Expression of E1A in Terminally Differentiated Muscle Cells Reactivates the Cell Cycle and Suppresses Tissue-Specific Genes by Separable Mechanisms†

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Terminally differentiated cells are characterized by permanent withdrawal from the cell cycle; they do not enter S phase even when stimulated by growth factors or retroviral oncogenes. We have shown, however, that the adenovirus E1A oncogene can reactivate the cell cycle in terminally differentiated cells. In this report, we describe the molecular events triggered by E1A in terminally differentiated skeletal muscle cells. We found that in myotubes infected with the adenovirus mutant *dl520*, 12S E1A bypasses the early G₁ phase and activates the expression of late-G₁ genes, such as the cyclin E and cyclin A genes, *cdk2*, *PCNA*, and *B-myb*. Of these, the cyclin E gene and *cdk2* were significantly overexpressed in comparison with levels in proliferating, undifferentiated myoblasts. p130 and pRb were phosphorylated before the infected myotubes entered S phase, despite the high expression of the cyclin-dependent kinase inhibitor p21, and E2F was released. Our results suggest that one of the mechanisms that E1A uses to overcome the proliferative block of terminally differentiated cells involves coordinated overexpression of cyclin E and *cdk2*. Following E1A expression, the myogenic transcription factors MyoD and myogenin and the muscle-specific structural genes encoding muscle creatine kinase and myosin heavy chain were downregulated. The muscle regulatory factors were also silenced in myotubes infected with adenovirus E1A mutants incapable of reactivating the cell cycle in terminally differentiated muscle cells. Thus, the suppression of the differentiation program is not a consequence of cell cycle reactivation in myotubes, and it is induced by an independent mechanism. Our results show that E1A reactivates the cell cycle and suppresses tissue-specific gene expression in terminally differentiated muscle cells, thus causing dedifferentiation.

Terminally differentiated cells, such as skeletal and cardiac muscle cells, white fat adipocytes, keratinocytes, and neurons, are characterized by their permanent withdrawal from the cell cycle (2). These cells cannot enter S phase even when exposed to various mitogenic stimuli, such as growth factors or overexpression of activated cellular oncogenes and cell cycle-regulatory genes (15, 64).

The molecular mechanisms responsible for the postmitotic state in terminally differentiated cells are not clearly understood. A role in the induction and/or maintenance of growth arrest has been suggested for such cell cycle regulators as pRb (7, 14, 17, 18, 31, 35, 55) and p21^{Waf1/Cip1} (20, 21, 41). It has been shown that pRb^{-/-} mice die during embryonal development, showing abnormalities in neuronal and hematopoietic cell differentiation (7, 31, 35). Cell death and a large number of ectopic mitoses in the nervous system suggest that pRb is important in maintaining the postmitotic state (7, 31, 35). Moreover, multinucleated skeletal muscle myotubes established in vitro from pRb^{-/-} mouse embryo myoblasts can enter S phase when challenged with growth factors (55). This finding further indicates that in the absence of pRb, differentiation takes place but is no longer terminal. In neurons, hematopoietic and skeletal muscle cells, upon terminal differentiation,

high levels of unphosphorylated or hypophosphorylated pRb are expressed (53, 55, 57). In its unphosphorylated form, pRb binds members of the E2F family of transcription factors, thus inhibiting the transcriptional activation of several G₁/S boundary genes and progression of the cell cycle (reviewed in references 45 and 70).

During the normal cell cycle, phosphorylation of pRb by specific cyclin-dependent kinases (cdks) in late G₁ allows progression into S phase (reviewed in reference 70). p21 is a universal inhibitor of cdk-cyclin-proliferating cell nuclear antigen (PCNA) complexes (19, 26, 73). High levels of p21 are induced during terminal differentiation of myocytes, keratinocytes, and hematopoietic cells (20, 21, 41, 61). It has been shown that upregulation of p21 in differentiated skeletal muscle cells is accompanied by decreases in cdk2- and p21-associated kinase activities (41). It has thus been suggested that p21 contributes to the establishment and/or maintenance of the postmitotic state by inactivating cdks during differentiation.

The proliferative block of terminally differentiated cells can be overcome by at least some DNA tumor virus oncogenes, such as the simian virus 40 large T antigen (16, 30, 46) and adenovirus E1A (12) genes. We have previously shown that E1A has the ability to reactivate the cell cycle in terminally differentiated skeletal muscle cells and adipocytes (12). This process includes DNA synthesis, mitosis, and cytokinesis. In our conditions, cell cycle reactivation in myotubes is generally followed by cell death, beginning 4 days after infection (12a). Rare myotubes appear capable of escaping death and proliferating (11). By means of adenovirus infection, E1A can be easily expressed in virtually all cell types, including terminally differentiated and primary cells that are often refractory to

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† M.C. dedicates this paper to Harold Weintraub with sorrow for his untimely loss and with regret for having had too few opportunities to meet him.

other transduction methods (36, 51). E1A can interfere with regulation of the cell cycle by forming complexes with the cellular proteins pRb, p107, p130 (also referred to as pocket proteins), and p300 (25, 65, 72). Different mutations in the conserved regions or in the N terminus of E1A selectively abolish these bindings (65, 75). Thus, appropriate mutant adenoviruses can be used to define the roles played by the cellular regulators in the growth arrest of terminally differentiated cells.

Skeletal muscle cells provide a well-established in vitro model of terminal cell differentiation (48). Proliferating precursors (myoblasts) express at least one muscle-regulatory factor belonging to the MyoD family of basic helix-loop-helix (bHLH) transcription factors (71). Myoblasts can be induced to differentiate in vitro by growth factor deprivation. Growth factor-starved myoblasts exit permanently from the cell cycle (commitment) and express an array of muscle-specific genes (biochemical differentiation), thus becoming terminally differentiated myocytes (48). Genes expressed upon differentiation include myogenin, a regulator belonging to the MyoD family, and a large number of structural genes, including the muscle creatine kinase (MCK) and myosin heavy chain (MHC) genes (49). The final phase of muscle cell differentiation involves myocyte fusion into multinucleated myotubes (48).

In this study, we investigated the molecular mechanisms by which the E1A oncogene can overcome the proliferative block of myotubes. We expressed E1A in mouse skeletal myotubes by means of adenovirus infection and examined which regulatory genes E1A activates in order to force postmitotic cells to reenter the cell cycle. Moreover, since proliferation and terminal differentiation are mutually exclusive phenomena, we investigated whether E1A interferes with the expression of muscle-specific genes in the course of reactivating the cell cycle. Finally, by using different E1A mutants, we studied whether E1A interferes with differentiation as a consequence of inducing proliferation or through a pathway separable from that used to reactivate the cell cycle. We show that one of the mechanisms by which E1A forces terminally differentiated muscle cells to reenter the cell cycle involves overexpression of cyclin E and cdk2; we also show that induction of proliferation is accompanied by downregulation of muscle specific genes and that these two effects are achieved by E1A through separable mechanisms.

MATERIALS AND METHODS

Cells. The mouse myoblast line C2C12 (4, 74) was cultured in growth medium (GM; Dulbecco's modified Eagle medium [DMEM] containing 10% fetal calf serum and antibiotics). Differentiation was obtained by plating the cells into collagen-coated dishes in serum-free medium (DMEM supplemented with Redu-Ser [Upstate Biotechnology Incorporated] to final concentrations of 5 μ g of human insulin, 5 μ g of human holo-transferrin, and 5 ng of sodium selenite per ml) containing 50 μ M cytosine β -D-arabinofuranoside to eliminate undifferentiated cells. The adipocyte cell line Adipo 5-2 (12) was established by treating mouse C3H-10T1/2 fibroblasts with 5-azacytidine (63) and was cultured in GM. Differentiation was induced by treating confluent cells for 7 days with 10% newborn calf serum in DMEM supplemented with 10 μ g of human insulin per ml. Mouse C3H-10T1/2 fibroblasts were grown in GM and brought into quiescence by a 48-h incubation in DMEM containing 0.5% fetal calf serum.

Viruses. The adenovirus mutants *d*520, 12S.928, 12S.RG2, 12S.YH47/928, 12S.RG2/928, and *d*312 have been described previously (22, 65, 75). All viruses were grown and titers were determined in permissive 293 cells (27). Infections at the appropriate multiplicity of infection were performed for 2 h in DMEM, after which the cells were shifted to either serum-free medium or GM.

Immunofluorescence. The following monoclonal antibodies (MAbs) or antisera were used for immunofluorescence: anti-5-bromo-2'-deoxyuridine (BrdU) MAb Bu20a (Dako); rabbit anti-MHC antiserum (a kind gift of G. Salvatori and G. Cossu); rabbit anti-cyclin A (a kind gift of G. Draetta); and anti-human pRb MAb G3-245 (Pharmingen). MAbs were detected by fluorescein-conjugated, affinity-purified, goat serum anti-mouse immunoglobulin G (Organon Teknika). Reaction of rabbit antisera was detected by a rhodamine-conjugated, affinity-

purified, goat serum anti-rabbit immunoglobulin G (Organon Teknika). After immunofluorescence treatments, nuclei were stained by a 3-min incubation in a 0.1- μ g/ml solution of Hoechst 33258 dye in phosphate-buffered saline. To study pRb phosphorylation in situ, we treated the cells with an ice-cold hypotonic solution containing 0.1% Triton X-100 as previously described by Mittnacht and Weinberg (42). After the extraction, the cells were fixed and processed for anti-pRb immunofluorescence as described above.

Northern (RNA) blot analysis. For extraction of total cellular RNA, 5×10^6 C2C12 cells were plated into 150-mm-diameter collagen-coated dishes, induced to differentiate, and infected as described above. Samples of 20 μ g were run on formaldehyde gels, transferred, blotted, and hybridized according to standard protocols (54).

Western blot (immunoblot) analysis. For Western blot analysis, whole-cell extracts were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and electroblotted onto a polyvinylidene difluoride membrane (Du Pont) as described previously (60). Expression of proteins was analyzed by using the following antibodies: polyclonal rabbit antisera specific for cyclin A, cdk2, and cdc2, kindly provided by M. Pagano; MAbs specific for cyclin D1, kindly provided by J. Bartek; MAbs to the N terminus of E1A, kindly provided by E. Harlow; MAbs specific for PCNA, obtained from Boehringer Mannheim; polyclonal rabbit antibodies specific for cyclin E and p27, obtained from Santa Cruz Biotechnology; MAbs specific for p130, obtained from Transduction Laboratories; MAbs specific for pRb, obtained from Pharmingen; and polyclonal rabbit antisera specific for p21, kindly provided by C. Schneider. Immunoreactive proteins were detected by peroxidase-coupled secondary antibodies and enhanced chemiluminescence (Amersham).

Kinase activity assays. Cells were extracted for 10 min at 4°C in 1 ml of lysis buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 250 mM NaCl, 0.25% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM sodium orthovanadate, 10 mM NaF, and 1 mM dithiothreitol. Cell lysates were centrifuged to remove cell debris, and supernatants were precleared by a 1-h incubation with protein A-agarose (Santa Cruz Biotechnology). Precleared lysates were then subjected to immunoprecipitation using protein A-agarose-bound polyclonal antibodies specific for cyclin A and cyclin E. After extensive washing, immunoprecipitates were resuspended in kinase buffer (50 mM HEPES [pH 7.9], 10 mM MgCl₂, 5 mM MnCl₂, 1 mM dithiothreitol) supplemented with 100 μ g of histone H1 per ml, 2.5 mM ATP, and 100 μ Ci of [γ -³²P]ATP per ml and incubated for 20 min at 30°C. The reactions were terminated by the addition of 2 $\times$ sample buffer, and labeled proteins were resolved on an SDS-10% polyacrylamide gel and detected by autoradiography.

E2F electromobility shift assay. Protein extracts were prepared and incubated with a labeled, double-stranded oligonucleotide containing a single E2F binding site from the adenovirus E2 promoter as described previously (60). To demonstrate the specificity of the E2F complexes, competitions were performed with 100-fold molar excesses of oligonucleotides containing the wild-type or a mutated E2F site. E2F-associated proteins were analyzed by incubating the band shift reaction mixtures with specific antibodies on ice for 40 min. The following antibodies were used: p130-specific polyclonal antiserum (Santa Cruz Biotechnology), MAb XZ55 to pRb (Dianova); MAb SD15 to p107 (kindly provided by E. Harlow), and MAb TFD10 to DP1 (kindly provided by K. Helin).

RESULTS

Immediate-early genes are downregulated by E1A in terminally differentiated myotubes. To study the expression of cell cycle-regulatory genes in E1A-activated myotubes, we performed time course analyses of RNA and proteins of murine C2C12 myotubes infected with adenovirus *d*520. This replication-defective (43) mutant adenovirus expresses only the 12S E1A mRNA, and it was used to avoid the cytopathic effect associated with the 13S mRNA product (12). Since the expression of most adenovirus genes depends on the 13S product and on viral replication, this mutant expresses few other adenovirus genes and generally only at low levels (22). We have shown through genetic analysis (12) and plasmid-mediated transfection of E1A alone (64) that 12S E1A is sufficient to bring about cell cycle reactivation in myotubes. Myotubes were infected with *d*520 at an approximate multiplicity of infection of 300, in order to activate S phase in about 90% of the cells. The proportion of myotubes in S phase was determined by double immunofluorescence staining for BrdU and MHC (Fig. 1; see also Fig. 4).

We analyzed the expression of several cell cycle-regulatory genes before and at various times after adenovirus infection. Proliferating myoblasts were included for comparison. Express-

FIG. 1. Time course analysis of BrdU incorporation by C2C12 myotubes infected with adenovirus *dl520*. Two hundred MHC-positive cells per point were analyzed.

sion of E1A became appreciable approximately 15 h postinfection (p.i.) (Fig. 2 and 3A) and continued to increase until at least 48 h p.i., the last point in our time course. E1A expression was followed by downregulation of *c-fos* and *c-myc*, while *c-jun* expression remained essentially unaltered (Fig. 2).

The expression of the HLH protein Id-1 is high in proliferating cells and low in quiescent and differentiated cells (1, 3). It has been shown previously that serum stimulation of quiescent fibroblasts (1, 24) and myoblasts as well as terminally differentiated myotubes (64) induces rapid expression of Id-1. Id-1 reaches its peak after 1 h of serum stimulation, similarly to a number of immediate-early genes (24, 64). Figure 2 shows that Id-1 expression was not activated by E1A in myotubes; on the contrary, Id-1 mRNA, already present at low levels, was further downregulated. Thus, the mechanism by which E1A induces myotubes to enter the cell cycle does not involve activation of Id-1 or a number of growth factor-inducible early genes.

E1A activates expression of late- G_1 genes. In normal cells, entering into S phase requires phosphorylation of pRb. This is performed by cdk complexes, such as cyclin D1-cdk4, cyclin E-cdk2, and cyclin A-cdk2 (52, 70). We analyzed the expression of these cyclins and cdk in C2C12 myotubes at various times after *dl520* infection (Fig. 2 and 3A). Low levels of cyclin D1 mRNA and protein were found in myotubes. After E1A expression, cyclin D1 mRNA was further downregulated (Fig. 2), as previously reported for rodent and human fibroblasts (60). Cyclin D1 protein, however, already much lower in myotubes than in myoblasts, did not change significantly in myotubes after adenovirus infection (Fig. 3A). On the contrary, starting from 18 h p.i., E1A induced a striking overexpression of cyclin E as both mRNA and protein. cdk2, one of the kinases regulated by cyclin E, also accumulated significantly above the levels found in proliferating myoblasts. Both cyclin E and cdk2 levels continued to increase until at least 31 h p.i. (Fig. 3A). Induction of cyclin A expression by E1A took place 24 h p.i. (Fig. 3A), and the cyclin A content of the infected myotubes was comparable with that of myoblasts. To confirm cyclin A expression and proper nuclear localization in the infected myotubes, we performed immunofluorescence staining of cyclin A in *dl520*-infected myotubes at 27 h p.i. Figure 4 shows nuclei of *dl520*-infected myotubes stained brightly for cyclin A.

The beginning of S phase is marked by the transcriptional

FIG. 2. Time course Northern analysis. (A) Expression of E1A and various cell cycle regulators in myotubes infected with *dl520*. Each blot should be compared with the corresponding glyceraldehyde-3-phosphate dehydrogenase hybridization (B), indicated by a capital letter in parentheses. prolif., proliferating, undifferentiated myoblasts.

activation of such genes as *PCNA* and *B-myb*, whose expression starts at the G_1/S -boundary (13). In *dl520*-infected myotubes, we detected expression of both genes at 24 h p.i. at the mRNA (Fig. 2) and protein (*PCNA* [Fig. 3A]) levels. The induction of the late- G_1 genes analyzed occurred 9 to 16 h after the first appearance of E1A (Fig. 2 and 3A). The expression of these genes coincided with the synchronous entry into S phase of myotubes infected with *dl520*, between 24 and 38 h p.i., as demonstrated by time course analysis of BrdU incorporation (Fig. 1). Finally, we found expression of *cdc2*, a G_2 kinase, 31 h p.i. (Fig. 3A), consistent with our previous finding that myotubes undergo mitosis after adenovirus infection (12).

E1A-dependent overexpression of cyclin E coincides with pRb phosphorylation in the presence of high levels of p21. pRb and p21 are expressed at higher levels in terminally differentiated muscle cells than in proliferating myoblasts (41, 55) (Fig. 2 and 3). In principle, reentry of such cells into the cell cycle might involve the downregulation of the negative regulators

FIG. 3. Time course Western analysis. (A) Protein expression of E1A and various cell cycle regulators in myotubes infected with *dl520*. prolif., proliferating, undifferentiated myoblasts; N.D., not done. In the p130 panel, the upper band is nonspecific (data not shown). (B) Lighter exposure of the pRb blot in panel A.

pRb and p21 by E1A. However, E1A-activated myotubes did not show notable reductions in the mRNA and protein levels of pRb and p21 (Fig. 2 and 3). In fact, p21 and pRb protein levels increased between 24 and 31 h p.i. (Fig. 3); this occurred at a time when pRb was being phosphorylated (Fig. 3B; see also below) and the myotubes were beginning to enter S phase (Fig. 1). Expression of the cyclin-dependent kinase inhibitor p27 was not appreciably modified by differentiation or by E1A expression (Fig. 3A).

In spite of the large amounts of p21, which should inhibit cdk2, pRb became phosphorylated in myotubes infected with *dl520*. A shift in the mobility of the pRb band, diagnostic of pRb hyperphosphorylation, was first seen 24 h p.i. (Fig. 3B), slightly preceding entry into S phase (Fig. 1). To confirm that pRb phosphorylation takes place in *dl520*-reactivated myotubes, we took advantage of a recently described *in situ* technique (42). At 48 h p.i., *dl520*-infected myotubes were subjected to a

hypotonic wash, which extracts hyperphosphorylated but not hypophosphorylated pRb. Figure 4 shows that the nuclei of *dl520*-infected myotubes were not stained by an anti-pRb MAb, confirming pRb phosphorylation at the single-cell level.

Phosphorylation of pRb, in spite of the presence of p21, raises the possibility that overexpression of cyclin E, together with increased cdk2 expression, is a mechanism to overwhelm p21 or p21-like cell cycle inhibitors and promote pRb phosphorylation. To verify that cyclin E was part of functional complexes, we assessed cyclin E-specific kinase activity. Already at 18 h p.i., kinases coimmunoprecipitating with this cyclin showed activity on histone H1; at 24 h p.i., this kinase activity was as high as in proliferating myoblasts (Fig. 5). Activation of cyclin A-associated kinases followed with a delay of several hours.

p130 is one of the so-called pocket proteins bound by E1A. It also binds E2F and thus may have an important role in the control of the cell cycle (8, 28). We detected p130 protein in differentiated cells as a single band, whose migration became retarded 18 h after infection (lower band in the p130 panel of Fig. 3A). This shift suggests that p130, like pRb, becomes phosphorylated after infection.

E2F binding activity changes during E1A-induced reentry of myotubes into the cell cycle. Multiple changes have been reported in E2F function and association with cellular proteins upon muscle differentiation (10, 56, 66). Distinct E2F-containing complexes were detected in proliferating myoblasts and differentiated myotubes. Since E1A is known to disrupt the association of the E2F proteins with the pRb family members, we investigated the variations of E2F binding activity in the course of adenovirus infection of myotubes. Proteins were extracted from myoblasts, myotubes, and myotubes at various times after *dl520* infection and incubated with an oligonucleotide containing an E2F binding site (see Materials and Methods for details). In proliferating C2C12 myoblasts, we found three major complexes, indicated as A, B, and C, and a minor complex, d (Fig. 6A). These complexes were challenged with antibodies specifically recognizing the three pocket proteins. Complexes B and C could not be supershifted by any of these antibodies. Moreover, they could not be disrupted by the addition of purified E1A protein to the binding reaction, whereas purified glutathione *S*-transferase-pRb supershifted both complexes (data not shown). Thus, complexes B and C are regarded as "free" E2F. Complex A could be shifted significantly by the anti-p107 and, to a minor extent, the anti-p130 antibodies. All observations are consistent with what was reported by other groups for C2 and C2C12 myoblasts (10, 56). The d complex was supershifted by the anti-pRb antibody. Complexes B, C, and d and a significant part of complex A could be disrupted by an antibody directed to the E2F partner DP1.

In myotubes, three E2F complexes, E, B, and d, were evident (Fig. 6A). As expected, the free E2F complexes were much reduced in comparison with proliferating cells: the B band was markedly less intense, and the C complex was virtually absent. Complex E is the major complex present in myotubes and contains p130, as demonstrated by the supershift produced by the anti-p130 antibody. Complex d was completely supershifted by the anti-pRb antibody. Thus, in our experimental conditions, we can detect pRb-E2F complexes even in terminally differentiated myotubes. Infected myotubes, at 18, 24, and 31 h p.i., all showed two major bands, B and F. Band B, representing free E2F, increased severalfold compared with uninfected myotubes. Complex F had a slightly faster mobility than complex E, did not appear to contain any pocket proteins, and could not be supershifted by an anti-cdk2 antibody which supershifted complex A (data not shown). Thus, the composi-

FIG. 4. Late- G_1 events induced by E1A in *dl520*-reactivated myotubes. Shown are examples of MHC-positive myotubes (c) that have incorporated BrdU (b) 48 h p.i. Phase contrast of the same field is shown in panel a. (e) Cyclin A expression in myotubes 27 h p.i. (d and f) Phase contrast (d) and Hoechst staining (f) of the same field. (g to i) pRb phosphorylation in *dl520*-infected myotubes. A multinucleated myotube (large arrowhead) shows no pRb staining (h) after a hypotonic wash, indicating hyperphosphorylation of pRb. In the same field, a binucleated myotube (small arrowhead) is still pRb positive, indicating hypophosphorylation of pRb. In this experiment, the multiplicity of infection (about 100) was chosen so that only a fraction of the myotubes reentered the cell cycle. (g and i) Phase contrast (g) and Hoechst staining (i) of the same field. The poor appearance of the cells in panels g to i is due to the hypotonic treatment. Bar, 25 μ m.

tion of complex F, which exists only in infected myotubes, remains to be determined. Complex d, barely detectable at 18 h p.i., became slightly more evident at later times. In infected and uninfected myotubes, the anti-DP1 antibody disrupted essentially all complexes, indicating that DP1 is a major component of all of them.

Finally, since E2F is capable of transcriptional autoactivation (29), we reasoned that the E2F-1 mRNA might increase upon myotube infection and consequent freeing of existing

E2F. Northern analysis of poly(A)⁺ RNA of uninfected and infected muscle cells showed that E2F mRNA was below the detection threshold in myoblasts and differentiated cells but was strongly upregulated in infected myotubes (Fig. 6B).

Altogether, these results indicate that infection of myotubes frees E2F from the control of pocket proteins, mainly p130, and suggest that this initiates a positive autoregulatory loop.

Muscle-specific gene expression is downregulated when myotubes reenter the cell cycle. Since cell differentiation and

FIG. 5. Time course analysis of cyclin E- and A-specific kinase activities in *dl520*-infected myotubes assessed by the ability to phosphorylate histone H1. I.P., immunoprecipitation; Prolif, proliferating, undifferentiated myoblasts.

proliferation in skeletal muscle are mutually exclusive, we analyzed expression of muscle-specific genes in myotubes reentering the cell cycle. We found that expression of the muscle-specific transcription factors MyoD and myogenin was silenced soon after E1A appeared in the myotubes, i.e., within 24 h p.i. (Fig. 7). This eventually led to downregulation of the muscle structural genes, of which we analyzed the MCK and MHC genes (Fig. 7). The mRNA levels of these two genes decreased between 24 and 48 h p.i. The residual mRNA levels might be attributed to the known long half-life of most structural gene products in myotubes (44). Taken together, reentry into the cell cycle and suppression of muscle-specific gene expression strongly indicate that E1A can induce total dedifferentiation of previously terminally differentiated myotubes.

E1A induces proliferation and suppresses muscle-specific markers by independent mechanisms. To investigate which determinants in the E1A molecule are required to reactivate the cell cycle in terminally differentiated cells, we infected the myotubes with several adenovirus type 5 mutants expressing mutant forms of the E1A 12S mRNA. Their respective protein products bind differentially a number of cellular proteins with which wild-type E1A forms complexes. Table 1 shows the capacity of each mutant E1A to bind pRb, p107, p130, and p300. We analyzed the abilities of the mutants to induce DNA synthesis in myotubes and performed time course mRNA analyses

FIG. 7. Time course Northern analysis of muscle-specific gene expression in *dl520*-infected myotubes. For details, see the legend to Fig. 2.

to study their effects on the expression of critical regulatory genes.

DNA synthesis was assessed by double immunofluorescence staining of the myotubes with anti-MHC and anti-BrdU antibodies. Table 2 shows that wild-type 12S E1A could efficiently reactivate DNA synthesis in myotubes. The RG2 mutant, capable of binding the pRb family members but not p300, was also able to activate DNA synthesis, albeit with a somewhat reduced efficiency. The other viruses, 12S.928, 12S.YH47/928, and 12S.RG2/928, which are unable to bind some or all of the pocket proteins (Table 1), were incapable of inducing DNA synthesis in myotubes. Similar results were obtained when other terminally differentiated cells, adipocytes, were infected with the same set of mutants (Table 2). To ascertain whether our findings pertain only to terminally differentiated cells, as opposed to reversibly quiescent ones, we infected serum-starved, resting 10T1/2 mouse fibroblasts. Table 2 shows that the results of these experiments were essentially superimposable with those obtained with C2C12 myotubes. Similar results were also obtained by infecting resting NIH 3T3 cells (not shown).

To study whether E1A induces proliferation and suppresses

FIG. 6. E2F complexes and expression in *dl520*-infected myotubes. (A) Electromobility shift assay of extracts from proliferating myoblasts (prolif.), uninfected myotubes, and myotubes at various times p.i. Ad E2 wt and Ad E2 mut, wild-type and mutant adenovirus E2. (B) Northern blot analysis of E2F-1 expression. Poly(A)⁺ mRNA samples (1.5 µg per lane) were probed with a human E2F-1 probe at low stringency. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

TABLE 1. Binding specificities of adenovirus E1A mutants

Mutant	Mutation(s) in E1A 12S	Binding			
		p300	pRb	p107	p130
<i>dl520</i>		+	+	+	+
12S.RG2	Arg-2→Gly	—	+	+	+
12S.928	Arg-124→Gly	+	—	+	+/-
12S.YH47/928	Tyr-47→His, Arg-124→Gly	+	—	—	—
12S.RG2/928	Arg-2→Gly, Arg-124→Gly	—	—	—	—

sion of muscle-specific genes by the same mechanism, we performed time course mRNA analyses of myotubes infected with the mutant adenoviruses. We studied the expression of cyclin E as a marker of cell cycle progression, whereas suppression of the mRNA for the key muscle differentiation factor, myogenin, was assessed to determine the effects of the mutants on differentiation (Fig. 8). We found that 12S.RG2, the only mutant capable of inducing DNA synthesis, activated as high levels of cyclin E expression as *dl520*, although at a slightly later time. This mutant also showed delayed downregulation of myogenin compared with *dl520*. The other mutants, unable to induce DNA synthesis in myotubes, showed no *dl520*-like activation of cyclin E expression, although 12S.928 induced this mRNA in a weak and transient fashion. On the other hand, all of the mutants possessing binding activities reduced myogenin expression more than 65-fold, though more slowly than *dl520*. The 12S.RG2/928 mutant, unable to bind p300 or pRb family proteins, reduced myogenin mRNA levels less than sevenfold, possibly because of some residual E1A activity on myogenin expression or to other adenovirus gene products. To distinguish between these two possibilities, C2C12 myotubes were infected with the *dl312* mutant, which expresses no E1A (32). *dl312* infection reduced myogenin expression less than threefold (Fig. 8). Thus, the vast majority of the myogenin-suppressing activity can be ascribed to E1A.

In summary, E1A mutants capable of binding either p300 or some pRb family members are all able to downregulate muscle regulatory genes. In contrast, the capacity to reactivate the cell cycle segregates with the ability of E1A to bind pocket proteins, while binding p300 is not required. Thus, E1A induces loss of muscle-specific gene expression independently of its capacity to induce reentry into the cell cycle.

DISCUSSION

In this study, we investigated how E1A, unlike a variety of retroviral oncogenes, is capable of reactivating the cell cycle in terminally differentiated cells and whether it affects their differentiation program. We show that the mechanism by which E1A reactivates proliferation of terminally differentiated muscle cells includes overexpression of cyclin E and cdk2. In ad-

dition, we present evidence that E1A, at the same time as it forces myotubes to reenter the cell cycle, independently suppresses the muscle differentiation program. The reversal of growth arrest and suppression of muscle-specific gene expression leads to the conclusion that E1A induces full dedifferentiation.

E1A downregulates early- G_1 genes in terminally differentiated myotubes. It is not known how E1A impinges on the cell cycle in terminally differentiated cells. In particular, we examined which cell cycle-regulatory genes are activated by E1A in the course of forcing terminally differentiated cells to return to proliferation. The present data strongly suggest that E1A bypasses the early G_1 phase: the immediate-early genes *c-myc* and *c-fos*, the Id-1 gene, and the delayed-early cyclin D1 gene were downregulated soon after E1A expression. This effect is due not to an inability of myotubes to activate early- G_1 genes, since they can do it in response to serum stimulation (64), but rather to the mechanisms through which E1A activates the cell cycle. Our results are in agreement with previous observations indicating that E1A elicits proliferation of quiescent fibroblasts without activating a number of early- G_1 genes (23, 60). This finding suggests that both quiescent and terminally differentiated cells respond similarly to E1A and that E1A bypasses and/or represses the differentiation-specific mechanisms that render postmitotic myotubes unresponsive to numerous other proliferative signals.

Id-1 expression is low in terminally differentiated muscle cells, and we found it to be further downregulated in *dl520*-infected myotubes. When quiescent cells or terminally differentiated myotubes are stimulated with growth factors, Id-1 expression is swiftly induced. Although Id-1 has been shown to be necessary for progression through G_1 (1), our results demonstrate that E1A can force myotubes to reenter the cell cycle in the virtual absence of Id-1 mRNA and certainly without inducing its upregulation.

The roles of the immediate-early genes in cell cycle progression are not clearly defined. However, cyclin D1, in association with cdk4 or cdk6, is believed to contribute importantly to pRb phosphorylation (70). While cyclin D1 is required for passing the G_1 restriction point and entering S phase, it is dispensable in cells lacking functional pRb (38, 39). Our findings with terminally differentiated cells and those with quiescent fibroblasts (60) are in accordance with these results: when pRb is inactivated by E1A, cyclin D1 is not necessary to enter S phase.

E1A-induced S phase is preceded by overexpression of cyclin E and cdk2 in terminally differentiated muscle cells. In contrast to activation of the early- G_1 genes, activation of late- G_1 genes occurred in myotubes infected with *dl520*. This appeared to take place in a coordinated temporal sequence, suggesting that E1A triggers a cascade of events and does not induce simultaneous, independent transcription of a number of genes involved in the G_1/S transition. Moreover, we found that pRb became phosphorylated shortly before entry into S phase. Surprisingly, phosphorylation took place in the presence of high levels of p21, which should inhibit cdk activities. However, in myotubes infected with *dl520*, the expression levels of cyclin E and, to a lesser extent, cdk2 were also high in coincidence with pRb phosphorylation. We also showed that in the presence of high levels of p21, cyclin E-specific kinase activity was present in infected myotubes. Remarkably, despite the fact that *dl520*-infected myotubes expressed higher amounts of cyclin E and cdk2 than myoblasts, their cyclin E-associated kinase activity was not increased, suggesting that major proportions of these proteins are not engaged in active kinase complexes; instead, excesses of cyclin E and cdk2 might be required to titrate p21 or other kinase inhibitors. It has been shown that cyclin E is

TABLE 2. Induction of DNA synthesis by adenovirus mutants^a

Mutant	% BrdU-positive cells		
	C2C12 myotubes	Adipocytes	10T1/2 fibroblasts
<i>dl520</i>	91	46	63
12S.RG2	68	49	39
12S.928	2	1	4
12S.YH47/928	1	1	4
12S.RG2/928	1	<1	2.5
Mock	<1	<1	3.5

^a Multiplicities of infection used: C2C12 and 10T1/2, 400; adipocytes, 2,000.

FIG. 8. Effects of mutant adenovirus infections of myotubes on cyclin E and myogenin expression. (A) Time course Northern analysis of cyclin E and myogenin expression in myotubes infected with adenoviruses expressing wild-type (dl520) or mutant 12S E1A. See Table 1 for descriptions of the mutants and Table 2 for their abilities to induce DNA synthesis in various cell types. dl312 expresses no E1A as a consequence of a large deletion in the E1A region. prolif., proliferating, undifferentiated myoblasts; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (B) Densitometric evaluation of the influence of the E1A mutants on myogenin mRNA. For each virus, histograms compare the expression of myogenin at 38 h p.i. with that of uninfected myotubes (0 h in panel A) and express the differences as fold reduction. All densities are normalized to the value for glyceraldehyde-3-phosphate dehydrogenase.

necessary for entry into S phase and, in contrast to cyclin D1, is required for G₁/S transition even in cells lacking pRb function (47). Our results suggest that one of the strategies that E1A uses to overcome the antiproliferative activity of pRb and p21 is overexpression of cyclin E and cdk2. However, this need not be the only mechanism used by E1A. For example, it has been recently demonstrated that E1A can bind and inactivate p27 (40). This interaction or a similar one, with p21 or another cdk inhibitor, could be responsible, at least in part, for the reactivation of the cell cycle.

We also observed that p130 became phosphorylated rapidly following E1A expression in myotubes, approximately in coincidence with the first appearance of cyclin E and cdk2 as well as cyclin E-specific kinase activity. Moreover, it has been shown that p130 can interact with cdk2 and cyclin E complexes and that it is a substrate for their kinase activity (37). In terminally differentiated cells, pRb and p130 can be found in complexes with E2F (9, 56). The major pool of E2F, however, is bound by p130, which thus may have an important role in the control of the cell cycle (9, 56). E1A can directly bind the pRb family members and release E2F, which then contributes to the activation of G₁/S boundary genes (70). However, we demonstrate here that in infected myotubes, pRb and p130 not only were bound by E1A (not shown) but also became phosphorylated. Thus, both a direct and an indirect mechanism to inactivate pocket proteins appear to act in adenovirus-infected myotubes. It will be interesting to determine whether both are needed to ensure restart of the cell cycle in myotubes, or whether pRb phosphorylation is merely a dispensable by-product of the general reactivation of the cell cycle machinery.

Effects of adenovirus infection on E2F. Analysis of E2F binding abilities and complex formation in myoblasts and uninfected myotubes gave results essentially in agreement with the literature. However, at variance with other reports (56), we were able to detect E2F complexes containing pRb in terminally differentiated myotubes. This could be explained by differences among the cell lines used in the diverse studies or to our sensitive assay conditions, which allowed us to detect minor E2F-containing complexes even if represented in small amounts.

Adenovirus infection and E1A expression induced conspicuous changes. E2F, largely bound to p130 in differentiated cells, became mostly free at 18 h p.i. At this time, a pRb-containing complex could not be detected, although pRb was still hypophosphorylated (Fig. 3B). At later time points, traces of supershiftable, pRb-containing complexes could be detected, even though most pRb molecules were hyperphosphorylated. This finding can be explained by the striking increase in the amount of pRb protein that follows infection (Fig. 3B). A minor fraction of the pRb molecules might escape phosphorylation and be able to form complexes with E2F. It remains to be determined whether E2F plays a role in the suppression of muscle-specific gene expression, since it has been shown that it can inhibit differentiation when expressed in myoblasts (66).

E1A-induced S phase in myotubes is independent of p300 binding. By infecting myotubes with a set of adenoviruses expressing mutated forms of E1A, we found that induction of DNA synthesis cosegregated with E1A's ability to bind pRb family members, whereas binding p300 was not required. The inability of the YH47/928 mutant, which binds only p300, to induce DNA synthesis in terminally differentiated muscle cells and adipocytes as well as in quiescent fibroblasts contrasts with the results obtained with quiescent baby rat kidney cells. In these cells, the E1A regions responsible for p300 binding are sufficient alone to induce DNA synthesis (75). This apparent discrepancy is possibly explained by different controls of cell growth in baby rat kidney cells, as already evidenced by others (59). In myotubes, the mutant YH47/928 cannot activate expression of the cyclin E gene (Fig. 8) or of the other G₁/S boundary genes (data not shown), which explains mechanistic

cally why S phase is not induced. In agreement with these results, the only viruses capable of eliciting pRb phosphorylation are *dl520* and RG2 (data not shown). Our results indicate that p300 does not play a major role in the maintenance of the postmitotic state in terminally differentiated cells. Rather, p300 might contribute to the induction of growth arrest in the initial phases of terminal differentiation by upregulating p21, as suggested by Missero et al. (41).

E1A suppresses muscle-specific gene expression in myotubes. Although E1A interferes with differentiation of various cell types (33, 41, 57, 68, 69), including myoblasts (5, 6, 34, 67), it is not known whether it can exert similar effects when expressed in cells that have already undergone terminal differentiation. In this study, we expressed E1A in postmitotic myotubes and assessed directly its effects on the maintenance of the differentiated state. We found that expression of the muscle-specific transcription factors MyoD and myogenin was silenced immediately after the beginning of E1A expression in C2C12 myotubes. This was followed by downregulation of the muscle structural MCK and MHC genes, presumably as a consequence of the lack of necessary myogenic transcription factors. The downregulation of muscle structural gene mRNAs is in agreement with the morphological features of *dl520*-infected myotubes: flattening, loss of refringency, disorganization of contractile structures, and loss of MHC expression, as revealed by immunofluorescence (12).

Interference of E1A with differentiation is independent of reactivation of the cell cycle. Having determined that E1A induces loss of muscle-specific gene expression, an important question was whether this occurs as a consequence of reentry into the cell cycle. In this view, the unknown mechanisms responsible for the mutual exclusion of proliferation and differentiation in skeletal muscle cells would be activated by E1A-induced proliferation, which would then lead to downregulation of muscle-specific genes. Alternatively, E1A might act directly to suppress the muscle differentiation program.

Our finding that downregulation of MyoD and myogenin began as soon as E1A appeared, and well before entry into S phase, supports the latter possibility. Accordingly, we found that E1A mutants that were unable to reactivate the cell cycle were capable of downregulating muscle-specific genes. These results clearly show that the interference with the differentiation program is independent of the ability to reactivate the cell cycle.

E1A mutants able to bind either p300 or pRb family members were both capable of suppressing the expression of myogenin (Fig. 8) and MyoD (not shown) in terminally differentiated muscle cells. This activity can be attributed primarily to E1A, since the E1A-deleted *dl312* mutant only showed a slight tendency to repress muscle-specific gene expression (Fig. 8). This was presumably due to nonspecific effects of adenovirus infection or to uncharacterized, weak suppressive activities of other adenovirus gene products. In proliferating myoblasts, E1A can interfere with the MyoD gene family in at least two ways: inhibition of transcription of its genes and suppression of the *trans*-acting capacities of its gene products (67). Transcriptional inhibition correlates with the ability of E1A to bind p300 (6), while interference with *trans*-acting properties apparently requires the oncogene to bind pocket proteins (6, 18). Since the muscle-regulatory factors are capable of auto- and cross-activation, interference with their *trans*-acting capacity can lead to their downregulation (50). Thus, our finding that mutants binding either set of cellular proteins suppressed muscle-regulatory factor expression suggests that the molecular mechanisms leading to loss of differentiation characteristics in adenovirus-infected myotubes are similar to those inhibiting

differentiation in myoblasts. E1A has been shown to bind bHLH proteins through its amino terminus, and this could constitute a third mechanism by which it inhibits muscle differentiation (62). To our knowledge, it is not known whether the RG2 mutation interferes with this binding. Therefore, with the mutants used in this work, we cannot determine whether E1A-bHLH binding plays a role in reversing muscle differentiation. The fact that the 12S.RG2/928 mutant, unable to bind p300 and pRb family proteins, still retains some ability to downregulate myogenin (Fig. 8; compare 12S.RG2/928 with *dl312*) suggests that E1A-bHLH interactions might be involved. It is, of course, also possible that other, unknown activities of E1A determine the residual suppression of muscle-specific genes.

Terminal differentiation is defined by permanent withdrawal from the cell cycle and simultaneous expression of tissue-specific genes. We would like to stress that by suppressing both of these essential characteristics in skeletal myotubes, E1A causes full dedifferentiation.

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