CHAM (47), was obtained from R. Eckner (Dana-Farber Cancer Institute, Boston), and the blank pCMV expression plasmid was the gift of Frank Rauscher (Wistar Institute, Philadelphia). cDNA probes encoding murine skeletal α-actin, cardiac α-actin, and human β-actin have been described previously (17, 43); a human histone 3.1 cDNA probe was the kind gift of Larry Kedes (University of Southern California, Institute of Genetic Medicine, Los Angeles). A CMV expression vector encoding bel-2 was generously provided by Dr. Michael Kiefer (LXR Biotechnology, Richmond, CA).

Transfection—Cardiac myocytes were transfected using an adaptation of the calcium phosphate method on the day following plating, as described previously (45). Equal numbers of myocytes were co-transfected with 5–10 μg of reporter plasmid and either an E1A expression vector or an equal amount of blank plasmid vector, using the calcium phosphate technique. DNA/calcium precipitates were allowed to remain on the cells overnight. On the following day (day two of culture) plates were washed two times and MEM, 5% FCS was replenished. Cells were incubated for a further 40 h prior to harvesting and assay for chloramphenicol acetyltransferase as described previously (48). CAT values were expressed as percent conversion of chloramphenicol to mono- and diacetylated forms corrected for lysate protein content. For p300 co-transfection, 5 μg of reporter/CAT construct was co-transfected with and without 2 μg of pWTE1Awt in the presence or absence of 5 μg of pCMVβ-p300CHAM; total transfected DNA remained constant within individual experiments by addition of appropriate amounts of the blank CMV expression vector and/or pUC18 as appropriate.

Virus Culture and Infection—Viruses were grown from seed stocks on 293 cell monolayers and titrated by plaque assay (49). All viruses grew with approximately equivalent efficiency. For infections, cardiac myocytes were infected at a multiplicity of 10 pfu/cell on day 5 of culture. Virus was added to the medium and allowed to adsorb for 1 h at 37 °C in a humidified incubator. The media was then replaced with MEM, 5% FCS, and the infected cells were maintained at 37 °C for the rest of the experiment. For BrdU labeling, the replacement medium was supplemented with 0.1 mg/ml BrdU for 24 h prior to cell fixation.

RNA Blot Analysis—Cardiac myocytes were infected as described above with one of several recombinant E1Awt adenoviruses, and harvested 48 h later. Total RNA was prepared using RNAzol B, separated on formalin-agarose gels, transferred overnight to nylon membranes, and cross-linked in a Stratalinker (Stratagene), exactly as described previously (50). The blots were probed sequentially with three or more cDNA probes that were radiolabeled by random priming to >10^6 cpm. Autoradiography was performed and quantitated on a Lynx 2-D densitometer.

Immunoprecipitation—Immunoprecipitations were essentially as described in (51), with minor modifications. Infected cardiac myocytes were metabolically labeled for 18–20 h with Trasylol-S-label (0.2 mCi; ICN) in 4 ml of MEM, 5% FCS lacking methionine. Cell monolayers were lysed in situ in a buffer containing 50 mM Tris (pH 7.5), 250 mM NaCl, 0.1% Triton X-100, and 5 mM EDTA, supplemented with apotinin, leupeptin, and pepstatin (each at 1 μg/ml), and phenylmethylsulfon- fyl fluoride (257 μg/ml, U. S. Biochemical Corp.), for 30 min at 4 °C. Lysates were precleared with 100 μl of IgSorb (Enzyme Center, Malden, MA); counts/min/ml were determined in a scintillation counter and normalized accordingly. Proteins were immunoprecipitated using an E1A-specific mouse mononclonal antibody M73 (44) at 4 °C for 1 h, followed by purification on protein A-Sepharose beads (Pharmacia Biotech Inc.). Proteins were released by boiling in 30 μl of 2 × Laemmli buffer, separated on 6% SDS-polyacrylamide gels, and examined by autoradiography.

Immunoblotting—Cardiac myocytes were transfected as described above with 10 μg of wild-type or mutant E1Awt expression plasmids. After 2 days, cells were harvested and resuspended in Nonidet P-40 lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF) with freshly added 1 mM Na3VO4, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 25 μg/ml apro- tinin. Cells were lysed in a Dounce homogenizer with piston A for 20 strokes, and the resulting lysates were centrifuged for 10 min. 100 μg of each lysate supernatant was fractioned on a 12% SDS-polyacrylamide gel and electroeluted onto nitrocellulose filters. Filters were blocked in 5% non-fat milk and incubated with mononclonal antibody M73. Proteins were detected by enhanced chemiluminescence (ECL, Amersham Corp.)

Analysis of Apoptosis—Cells were analyzed for apoptosis by visualization of nuclear chromatin morphology using DNA-binding dyes H33342 and propidium iodide. Cardiac myocytes on day 3 of culture in 5% serum-containing medium were infected with the mutant viruses described above and evaluated for the percentage of fragmented and condensed myocyte nuclei. Control and wt- and d2–36-infected cells were examined at both 24 and 48 h, while cells infected with YH47/928, Rg2, Rg2/928, m928, and d15–35 were examined at 48 h. At the end of 24 or 48 h, infected cells were rinsed with phosphate-buffered saline and fixed in a 1:1 mixture of cold ethanol and 30% cold trichloroacetic acid. Cells were then incubated with 5 μg/ml H33342 and 5 μg/ml propidium iodide for 30 min. Individual nuclei were examined at × 400 on a Zeiss Axioscop fluorescence microscope using paired phase and fluorescence imaging and scored for the presence or absence of apoptotic features. Propidium iodide was used to identify non-viable cells. Cells that stained positive for propidium iodide and exhibited nuclear characteristics of apoptosis were scored as apoptotic, while propidium iodide-positive cells with near-normal chromatin were counted as necrotic. To quantitate apoptosis, an average of 200 nuclei from random fields were analyzed, and counts were expressed as (apoptotic nuclei/total nuclei) × 100% to obtain the percentage of apoptotic nuclei. Samples were numbered to conceal the identity of the different treatment groups during scoring, and at least three samples were scored per group.

Analysis of DNA Synthesis—Cardiac myocytes grown on 2-well coverslips (Nunc) were incubated with [H]thymidine (1 μCi/ml final concentration) for 24 h, beginning 24 h after infection with one of several recombiant adenoviruses. At the end of the labeling period, cells were fixed in a mixture of 70% ethanol, 15% formalin, 15% acetic acid at ~20 °C, for 5 min and then incubated in 1.5 μl HCl for 30 min at room temperature. Cells were next rinsed, air-dried, and reacted sequentially with mouse anti-BrdU monoclonal antibody (Sigma), biotinylated anti-mouse IgG, and fluorescein-conjugated avidin D (both from Vector Laboratories, Burlingame, CA). In some cases cells were also reacted with a polyclonal anti-rat desmin antibody followed by Texas Red (Vector Laboratories)- or Cy-3 (Biological Detection Systems, Inc., Pittsburgh)-conjugated goat anti-rabbit IgG. Cells were visualized on a Zeiss Axioscop fluorescence microscope using paired phase and fluorescence imaging and recorded on a dedicated 35-mm camera using Kodak P1600 color reversal film.

Statistics—Analysis of variance with multiple column comparisons was performed as described (50) using InStat software for Macintosh (GraphPad, San Diego, CA).
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RESULTS

Primary cultures of neonatal rat cardiac myocytes were co-transfected with increasing amounts of the wild-type Ad2/5 E1A gene (encoding both 13 and 12 S proteins) and plasmids encoding the CAT reporter gene under the control of one of several tissue-specific or ubiquitous promoters, including human skeletal (hSA) and cardiac (hCA) actin and murine c-fos and rat α-myosin heavy chain (α-MHC) promoters. The muscle-specific promoters hSA, hCA, and α-MHC were strongly (>90%) inhibited by co-expression of E1A (Fig. 1A). Transcriptional repression by E1A was dose-dependent and was maximal at a reporter:E1A ratio of 1:1 (Fig. 1A). In contrast, the c-fos promoter and a basal hSA promoter truncated at −87 were only partially inhibited at the maximal dose of E1A (Fig. 1A). Thus, E1A selectively inhibited the expression of muscle-specific promoters in cardiac myocytes, closely paralleling its effects in skeletal myocytes (17).

Two functionally distinct proteins of 12 and 13 S are generated through alternate splicing of the primary E1A gene transcript (52, 53). The 13 S protein includes a COOH-terminal exon with transforming and transcriptional activating properties (25, 54–56), while the 12 S protein is thought to function primarily as a transcriptional repressor. To verify that the 12 S E1A product was responsible for cardiac myocyte transcriptional repression, we repeated the co-transfections using an E1A expression plasmid encoding only the 12 S protein (Fig. 1B). The 12 S protein was an even more potent repressor than the 13 S construct, and transcriptional repression was still markedly selective for muscle-specific promoters. The hSA promoter was significantly repressed by <1 μg of co-transfected E1A12S (reporter:E1A ratio of 5:1) and was maximally repressed at a reporter:E1A ratio of 5:2 (Fig. 1B, closed circles). About 20-fold more E1A12S was required to inhibit the ubiquitously expressed β-actin promoter (Fig. 1B, shaded squares). The exact molar ratio of E1A to reporter construct was variable under these conditions because of the differing lengths of these promoters. However, this variation did not account for the differential sensitivity of the promoters. For example, the β-actin promoter construct is approximately 1000 bp larger than the 2000 bp hSA construct, so that on a molar basis there was approximately 11% more E1A for each β-actin plasmid than for each hSA plasmid transfected. Hence, the β-actin promoter was more active than the hSA promoter in the presence of a greater proportion of E1A. In another series of experiments, E1A12S repressed the muscle-specific hSA and α-MHC promoters by >90% and 95%, respectively (n = 3). In contrast, the ubiquitously expressed β-actin and c-fos promoters were only partially repressed, and high level expression from the proenkephalin promoter was not affected (Fig. 1C). Thus, transcriptional inhibition by E1A12S was selective for cardiac-restricted genes.

The E1A proteins affect cell growth and differentiation by interacting with a large number of cell regulatory proteins, expressing the 12 S E1A and E1B proteins from the E1A promoter (42) was transfected with the indicated promoter/CAT constructs at a ratio of 2 μg of E1A:5 μg of marker. Plasmids encoding muscle-specific skeletal actin/CAT (hSA2000CAT, black circles) or a human β-actin CAT (βACTCAT, black squares) were co-transfected into cardiac myocytes with increasing amounts of the 12 S E1A plasmid, and CAT activity in extracts was quantitated as described in the legend to A. Data are expressed as a percentage of maximal CAT activity in the absence of E1A, and represent a single experiment that was repeated three times with similar results. C, E1A12S selectively inhibits cardiac-specific promoters. The indicated promoters were co-transfected with the E1A12S plasmid at a ratio of 5:2 (micrograms of marker:E1A) and CAT activity determined in cell lysates as above. These data represent the mean of three separate experiments.
including p300, CBP, AP-1, and AP-2 (57–59) and the pocket proteins p105Rb, p107, and p130. Specific domains of E1A<sub>12S</sub> protein that interact with these cell regulatory proteins have been mapped by deletion and point mutagenesis (29–33, 41, 42) (Fig. 2). One E1A domain contains the amino terminus and the COOH-terminal portion of conserved region 1 (CRI) and binds the related proteins p300 and CBP. A second domain contains binding sites for the pocket proteins and is comprised of amino-terminal domain 1 = conserved region 1. 2 = conserved region 2. Hatched areas are included in domain I, white areas in domain II.

To examine cardiac myocyte proteins interacting with E1A, we infected cardiac myocyte cultures with viruses expressing the wild-type or mutant E1A<sub>12S</sub> genes indicated in Fig. 2 (41, 42). Cardiac myocyte-E1A protein complexes were immunoprecipitated from cell lysates using an antibody against a common procedures. Lysates from 293 cells, which constitutively express E1A epitope (M73 (44)) as described under “Experimental Procedures,” and cell lysates were immunoprecipitated with either antibody M73 (first lane and third through seventh lanes in B) or an anti-c-fos antibody (second lane). Equal counts/min were loaded in each lane. Arrows indicate the positions of major E1A-associated 293 cell proteins. B, cardiac myocyte proteins associated with E1A. Cardiac myocytes were infected with one of the indicated recombinant adenoviruses expressing wt or mutant 12SE1A and metabolically labeled with Tr<sup>35S</sup>-label for 18 h postinfection. Infected myocyte lysates (first through fourth lanes and sixth through eighth lanes) and 293 cell lysates (fifth lane) were immunoprecipitated with an E1A-specific mouse monoclonal antibody M73 (44) or an anti-c-fos antibody (not shown) followed by purification on protein A-Sepharose beads (Pharmacia). First and second lanes, 12SE1A mutant RG2, 10 and 50 pfu/ml infectivity, respectively. Third and fourth lanes, mutant YH47/928, also at 10 and 50 pfu/ml infectivity, respectively. Fifth lane, uninfected human 293 cells. Sixth lane, cardiac myocytes infected with a wt 12SE1A virus at 20 pfu/ml. Seventh through ninth lanes, the same, but infected with viruses expressing d15–35 (seventh lane), d2–36 (eighth lane), or RG2/928 E1A mutants (nineteenth lane). Tenth lane, uninfected cardiac myocytes. Top arrow, a cardiac nuclear protein co-migrating with p300 in 293 cell extracts; lower arrows, proteins with mobility consistent with pocket proteins p130 and p107/p105. Differences in mobility between these rat-derived proteins and the corresponding 293 proteins may reflect species differences.

larly, binding by the presumptive cardiac myocyte p300 was sharply reduced or eliminated by point mutation of arginine at position 2 (Fig. 3B, lanes 1, 2, 9) or by deletion of E1A residues 15–35 or 2–36 (Fig. 3B, lanes 7 and 8).

These results indicate that cardiac proteins complexing with E1A are similar in size and binding properties to those previously described in other cell types (41, 42) and are likely to be cardiac homologues of p300 and the pocket proteins. Despite the presence of a small number of non-myocardial cells in the culture (≤5% of the total, mainly fibroblasts), it is also probable that the protein bands identified on these gels originate primarily from cardiac myocytes. We detected no p300, p105 and p107, and very little p130, in selectively plated non-myocytes infected and labeled under the same protocol (not shown). This may simply reflect the existence of different labeling kinetics for these factors in non-myocytes following infection, rather than their absence. In either case, the contribution of
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Cardiac myocytes were transfected with plasmid vectors encoding the indicated 12 S E1A protein variants together with either the human skeletal actin promoter hSA2000CAT (sACT, black bars) or the human β-actin promoter βACTCAT (βACT, shaded bars), on day 1 of culture, and harvested 48 h later. CAT activity was determined as in Fig. 1. Data are expressed as the absolute amount of promoter inhibition as a percentage of control activity in the absence of wt 12 S E1A. These data represent the mean ± S.E. of at least five experiments with replicate plates, in which a minimum of two different plasmid preparations was used for each vector. Asterisks indicate significant divergence from wt inhibitory activity (p < 0.01).

To determine whether binding of E1A to specific cellular proteins correlated with its transcriptional repression functions, plasmid expression vectors encoding individual E1A.128 mutants were co-transfected into cardiac myocytes with either the human skeletal α-actin (hSA) or the human β-actin (βACT) promoter. These promoters are derived from two closely related actin isogenes that differ in their tissue distribution: α-actins are striated muscle-specific, while β (cytoplasmic)-actin is ubiquitously expressed. As shown in Fig. 4, the two promoters were differentially repressed by specific E1A mutations. Transcriptional repression of βACT (shaded bars in Fig. 4) by E1A protein was maximal at 54.2 ± 6% inhibition (n = 6) and absolutely required an intact pocket protein binding domain. An E1A protein mutated at residues 47 and 124, and lacking all known binding ability in the pocket domain (YH47/928), did not inhibit βACT (p < 0.01). Two other mutants with single point mutations in the pocket domain (m.928, RG2/928) were partially impaired, as were the amino-terminal deletion mutants d2–36 and d15–35 (Fig. 3B, lanes 7 and 8). Point mutation of the p300 binding site at residue 2 or 20 did not affect either hSA (Fig. 4, black bars) or βACT promoter inhibition. These results indicate that pocket protein interactions are essential for βACT transcriptional repression by E1A. In addition, it is clear that portions of the E1A amino terminus are required for optimal inhibition of βACT, suggesting that multiple proteins contribute to the regulation of βACT in cardiac myocytes.

There were striking differences in the effects of discrete E1A mutations on hSA versus βACT promoter inhibition. The pocket domain mutant YH47/928, which was defective for βACT repression, was still a potent inhibitor of hSA expression (Fig. 4). This observation alone indicated that different E1A functions were required for inhibition of constitutive and cardiac specific promoters. The d15–35 and d2–36 mutants also had significantly different effects on the two promoters: while both deletion mutants were equally defective for βACT inhibition, the d15–35 mutant had wild-type inhibitory activity for the hSA promoter. In fact, of all the constructs tested, only the large amino-terminal deletion d2–36 lacked inhibitory activity against hSA. The same mutant was also defective for inhibition of two other tissue-specific promoters, cardiac α-actin and α-MHC (data not shown).

As shown above, the d2–36 mutant did not bind p300, but retained affinity for pocket proteins (Fig. 3B, lane 7). However, loss of p300 binding did not account for the transcriptional repression defect of this mutant. Three other mutants (RG2, Δ15–35, and RG2/928) that did not bind p300 (cf. Fig. 3B, lanes 1, 2, 8, and 9) still inhibited hSA. Significantly, a double mutant defective for both p300 and pocket protein binding (RG2/928) also effectively repressed hSA. Consequently, neither p300 nor pRb-related pocket protein binding are necessary for cardiac-specific gene inhibition, but residues 2–15 are required.

Infection by E1A-expressing adenovirus has been reported to induce apoptosis in cardiac myocytes (62, 63). Thus, it is possible that the reduced expression of muscle-specific proteins in the presence of E1A is due to the induction of apoptosis. To exclude this possibility, we determined the apoptotic potential of the different E1A mutants under the culture conditions used in our experiments. We found that apoptosis potential did not segregate with the ability to inhibit either β- or α-actin expression (cf. Fig. 5A). Mutant RG2 was the most defective for apoptosis, but displayed wild-type levels of skeletal actin repression (cf. Fig. 4). Furthermore, mutants YH47/928, 928, and RG2/928 had approximately equivalent apoptosis potential, but only YH47/928 was defective for βACT repression. In fact, all mutant E1A species tested were much weaker inducers of apoptosis than wild-type E1A. Thus, transcriptional repression by E1A could be readily dissociated from its effects on programmed cell death.

To confirm this, we measured the ability of Bcl-2, a negative modulator of apoptosis (64–66), to block transcriptional repression by E1A. Bcl-2, a member of a family of genes involved in the regulation of programmed cell death, is a functional homologue of adenovirus E1B (67) and is able to block the induction of apoptosis by a wide variety of stimuli. Co-expression of Bcl-2 had no effect on E1A repression of the skeletal actin promoter (Fig. 5, B and C), over a range of doses of both E1A and Bcl-2. The primary effect of Bcl-2 appeared to be a strong, highly dose-dependent transactivation of the actin promoter that was reversed by E1A (Fig. 5B). These data further support the conclusion that apoptosis does not account for E1A-mediated cardiac myocyte transcriptional repression.

The absence of transcriptional repressor activity by the d2–36 mutant could be due to absence of the protein, either because of instability or low expression from the virus. We addressed this issue in four ways. First, we re-analyzed d2–36 virus-infected cardiac myocyte proteins on a higher percentage acrylamide gel to resolve the d2–36 E1A protein from a strong background band. The results, shown in Fig. 6A, indicate that the protein is readily detected in infected myocytes. Second, Western blots were performed on extracts of cardiac myocytes transfected with one of several E1A expression plasmids. As seen in Fig. 6B, the plasmid-encoded d2–36 protein was expressed at levels similar to both wt and d15–35 protein. Significantly, three E1A mutants that exhibited significant transcriptional repression were present at much lower levels (Fig. 6B), suggesting that even small amounts of these proteins are sufficient to saturate their biological targets.

Third, if reduced transcriptional repression is due to low protein abundance, it should be possible to overcome both deficiencies by increasing the amount of transfected E1A DNA. Accordingly, we evaluated d2–36 mutant repression of hSA transcription over an extended dose range. Comparative dose-response curves for the wt, d2–36, and several other mutant E1A plasmids are shown in Fig. 6C. Increasing the amount of
Axioscop fluorescence microscope was used for phase and fluorescence of the total amount of apoptosis occurring in each sample. A Zeiss initial stages of apoptosis and thus represents a conservative estimate biguous features were scored as non-apoptotic. This procedure tation as described under “Experimental Procedures.” Cells with am-

**Fig. 5. Apoptosis does not account for differential promoter inhibition by E1A.** A, apoptosis induced by recombinant E1A mutant-expressing viruses. Apoptosis was quantitated by examination of fixed monolayers of cardiac myocytes infected for 24 or 48 h with the indicated E1A12S virus and scoring of nuclei for condensation and fragmen-tation as described under “Experimental Procedures.” Cells with am-

of co-transfected Bcl-2 expression vector, in the presence of 2 μg of E1A12S plasmid (dotted line) or of the blank pUC18 vector (solid line). The total amount of DNA was kept constant by addition of decreasing amounts of the parental CMV expression vector. Note the strong transcriptional activation of the hSA promoter by Bcl-2, an effect that is nearly eliminated by E1A. Upper graph, percent promoter inhibition was determined as the ratio of absolute CAT activity in the presence and absence of E1A, at each level of co-transfected Bcl-2 vector. This percentage does not vary significantly with the co-expression of Bcl-2. These results are representative of three independent experiments. C, effect of Bcl-2 co-expression on E1A dose-response curve. The effect of increasing amounts of co-transfected E1A12S plasmid on absolute CAT activity from the hSA2000CAT construct was determined in the pres-

available protein in this manner did not reveal any latent capacity for transcriptional repression in the d2–36 mutant, nor did it account for the differential effects of the other mu-
tants. These results suggest that viral or plasmid-mediated expression of E1A results in levels of protein considerably in excess of what is required to saturate its intracellular targets.

A final question is whether the d2–36 protein is biologically active in a relevant assay. Since this mutant is competent to bind pRb-related proteins, we examined its ability to induce histone mRNA levels, which are normally tightly coordinated with DNA synthesis (61). In cardiac myocytes infected for 48 h, the d2–36 mutant virus induced histone mRNA transcript lev-

effects (Fig. 6D). In aggregate, these results suggest that the absence of transcriptional repression by the d2–36 protein is not due to a deficiency in its production or stability. Furthermore, apart from transcriptional repression, the d2–36 protein is biologi-
cally equivalent to other E1A proteins, including the d15–35 mutant.

The effects of E1A on transient promoter expression were reflected by changes in endogenous gene expression. Northern analyses of RNA from wild-type and mutant 12 S adenovirus-infected cells revealed a modest but consistent reduction in steady-state α-skeletal actin transcript levels (0.77-fold ± 0.27) at 24 h after infection (Fig. 7). This effect is consistent with the previously observed transcriptional inhibition of the skeletal actin promoter and the long (≥12 h) half-life of α-skeletal actin mRNA (data not shown). Significantly, infection with the deletion mutant virus d2–36 failed to reduce α-skeletal actin mRNA levels and, in fact, caused a slight induction (2.90-fold ± 0.65, p < 0.05). This induction was not observed with the pocket domain mutant YH47/928. In contrast, β-actin mRNA levels were induced by all three E1A viruses in cardiac myocytes, although significantly less so by YH47/928 (p < 0.05) (Fig. 7). This effect on β-actin expression was not seen in similarly infected non-myocytes (Fig. 7, NMC), despite equiv-
alent expression of the viral E1A protein (not shown).

In addition to changes in endogenous gene expression, car-
diac myocytes infected with wild-type 12 S adenovirus de
doped distinctive, qualitative growth abnormalities by day 5 (Fig. 8). Under our growth and culture conditions (43), cardiac myocytes form synchronously contracting, multicellular clusters with cytoplasmic extensions bridging the individual clusters (Fig. 8A). This characteristic architecture was lost 72 h postinfection with wt 12 S E1A virus (Fig. 8G), or mutant RG2/928 (Fig. 8E). Similar results were obtained with mutants m.928 and YH47/928 (not shown). Morphological differences did not originate with differences in confluence, as the mono-

layer culture confluence and morphology were initially identi-
cal in all plates. In contrast, cells infected with the d2–36 mutant had growth properties indistinguishable from un-

**Fig. 6. Transient promoter expression is inhibited by E1A.** A, percent CAT activity from the hSA2000CAT construct in the presence of increasing amounts of co-transfected E1A12S plasmid on absolute CAT activity from the hSA2000CAT construct was determined in the presence (solid line) or absence (dotted line) of 2 μg of Bcl-2 expression vector. Total transfected DNA was kept constant by addition of appropriate amounts of the respective parental vectors as described above. These results are representative of two independent determinations.
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Fig. 6. E1A mutant d2–36 is expressed and functional. A, comparative expression of E1A12S wild type and d2–36 proteins. Cardiac myocytes were infected with wt and d2–36 mutant E1A proteins, and lysates were immunoprecipitated with M73 antibody as described in the legend to Fig. 3B. The immunoprecipitates were electrophoretically separated on an 8% SDS-acrylamide gel to permit resolution of lower molecular mass bands. Upper arrow indicates the specific band corresponding to wt E1A in the first lane, and the lower arrow indicates the position of the d2–36 mutant protein. Other bands represent nonspecifically co-immunoprecipitated proteins also seen in uninfected cell lysates (Fig. 3B and not shown). B, Western analysis of E1A proteins in cells transfected with plasmid expression vectors. Cardiac myocytes were transfected as described in the legend to Fig. 4, using the indicated E1A-encoding expression vectors. Lysates were subjected to Western analysis as described under "Experimental Procedures," and E1A-reactive bands were visualized using a non-radioactive detection protocol (ECL, Amersham). Lysates from 293 cells were used as a control. No signal was detected in untransfected cardiac myocyte lysates (not shown). C, dose-response curves for transcriptional inhibition by E1A wt and mutant proteins. Dose-response curves for the indicated E1A12S plasmids were generated as described in the legend to Fig. 1C, using CAT expression from the hSA2000CAT construct as a marker of transcriptional activity. Each sample was transfected with 5 μg of marker DNA. Total DNA transfected was maintained by addition of E1A parental vector. D, comparative induction of histone mRNA by wt E1A and two 5′ deletion mutants. Cardiac myocytes in 5% serum-supplemented media were infected on day 1 of culture with one of three recombinant adenoviruses, and total RNA was prepared from the cells after 48 h. Northern analysis was performed as described previously. Top, representative blot probed sequentially with cDNA probes encoding murine skeletal α-actin, human β-actin, and the rRNA 18 S subunit. Bottom, densitometry data was taken from three separate experiments; mRNA levels are expressed as the mean percentage of control ± S.E.

Fig. 7. Differential E1A effects on skeletal α-actin and β-actin mRNA abundance in recombinant adenovirus-infected cells. Cardiac myocytes or selectively plated non-myocytes in 5% serum-supplemented media were infected on day 1 of culture with one of three recombinant adenoviruses, and total RNA was prepared from the cells after 48 h. Northern analysis was performed as described previously. Top, representative blot probed sequentially with cDNA probes encoding murine skeletal α-actin, human β-actin, and the rRNA 18 S subunit. Bottom, densitometry data was taken from three separate experiments; mRNA levels are expressed as the mean percentage of control ± S.E. Therefore, the structural abnormalities segregated more closely with muscle-specific (hSA) repression than with mutants defective in ubiquitous promoter inhibition. Interestingly, cardiac phenotypic repression did not correlate with the induction of DNA synthesis. In this series of experiments, all Ad wild-type and 12 S mutants tested were found to
augment DNA synthesis, although to different degrees (Fig. 8, D, F, and H). This was confirmed by quantitative analysis of DNA synthesis (Fig. 9; see “Experimental Procedures”). Cardiac myocyte DNA synthesis was identified by triple staining with anti-desmin and anti-BrdUrd antibodies and the nuclear stain Hoechst 33345 (Fig. 9A), confirming earlier reports (62, 63). E1A 12S virus and mutants RG2- and m.928-stimulated DNA synthesis in essentially all of the myocytes by 48 h (Fig. 9B). Mutants d15–35 and d2–36 were somewhat less efficacious in this regard, but mutant YH47/928 was markedly impaired, both in the presence and absence of serum. RG2/928 was also defective, but only in the absence of serum (Fig. 9B). Similar results were obtained with shorter BrdUrd labeling times, although the absolute numbers of positive cells decreased proportionately (not shown). Thus, the ability of individual mutants to induce DNA synthesis did not correlate with the development of structural abnormalities or with cardiac-specific transcriptional repression.

The E1A amino terminus may repress cardiac transcription by interacting with tissue-specific regulatory programs, as has been shown in other cell types (35, 59, 68). As an initial step toward identification of target proteins, we used two promoter deletion mutants to find the region of hSA that was susceptible to repression by E1A 12S. The proximal skeletal actin promoter between -2153 and the start of transcription contains a number of transcription factor binding sites, including sites for SRF, YY-1, and AP-1 (69–71); both Jun (AP-1) and SRF are potent activators of actin promoter transcription (69, 70, 72).2 Truncation of the promoter at -87 results in basal expression in cardiac myocytes (43). Fig. 10A shows that both the -2000 and -153 hSA constructs were repressed by wt E1A; low level expression from the -87 construct was not affected. It is reasonable to conclude from this that E1A-mediated repression involves one or more proteins interacting with the proximal promoter.

We next asked whether co-expression of Jun or SRF could interfere with the effects of E1A on hSA expression. We also tested the transcriptional co-activator p300 (47) in similar assays, reasoning that E1A could modulate p300 through mechanisms that do not require direct binding, as has been shown for pRb (51). As shown in Fig. 10B, 5 μg of Jun (AP-1) and 5 μg each Jun + SRF transactivated the hSA promoter by 5- and 7-fold, respectively, in the absence of E1A. However, 2 μg of E1A still efficiently (>80%) inhibited marker gene expression in the presence of these proteins (Fig. 10B). The same result was obtained with even higher concentrations of the transactivating vectors (10 μg each, not shown). In contrast, p300 blunted transcriptional repression by E1A. p300 reproducibly caused a modest (2–3-fold) transactivation of hSA and also reduced the ability of E1A to inhibit the hSA promoter over a range of E1A concentrations (Fig. 10, B and C). Transcriptional induction of hSA, by itself, did not account for the loss of repression by E1A, since SRF and Jun were both more potent activators of hSA than p300 (Fig. 10B). These data suggest that cardiac-specific transcriptional repression by E1A may be modulated in part by p300-related or associated proteins, although an intact p300 binding site on E1A does not appear to be required.

2 N. H. Bishopric, unpublished data.
DISCUSSION

In this study we have delineated two distinct, although contiguous, domains of E1A involved in the regulation of tissue-specific versus ubiquitously expressed promoters. This finding confirms that the cellular mechanisms for cardiac-specific and ubiquitous gene expression are also distinct. Amino-terminal residues 2–14 of E1A were required to disrupt cardiac-specific gene transcription, while inhibition of βACT, a non-tissue-restricted gene, required residues 15–35 as well as the binding domain for pocket proteins pRb and p107.

The E1A amino-terminal site (residues 2–14) involved in cardiac-specific repression is distinct from an amino-terminal site previously shown to modulate differentiation and muscle-specific gene expression in skeletal muscle. Comparison of our results with a previous study on skeletal RD myocytes (35)...

FIG. 9. Quantitation of DNA synthesis in infected cardiac myocytes in the presence and absence of serum. A, DNA synthesis in a E1A12S adenovirus-infected cardiac myocyte. Cardiac myocytes were cultured as described above, infected with wt 12S adenovirus on day 5 of culture, and labeled with Brdu for 48 h beginning on day 6. Using the anti-BrdU staining process described in the legend to Fig. 6, cells were also stained with a polyclonal anti-desmin stain followed by streptavidin-conjugated anti-IgG and Hoechst 33342. Multiple fields were recorded using single and double exposures of fluorescein, rhodamine, and UV channel illumination. B, percentage of cells labeling with Brdu in uninfected cells and following infection by the indicated mutant E1A virus. Cells were infected exactly as in A, except that replicate plates were infected in the presence (black bars) or absence (shaded bars) of 5% FCS. Nuclei were identified both by Hoechst staining and by the exclusion of staining in desmin-positive cells. Cardiac myocytes were scored for DNA synthesis only when specific nuclei could be localized to the cells. For each condition, at least 200 cells were scored from a minimum of five fields. The graph represents pooled data from three separate experiments.

FIG. 10. Molecular targets for E1A transcriptional repression. A, the proximal hSA promoter is repressed by E1A. Luciferase constructs containing 2100 bp (pluc1), 153 bp (p153luc1), or 87 bp (p87luc1) of the human skeletal α-actin promoter were co-transfected with a wild-type E1A expression vector or a blank vector. Transfections included 5 μg of the indicated promoter construct and 2 μg of E1A plasmid or pUC18. Methods were as described in the legend to Fig. 1. B, co-expression of p300, but not AP-1 or SRF, attenuated E1A-mediated inhibition of hSA. Cardiac myocytes were co-transfected with 5 μg of hSA2000CAT and 2 μg of wt E1A12S (+E1A) or its parental vector (−E1A), with or without 5 μg of pCMV vector (C) pCMVβ-p300CHAm (p300), pCMVJun (jun), or pCMVSRF (SRF). The total amount of transfecting DNA was kept constant at 17 μg. These data represent the mean of three separate experiments; error bars indicate S.E. C, p300 transactivates the hSA promoter and attenuates its repression by E1A. Dose-response curves for E1A repression of hSA in the presence (open circles) and absence (closed circles) of 5 μg of p300. The scale on the abscissa is logarithmic.
reveals that direct binding of E1A to p300 and pocket proteins was dispensable for tissue-specific transcriptional repression in both cell types. In the latter study, however, E1A-mediated transcriptional repression of the muscle-specific creatine kinase promoter was correlated with myogenin sequestration through an interaction with E1A residues 15–35. Thus, while residues 2–36 are implicated for both skeletal and cardiac-specific transcriptional inhibition, the specific sites within this domain appear to be different. By extension, if cardiac-specific homologues of skeletal bHLH proteins are responsible for hSA expression in cardiac myocytes, they do not bind to the same 12 S site. In support of this concept, there is evidence for at least two overlapping but distinct protein binding sites within the E1A amino terminus, including sites for transcriptional co-activators p300, CBP, and AP-2 (59, 78), in addition to the reported interaction with myogenin (35). Further analyses will be required to determine whether the target for E1A-mediated cardiac transcriptional repression is single or multiple.

In a previous report, transcriptional inhibition in cardiac myocytes was attributed to E1A complexing with either p300 or pocket proteins (62). The pathway outlined here is distinct from the latter, however, since inhibition of the tissue-specific skeletal actin promoter was independent of both p300 and pocket protein binding. Although our findings do not exclude a role for either p300 or pRB/p107 in one or more pathways of cardiac gene expression, it is more likely that the mechanism defined here involves tissue-specific factor(s). General functions targeted by E1A, such as availability of the TATA binding factor TFIID (79), would also be difficult to reconcile with the tissue-specific effects reported here. Both βACT and hSA promoters have classical TATA box elements that presumably would be equally sensitive to this type of interaction; hence, TFIID binding is unlikely to account for the promoter-specific repression described.

Similarly, a general effect on apoptosis is unlikely to account for the selective effects of E1A on cardiac-restricted genes. Transcriptional repression by E1A occurs both in the presence and absence of E1B (17, 62) and in immortalized cell lines that do not undergo appreciable levels of apoptosis (17, 59). Data presented in this paper show that apoptosis induction and transcriptional repression are dissociable functions (compare Figs. 4 and 5A) and that transcriptional repression is not reversed by overexpression of Bcl-2 (Fig. 5, B and C). Interestingly, we did observe significant transactivation of the skeletal actin promoter by low doses of Bcl-2. The mechanism of this transactivation is not clear, although it is likely that proteins such as Bcl-2 and E1B have additional cellular effects beyond the prevention of cell death (64).

We also show in this study that E1A alters endogenous β- and skeletal α-actin genes in a tissue- and gene-specific manner. The effects of E1A on endogenous α-skeletal actin expression, hSA promoter repression, and morphological abnormalities were all localized to the same amino-terminal E1A domain. Removal of this domain was associated with a reproducible induction of α-skeletal actin transcripts, possibly due to the unmasking of effects on mRNA stability or post-transcriptional regulation by E1A or later viral genes (80, 81). The same E1A viruses had roughly inverse effects on cardiac myocyte β-actin transcript levels. Both observations confirm that the net effect of E1A is a shift from muscle-specific to constitutive actin isoforms.

In contrast to previous reports in skeletal myocytes, cardiac-specific promoter repression did not appear to correlate with induction of DNA synthesis. Several mutants, including d15–35, d2–36, RG2/928, and YH47/928, were relatively compromised for DNA synthesis induction, consistent with a broadly distributed function (see Ref. 60). However, none of these mutations (apart from d2–36) significantly affected cardiac-specific gene expression. A role for p107 in the regulation of cardiac DNA synthesis is suggested by the observation that single mutation of residue 124 (=nucleotide 928) had no effect on DNA synthesis, in agreement with Liu and Kitsis (63), but mutation of both residues 47 and 124 did have a significant impact. Mutation of residue 47 is required to eliminate p107 binding in some cell types, whereas mutation of residue 124 abrogates binding by pRB and p130 (42).

Although binding to p300 was not required for cardiac-specific transcriptional repression, overexpression of p300 partially alleviated repression of the hSA promoter by E1A12S protein. There are several means by which p300/CBP could participate in cardiac transcriptional regulation. For example, we have previously shown that the skeletal actin promoter is activated by transcription factor AP1 (Fos/Jun) (70), and both p300 and CBP have been demonstrated to be co-factors for AP-1-mediated transcriptional activation (73, 74). Second, p300 overexpression may compete with or displace one or more cell type-specific proteins from complexes with E1A, a possibility that is not excluded by our co-transfection protocol. Third, p300 may have direct transcription activating properties as a component of TATA-binding or other protein-DNA complexes (60, 75, 76). Finally, E1A inhibits p300 phosphorylation in vitro (77), and this effect may not require direct interaction with p300. The phosphorylation state may have important effects on transcription factor activity and may be an important target for E1A in modulating gene expression through p300.

Our results implicate a site(s) within the amino-terminal domain of the adenovirus E1A125 protein in the transcriptional repression of the hSA promoter in cardiac myocytes. A number of factors have recently been associated with cardiac-specific transcription, including non-bHLH proteins GATA-4 (82–85), MEF2 (86–88), TEF-1 (89, 90) and Id, as well as HLH proteins E12 and E47 (16). Any of these, or other presently unidentified factors, may interact with this site in a direct or indirect manner. Among other possible interactions, the fact that ubiquitously expressed p300 can attenuate E1A-mediated transcriptional repression may mean that p300 is able to complex with these tissue-specific proteins to regulate cardiac-specific gene expression or that p300 competes with these factors for E1A reactive sites. Further studies will be required to determine the precise role of E1A-binding proteins in cardiac gene regulation.

Acknowledgments—We thank Dr. Elizabeth Moran for providing the E1A plasmids and viruses used in this paper and for many helpful discussions. We are also grateful to Dr. Richard Eckner for the gift of the p300 expression vector and to Dr. Ed Harlow for the M73 antibody.

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Adenovirus E1A Inhibits Cardiac-specific Transcription
Adenovirus E1A Inhibits Cardiac Myocyte-specific Gene Expression through Its Amino Terminus

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J. Biol. Chem. 1997, 272:20584-20594.
doi: 10.1074/jbc.272.33.20584

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