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Ectopic Expression of Cyclin D1 Prevents Activation of Gene Transcription by Myogenic Basic Helix-Loop-Helix Regulators

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Activation of muscle gene transcription in differentiating skeletal myoblasts requires their withdrawal from the cell cycle. The effects of ectopic cyclin expression on activation of muscle gene transcription by myogenic basic helix-loop-helix (bHLH) regulators were investigated. Ectopic expression of cyclin D1, but not cyclins A, B1, B2, C, D3, and E, inhibited transcriptional activation of muscle gene reporter constructs by myogenic bHLH regulators in a dose-dependent manner. Ectopic expression of cyclin D1 inhibited the activity of a myogenic bHLH regulator mutant lacking the basic region protein kinase C site, indicating that phosphorylation of this site is not relevant to the mechanism of inhibition. Analysis of cyclin D1 mutants revealed that the C-terminal acidic region was required for inhibition of myogenic bHLH regulator activity, whereas an intact N-terminal pRb binding motif was not essential. Together, these results implicate expression of cyclin D1 as a central determinant of a putatively novel mechanism that links positive control of cell cycle progression to negative regulation of genes expressed in differentiated myocytes.

Cyclin expression is attuned to the phases of the cell cycle, and different cyclin types control either progression through the cycle or specific transitions from one phase to another. The mammalian G_1 cyclins (D1, D2, D3, C, and E) are expressed most prominently during the G_1 phase of the cell cycle, and control progression through G_1 and entry into S phase (48, 60). Cyclins A and B are degraded late in M and do not accumulate to functional levels until early S phase. Cyclin A accumulation begins slightly earlier than that of cyclin B, reaching peak levels during S/G_2 (54). Cyclin A expression is essential for initiation of DNA synthesis, and for restricting the initiation of mitosis until the completion of S phase (63). Cyclin B accumulation reaches peak levels during G_2/M (48, 54) and is required for both entry into and maintenance of mitosis (49). Cyclins are also distinguished by the manner in which their expression is regulated. Expression of most cyclins (cyclins A, B1, B2, C, and E) is linked to intrinsic determinants of cell cycle progression, which are governed by parameters such as availability of nutrients, cell size, or completion of DNA replication (22, 24, 63). Expression of the D-type cyclins is regulated by extrinsic determinants, such as growth factor signals (26, 40, 42). The D-type cyclins are thought to integrate the extrinsic signals that act during G_1 to instruct cells to proliferate or to exit the cell cycle and differentiate with the autonomous events that drive the cell cycle from S phase through mitosis.

Cyclins are the regulatory subunits of the cyclin-dependent kinases (cdks). At least five isoforms of cdk (cdks 1 through 5) are present in mammalian cells, and their activity is regulated by cyclins in a type-specific manner. The D-type cyclins preferentially activate cdk4 (40), although activation of cdk2 and cdk5 has been observed (73, 74). Cyclin D1 controls the rate of cell progression through G_1 in some cells and is required for entry into S phase (3, 55), although as yet these functions have not been shown to be directly dependent on the assembly of an active complex with cdk4. The cyclin D1 cDNA was selected independently in three different screening procedures: (i) murine cyclin D1 (CYL1) was identified in a screen for delayed early response genes stimulated by colony-stimulating factor 1 (41, 42); (ii) human cyclin D1 was identified as the product of *PRAD1*, a gene rearranged in parathyroid tumors (45); and (iii) human cyclin D1 was also identified as the product of *CCND1*, a gene that could rescue a *Saccharomyces cerevisiae* mutant lacking functional *CLN* genes (35, 72). Each screening procedure demonstrated an important characteristic of cyclin D1: the first revealed that cyclin D1 expression is positively regulated by growth factors, the second indicated that cyclin D1 is involved in the control of cell proliferation and, when expressed inappropriately, can act as an oncogene, and the third demonstrated that cyclin D1 regulates cell cycle progression.

The molecular basis of skeletal muscle gene transcription has been studied in vivo, and a common element among most muscle gene enhancers is a binding site(s) for the myogenic basic helix-loop-helix (bHLH) regulators (66). Also referred to as the myoD family, four myogenic bHLH genes have been identified (the myoD, myogenin, myf-5, and MRF4/herculin/myf-6 genes [7, 8, 15, 43, 57, 71]). Members of this gene family share the functional property of effecting transdetermination to the myogenic phenotype upon forced expression in other cell types. Myogenic bHLH regulators activate muscle gene transcription in vivo as heterodimers with the E2A proteins (25, 33). Heterodimers can activate transcription when two or more proximal binding sites are present in the upstream region of the gene (65, 67, 69); however, in genes in which only a single binding site is present, transcription is activated when adjacent sites binding other transcription factors are also occupied (10, 59).

Myogenic bHLH regulators activate muscle gene transcription when expressed in cells cultured in low-mitogen medium, but their activity is suppressed by components of serum and embryo extracts, particularly transforming growth factor β (TGF-β) (39, 51) or basic fibroblast growth factor (bFGF) (11, 34, 62). Muscle genes in myogenic cell lines generated by permanent transfection with a myogenic bHLH regulator under the control of a constitutive promoter are regulated by growth factors equivalently to muscle genes in native myoblast cell lines (50). This observation indicates that posttranslational

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mechanisms control the activity of myogenic bHLH regulators. Some mechanisms of posttranslation inhibition alter the intrinsic DNA-binding characteristics of the bHLH dimers. Nuclear extracts from myoblasts contain myogenic bHLH regulators, but depending on the myoblast cell line and the culture conditions, these regulators display varying DNA-binding activities. Gel mobility shift assays of nuclear extracts from MM14 myoblasts, which are dependent on bFGF for growth, or C_2C_{12} myoblasts grown in the presence of bFGF, reveal low-to-undetectable levels of myogenic bHLH protein DNA-binding activity (10, 46). Recent studies have shown that one mechanism by which bFGF inhibits muscle gene transcription is through the activation of protein kinase C (PKC), which phosphorylates a conserved site in the basic regions of the myogenic bHLH proteins and thereby prevents binding to DNA (37). The DNA-binding activity of myogenic bHLH proteins can also be abrogated by members of the Id gene family, which are induced by serum (4). Id proteins consist of an HLH domain without the basic region required for contact with DNA; hence, heterodimers of Id with other bHLH proteins do not bind DNA. Expression of Id can negatively regulate myogenic bHLH protein activity either through the assembly of inactive heterodimers or by sequestering protein products of the E2A gene family, such as E47 (28). Recent studies have shown that covalently tethered MyoD-E47 dimers are resistant to negative regulation by serum factors, demonstrating the importance of Id in mediating suppression of muscle gene transcription (47).

TGF-β inhibits the activity of myogenic bHLH regulators via a mechanism that does not affect the ability of these regulators to bind DNA (9). In general, mechanisms of regulating myogenic bHLH protein activity that are not associated with changes in their intrinsic DNA-binding properties are not well understood and require further exploration. Indirect evidence has suggested that cyclin-cdk activities participate in the inhibition of myogenic bHLH regulators. Induction of muscle gene expression occurs in myoblasts that are growth arrested early in G_1; depletion of growth factors at other phases of the cell cycle (when intrinsic factors regulate cyclin expression) does not result in the induction of muscle gene transcription (reviewed in reference 50). Myoblasts stably transfected with a construct containing thermolabile simian virus 40 T antigen under the control of an inducible promoter were allowed to differentiate into myotubes and then induced to express T antigen at the permissive temperature (21). Induction of T-antigen expression resulted in both loss of muscle gene expression and induction of DNA synthesis in the myotube nuclei. Also observed was a rapid induction of cdk activity. These results were not obtained when a mutant simian virus 40 T antigen that does not bind the retinoblastoma protein (pRb [18, 38, 64]) was employed, suggesting that unphosphorylated, active pRb (or a functional homolog) is required for both growth suppression and activation of muscle gene transcription in differentiating myogenic cells. Elimination of cdk-mediated phosphorylation of at least one epitope on histone H1 is directly associated with terminal differentiation of skeletal myoblasts (13). Using a monoclonal antibody, 12D11, that binds an epitope on histone H1 only when it is cdk phosphorylated (reference 13 and unpublished observations), studies from this laboratory have shown by direct immunochemical methods that growth-arrested, undifferentiated mononuclear cells contain cdk-phosphorylated histone H1, whereas terminal differentiated myotubes do not. Since reversible growth arrest is associated with an attenuation of cdk activity (12, 70), these results also suggest that the action of phosphatases on cdk (and other) substrates may be necessary for transition to a terminally differentiated state. Indeed, inhibition of protein phosphatases 1 and 2A has been shown to prevent differentiation of myoblasts (31).

In this study, we first observed that expression of most cyclins, including cyclin D1, is reduced to weakly detectable or lower-than-detectable levels in differentiated myotubes. An exception was cyclin D3, which was induced by low-mitogen medium and expressed at significantly higher levels in differentiated myotubes. We then determined that ectopic expression of cyclin D1 prevents activation of muscle reporter gene constructs by MyoD. In conjunction with other studies that have shown that cyclin D1 controls the transition from G_1 to S, our results identify cyclin D1 expression as a functional link between the positive control of cell cycle progression and the transcriptional repression of genes whose expression is restricted to cells that have exited the cell cycle. In addition, we found that ectopic expression of cyclin D3 or other cyclins did not prevent muscle gene activation. This observation, as well as the opposing patterns of expression for cyclin D1 and D3 observed in myogenic cells, suggests that individual D-type cyclins may play divergent roles in the regulation of cell cycle progression and gene expression in different cellular contexts.

MATERIALS AND METHODS

Cell culture. C_2C_{12} myoblasts were acquired from the American Type Culture Collection. High-mitogen medium consisted of Dulbecco's modified Eagle medium (DMEM) supplemented with 15% fetal bovine serum and 0.5% chicken embryo extract. Low-mitogen medium consisted of DMEM supplemented with 2 to 4% horse serum. C3H10T1/2 (also referred to as 10T1/2) and C3H10T1/2-MyoD cells were acquired from H. Weintraub (Fred Hutchinson Cancer Research Center) and were cultured in DMEM supplemented with 10% fetal bovine serum or 15% fetal bovine serum and 0.5% chicken embryo extracts, respectively.

Plasmids. pEMSV-MyoD (15) and p4R(MCK)-TK-CAT (67) were given by H. Weintraub (Fred Hutchinson Cancer Research Center) and A. Lassar (Harvard Medical School); pEMSV-myogenin and pEMSV-myogenin T-N_{87} (37) were given by Eric Olson (M. D. Anderson Cancer Research Center); p1256MCK-CAT (27) was given by J. Buskin and S. Hauschka (University of Washington); pHCA177-CAT (59) was given by V. Sartorelli and L. Kedes (University of Southern California); expression constructs for cyclins A, B1, B2, C, D1, D3, and E in Rc-CMV (Invitrogen [23]) were obtained from P. Hinds and R. Weinberg (Whitehead Institute) and A. Arnold (Harvard Medical School); and pRSV-CAT (19, 20) was obtained from E. Johnson (Mount Sinai Medical School). CMV-cyclin D1-6 was generated by digesting CMV-cyclin D1 with *Not*I and *Apa*I, deleting from the 3′ end to base 745 of the cDNA with exonuclease III and mung bean nuclease, and ligating. pEMSV-cyclin D1 was constructed by blunt-end ligation of the *Hin*dIII-*Xba*I cyclin D1 fragment from Rc-CMV-cyclin D1 into the *Eco*RI site of pEMSVscribeα2 (15). pEMSV-CAT was constructed by ligating the *Sma*I fragment of p1256MCK-CAT (containing the chloramphenicol acetyltransferase [CAT] gene) into the *Eco*RI site (filled in with Klenow fragment) of pEMSVscribeα2. The structures and orientations of all new constructs were verified by DNA sequence analysis.

Northern blots. Total cellular RNA was extracted in guanidinium isothiocyanate and isolated by cesium chloride density centrifugation, and mRNA was purified by oligo-dT cellulose affinity chromatography according to standard methods (58). Formaldehyde-agarose gel electrophoresis, capillary transfer

to GeneScreen Plus (New England Nuclear), and Northern (RNA) blots were performed according to standard methods. Probes were generated from cyclin cDNAs, which were excised from the Rc-CMV expression vector by digestion with *Hin*dIII and *Xba*I. Cyclin and other cDNAs were radiolabelled by the random hexamer priming method with a kit sold by New England Biolabs.

Immunoprecipitations. A rabbit polyclonal cyclin D1 antibody was purchased from PharMingen. Transfected cells were radiolabelled with [^{35}S]methionine for 4 h, washed with phosphate-buffered saline (at ambient temperature), and scraped into IP buffer (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.6], 150 mM NaCl, 40 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 0.5% Triton X-100, 1 mM dithiothreitol, 0.5% bovine serum albumin, and protease inhibitors [20 μg of leupeptin ml^{-1}, 10 μg of aprotinin ml^{-1}, 1 mM benzamidine, 10 μg of bacitracin ml^{-1}] at 4°C). The scraped cells were vortexed (at top speed in 5-s bursts five times) and then pelleted in a microfuge (top speed) for 10 min. The supernatant was recovered and incubated for 18 h (4°C) with 2.5 μg of cyclin D1 antibody. Protein A coupled to agarose (Sigma) was added, and the suspension was incubated with shaking for 1 h (4°C). The suspension was centrifuged briefly in a microcentrifuge, and the pellet was washed four times with IP buffer lacking protease inhibitors. The bound proteins were released by incubating the agarose beads in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (100 mM Tris [pH 6.5], 2% SDS, 10% glycerol, 0.025% phenol red, 5% β-mercaptoethanol) for 5 min (100°C). Samples were resolved by SDS-PAGE, and bands were detected by autoradiography.

Transient expression assays. For each experiment, two T25 flasks of cells at 80% confluence (approximately 10^6 cells each) were used. A standard calcium phosphate precipitation method (58) was used to perform the transfections, and assays for CAT activity were performed with equivalent amounts of protein as described elsewhere (20). In each experiment, the mass of plasmid transfected into each culture was kept constant by adding the appropriate parent vectors (Rc-CMV for the CMV-cyclin constructs and pEMSVscribeα2 for myoD and myogenin constructs). For a typical experiment (specific quantities of plasmids used are detailed in the figure legends), one flask of 10T1/2 cells was transfected with 5 μg of p1256MKC-CAT, 5 μg of p4R(MCK)TK-CAT, 1.8 μg of pRSV-CAT, or 1.8 μg of pSV2-CAT plus 800 ng of pEMSV-MyoD and 750 ng of CMV-cyclin D1. Every experiment was performed in duplicate; hence, these quantities were doubled and used to generate 500 μl of precipitate. A 250-μl volume was then added to each T25 flask of cells. This dose was considered 750 ng per 10^6 cells or 1.5 μg per 2×10^6 cells. The cells were cultured with the precipitate for 18 h in DMEM supplemented with 10% fetal bovine serum and then in low-mitogen medium (DMEM supplemented with 2% horse serum) for 48 h prior to the assays for CAT activity. All procedures were performed in duplicate, and experiments were performed at least twice. The conversion rate of substrate to product in all experiments was 30% or below. Assays were quantified from thin-layer chromatographs by phosphorimage analysis. Quantifications were performed within the linear range of the analyzer.

RESULTS

Differentiation of skeletal myoblasts is associated with a loss of cyclin A, B2, C, and D1 expression, an increase in cyclin D3 expression, and a decrease in cdk4 expression. Differences in cyclin and cdk4 mRNA levels in asynchronous proliferating and differentiated C_2C_{12} skeletal myoblasts (6) were determined by Northern blot analysis using probes for cyclins A, B1, B2, C, D1, D3, and E, troponin I (a marker of myogenic differentiation [32]), cdk4, and glyceraldehyde phosphate dehydrogenase (a standard [52]). Differentiation was associated with significant reductions in the expression of cyclin A (which peaks during S and G_2 phases of the cell cycle [44, 53, 54]), cyclin B2 (which peaks during G_2 and M phases [44, 54]), cyclin C (which peaks slightly during early G_1 [35]), and cyclin D1 (Fig. 1). Reductions in the levels of A, B2, and C cyclin mRNAs are consistent with the progressive withdrawal of an asynchronous population of myoblasts from the cell cycle during differentiation. The expression of cyclin D1 has been shown in some cell types to be positively regulated by growth factors (reviewed in reference 60); hence, the reduction in cyclin D1 mRNA is consistent with the change from high- to low-mitogen medium used to induce differentiation of the myoblasts. Consistent with these results, we have observed that expression of cyclin D1 in myoblasts is stimulated by bFGF or TGF-β, albeit with different kinetics (56).

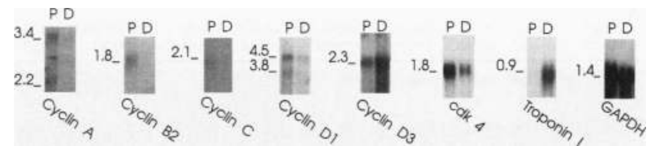

FIG. 1. Modulation of cyclin mRNAs in proliferating and differentiated cultures of myogenic cells. C_2C_{12} myoblasts were cultured in high-mitogen medium at low density (proliferating, P lanes) or in low-mitogen medium at high density for 48 h (differentiated, D lanes). poly(A)+ mRNA (8 μg) from each culture was analyzed by Northern blot with the following cDNA probes: cyclin A (54), cyclin B1 (53), cyclin B2 (23), cyclin C (35), cyclin D1 (45), cyclin D3 (35), cyclin E (35), cdk4 (40), troponin I (fast isoform, used as a differentiation marker [32]) and glyceraldehyde phosphate dehydrogenase (GAPDH; used as a loading control [52]). Signals for cyclin B1 and cyclin E were not detected in the asynchronous cells, although this does not rule out cell cycle phase-specific expression in these cells. Sizes of mRNAs (shown in kilobases) agree with previously reported values, except that cyclin A displayed an additional 3.4-kb form not observed in HeLa cells (54). Exposure times were as follows: GAPDH, 4 h; cyclin A, cyclin D1, cyclin D3, cdk4, and troponin I, 18 h; and cyclin B2 and cyclin C, 72 h.

Surprisingly, accumulation of cyclin D3 increased significantly in the differentiated cultures (Fig. 1). Cyclin D3 mRNA was not observed in 10T1/2 fibroblasts (data not shown) cultured in high- or low-mitogen medium. These observations indicate that induction of cyclin D3 expression in low-mitogen medium is a characteristic of the myogenic phenotype and that cyclins D1 and D3 probably perform distinct functions in myogenic cells. Expression of cdk4 was reduced in differentiated cultures; however, the short exposure time of the autoradiograph suggested that the level of cdk4 mRNA detected in differentiated cultures remained quite significant. Although cyclin B2 was detected, cyclin B1 expression was not detected by Northern blot in C_2C_{12} myoblasts. Western blot (immunoblot) analysis of cyclin B1 expression in proliferating myoblasts also revealed no detectable bands, under conditions that detected a strong signal for cyclin B1 in 10T1/2 cell lysates (data not shown). These results suggest that expression of B-type cyclins may be regulated in a cell type-specific manner.

Ectopic expression of cyclin D1 inhibits activation of muscle gene transcription by myogenic bHLH regulators. Muscle gene transcription can be activated in 10T1/2 fibroblasts by transfecting a myogenic bHLH regulator, such as myoD, in an

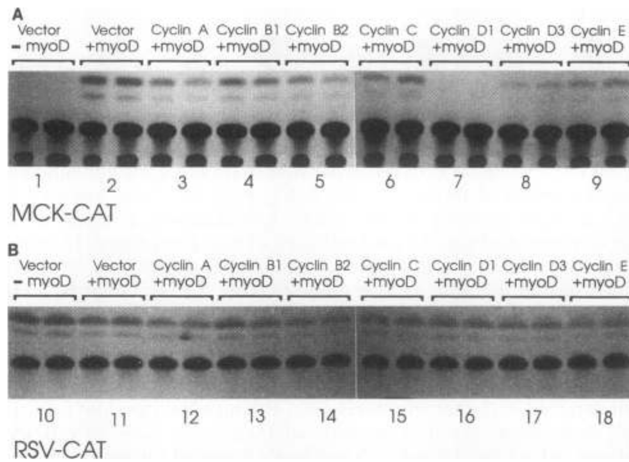

FIG. 2. Ectopic expression of cyclin D1 suppresses activation of muscle gene transcription by MyoD. Results from two independent experiments performed in parallel are shown. 10T1/2 or C_2C_{12} cells were transfected with the indicated plasmid constructs, cultured in growth medium for 24 h, and then cultured in low-mitogen medium for 48 h. In the experiments shown, the cells were cotransfected either with 5 μg of muscle-specific p1256MCK-CAT (derived from the MCK gene [27], indicated as MCK-CAT, sets 1 to 9) (A) or with the CAT gene under control of the RSV long terminal repeat (1.8 μg per flask; indicated as RSV-CAT, sets 10 to 18 [19]) (B). Total transfected plasmid in each transfection was held constant by the addition of the appropriate vector (lacking insert). Sets 1 and 10: 10T1/2 fibroblasts transfected with Rc-CMV expression vector (no insert) and pEMSVscribeα2 (no insert). Sets 2 and 11: 10T1/2 fibroblasts transfected with Rc-CMV expression vector (no insert) and pEMSV-MyoD (800 ng per flask [15]). Sets 3 to 9 and 12 to 18: 10T1/2 fibroblasts transfected with Rc-CMV expression constructs for cyclins A, B1, B2, C, D1, D3, and E (as indicated; 750 ng per flask [23]) and pEMSV-MyoD. Cotransfection of cyclin A, B1, B2, C, D3, and E cDNA expression constructs variably inhibited transcriptional activation of the MCK enhancer/promoter by MyoD, but in this and other experiments inhibition was only partial and not specific when scaled against inhibition of RSV-CAT. In contrast, cotransfection of the CMV-cyclin D1 construct reduced transcription of p1256MCK-CAT to basal level. The slight reduction in CAT activity in the CMV-cyclin D3 transfectants apparent here has not been observed in other experiments.

expression construct and culturing the cells in low-mitogen medium (33, 67). To determine the effects of ectopic cyclin expression from a constitutive promoter on myogenic bHLH regulator activity, 10T1/2 fibroblasts were cotransfected with the following plasmid constructs: a muscle-specific reporter construct consisting of the muscle creatine kinase (MCK) enhancer/promoter driving the CAT gene (p1256MCK-CAT [27]), murine myoD cDNA under control of the Moloney sarcoma virus long terminal repeat (pEMSV-MyoD [15]), and cDNAs for cyclin A, B1, B2, C, D1, D3, or E in an expression plasmid (Rc-CMV; Invitrogen) that utilizes the CMV promoter (23). The expression of this array of CMV-cyclin constructs has been characterized elsewhere (23). Independent experiments using a pEMSV-CAT reporter construct indicated that expression of genes from the pEMSVscribeα2 vector is not significantly affected by cotransfection of CMV-cyclin D1 in the quantities used here.

Baseline activity of the p1256MCK-CAT reporter was determined by transfection of 10T1/2 cells with p1256MCK-CAT, pEMSVscribeα2 (vector, with no insert) and Rc-CMV (vector, with no insert; Fig. 2, set 1). One hundred percent activation was determined from 10T1/2 cells transfected with p1256 MCK-CAT, pEMSV-MyoD, and Rc-CMV (Fig. 2, set 2). Transfection of cyclin A, B1, B2, C, D3, or E expression constructs (dose, 750 ng per 10^6 cells; see Materials and Methods) with pEMSV-MyoD only partly reduced activation of the p1256MCK-CAT reporter (Fig. 2, sets 3 to 6, 8, and 9). In contrast, ectopic expression of cyclin D1 completely eliminated myogenic bHLH regulator activity (Fig. 2, set 7). To determine the effects of ectopic cyclin expression on transcription from the Rous sarcoma virus long terminal repeat, the experiments were repeated, with substitution of pRSV-CAT (20) for p1256MCK-CAT. Variable reductions in Rous sarcoma virus CAT (RSV-CAT) activity to approximately 60% of the baseline value were associated with cotransfection of any CMV-cyclin construct (Fig. 2B, set 10). When scaled against inhibition of RSV-CAT, only inhibition of p1256MCK-CAT by CMV-cyclin D1 is significant. Transient expression assays also revealed inhibition of myogenic bHLH regulator activity by CMV-cyclin D1 when CAT reporter plasmids containing the proximal promoter of the human cardiac α-actin gene (pHCA177-CAT [59]) or four MyoD binding sites contiguous with the thymidine kinase promoter [p4R(MCK)-TK-CAT (67)] were substituted for p1256MCK-CAT (data not shown).

Inhibition of myogenic bHLH regulator activity by ectopic cyclin D1 is mediated by a putatively novel mechanism. Negative regulation of the activity of myogenic bHLH proteins by bFGF has been shown to result from activation of protein kinase C (PKC), which diminishes the DNA-binding activity of these transcriptional activators by phosphorylating a conserved threonine in a stretch of basic residues that mediates their contact with DNA (37). In addition, direct physical association between pRb and myogenic bHLH regulators (which is inhibited by phosphorylation of pRb) is thought to be important for transcriptional activation of muscle genes (21). A series of experiments was performed in order to determine whether ectopic expression of cyclin D1 indirectly inhibited myogenic bHLH regulator activity by influencing either of these events.

Elimination of the basic region PKC site on myogenin by changing threonine 87 to an asparagine (T-87 to N-87) generates a mutant that is active in cells cultured in the presence of bFGF (37). To determine whether cyclin D1 inhibits myogenic bHLH protein activity by a mechanism involving PKC phosphorylation of this site, the effect of ectopic cyclin D1 expression on muscle gene transcriptional activation by myogenin and mutant myogenin (T-87 to N-87) was evaluated. Transfection of CMV-cyclin D1 was equally effective in inhibiting transcriptional activation of p1256MCK-CAT by wild-type myogenin and by mutant myogenin (Fig. 3), indicating that the mechanism of inhibition by cyclin D1 lies downstream from the regulation of myogenic bHLH protein DNA binding by PKC phosphorylation. Similar results were also obtained with p4R(MCK)-TK-CAT as a reporter (data not shown).

Cyclin D1 binds the pRb "pocket" subdomain and thereby mediates phosphorylation of pRb by cdk4 (16, 17, 29). Myogenic bHLH regulators also bind the pRb pocket, and this association is thought to be important for their activity (21). Ectopic expression of cyclin D1 could negatively regulate the activity of myogenic bHLH proteins by inhibiting their binding to pRb through mechanisms involving either direct competition by cyclin D1 for the pocket subdomain or accentuated phosphorylation of pRb by cyclin D1-cdk4 complexes. A double point mutant of cyclin D1 (cyclin D1mu) with an altered N-terminal pRb consensus binding site (16) does not interact with pRb. Transfection of CMV-cyclin D1mu inhibited transcriptional activation of p1256MCK-CAT by MyoD as efficiently as transfection of CMV-cyclin D1 (wild type; Fig. 4). This result suggests that interactions between cyclin D1 and

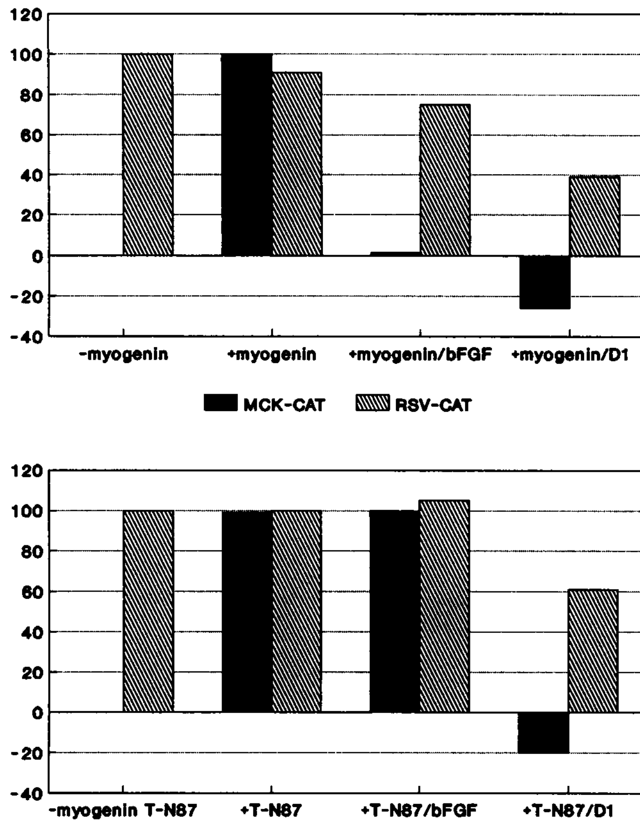

FIG. 3. Inhibition of myogenic bHLH regulator activity by cyclin D1 does not involve phosphorylation of the basic region by PKC. Myogenin and a myogenin mutant lacking the basic region PKC phosphorylation site (myogenin $T\text{-}N_{87}$ [37]) were used to activate transcription from p1256MCK-CAT (MCK-CAT; 5 μg per flask) in transient expression assays. Transfections with pRSV-CAT (1.8 μg per flask) were performed in parallel as a constitutive control. Total transfected plasmid in each transfection was held constant by the addition of the appropriate vector (lacking insert). Activation of p1256MCK-CAT transcription by cotransfection of pEMSV-myogenin (1.5 μg per flask) was inhibited by bFGF (+myogenin/bFGF), whereas the activity of pEMSV-myogenin $T\text{-}N_{87}$ (1.5 μg per flask) was not affected by bFGF (+T-N87/bFGF; as reported in reference 37). Ectopic expression of cyclin D1 (CMV-cyclin D1; 750 ng per flask) inhibited the activity of both myogenin and myogenin $T\text{-}N_{87}$ (+myogenin/D1 and +T-N87/D1, respectively). Mean values from duplicate experiments were quantified from thin-layer chromatography plates by phosphorimage analysis. The baseline value for MCK-CAT activity and the 100% value for RSV-CAT activity were derived from the −myogenin or −myogenin T-N87 values.

pRb are not required for inhibition of myogenic bHLH regulator activity, and this conclusion is supported by the observation that cyclin D3, which also binds pRb and mediates its phosphorylation by cdk4 (16, 17, 29), does not inhibit myogenic bHLH protein activity (as described above). Surprisingly, cyclin D1mu inhibited transcription from pRSV-CAT at doses that for wild-type cyclin D1 inhibited only myogenic bHLH regulator activity (as discussed below).

Differential inhibition of transcription by cyclin D1 mutants. Two mutant cyclin D1 expression constructs were tested for their ability to inhibit activation of p1256MCK-CAT transcription by MyoD: CMV-cyclin D1mu, which contains a double point mutation that eliminates the N-terminal pRb-binding consensus sequence (16); and CMV-cyclin D1-6, from

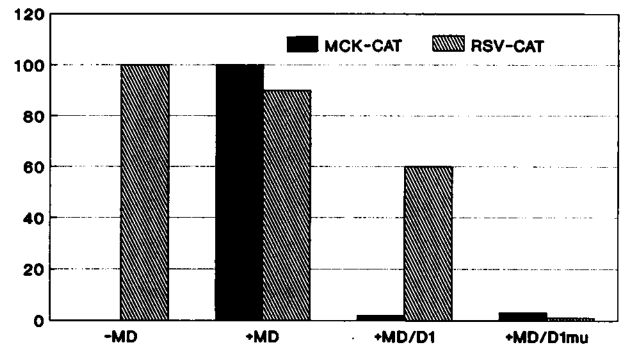

FIG. 4. Inhibition of myogenic bHLH regulator activity by mutant cyclin D1 lacking the pRb consensus binding site. 10T1/2 cells were transfected either with the p1256MCK-CAT (5 μg per flask; MCK-CAT) or pRSV-CAT (1.8 μg per flask) reporter construct alone (−MD), with reporter and pEMSV-MyoD (800 ng per flask) (+MD), with reporter, pEMSV-MyoD, and CMV-cyclin D1 (750 ng per flask) (+MD/D1), or with reporter, pEMSV-MyoD, and CMV-cyclin D1mu (which does not bind pRb; 750 ng per flask) (+MD/D1mu). Total transfected plasmid in each transfection was held constant by the addition of the appropriate vector (lacking insert). Mean values from duplicate experiments were quantified from thin-layer chromatography plates by phosphorimage analysis. The baseline value for MCK-CAT activity and the 100% value for RSV-CAT activity were derived from the −MD values.

which the acidic region from the C terminus to the cyclin box has been deleted (retaining residues 1 to 202; sequence from reference 45). Expression of the 23-kDa protein product of this deletion mutant construct in transfected 10T1/2 cells was determined by immunoprecipitation (Fig. 5). Different doses of CMV-cyclin D1, CMV-cyclin D1mu, and CMV-cyclin D1-6 were transfected with pEMSV-MyoD, and the effects on expression of p1256MCK-CAT and RSV-CAT were determined. At lower doses (0.5 to <2.0 μg per 2×10^6 cells; see Materials and Methods), CMV-cyclin D1 inhibited p1256 MCK-CAT without affecting transcription from pRSV-CAT; at higher doses (>2.0 μg per 2×10^6 cells), CMV-cyclin D1

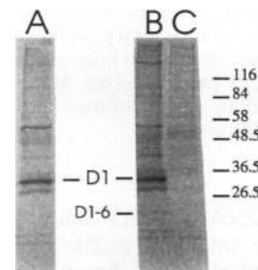

FIG. 5. Transient expression of CMV-cyclin D1-6 mutant in 10T1/2 cells. Proliferating 10T1/2 cells were transfected with 750 ng of Rc-CMV (lane A) or CMV-cyclin D1-6 (lanes B and C) per flask. After 18 h, the cells labelled metabolically with [^{35}S]methionine and cyclin D1 and cyclin D1-6 protein expression were assayed by immunoprecipitation with a cyclin D1 polyclonal antiserum (lanes A and B), SDS-PAGE, and autoradiography. Cyclin D1-6 migrated with an apparent mass of 23 kDa, in agreement with its predicted mass. Lane C is included as a control immunoprecipitation with an unrelated rabbit antibody. Molecular mass markers are indicated (in thousands) on the right. Bands migrating at positions corresponding to $p34^{cdk4}$ and p16 are present, presumably immunoprecipitated as complexes with cyclin D1 (74).

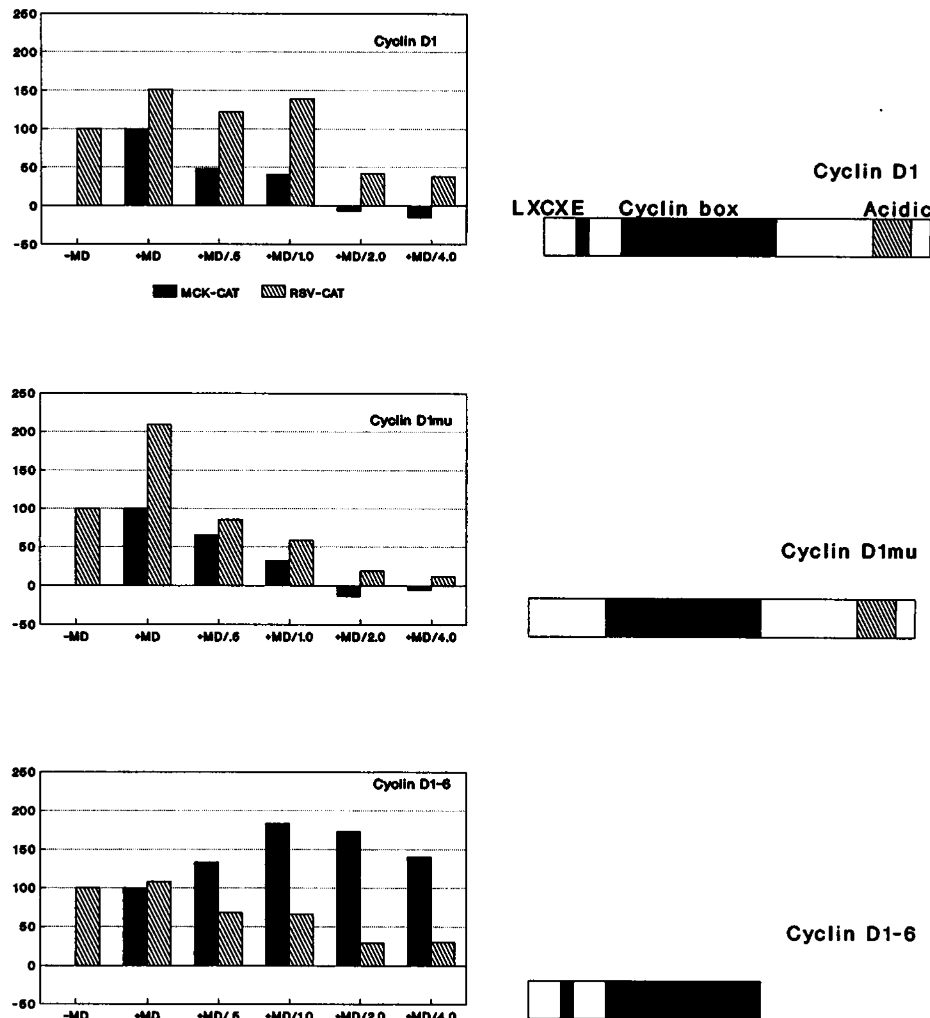

FIG. 6. Differential inhibition of MyoD activity by cyclin D1 mutants. The relative transcriptional inhibitory activities of CMV-cyclin D1, CMV-cyclin D1mu (23), and CMV-cyclin D1-6 were compared at different doses. Structural maps of cyclin D1 and the two mutants are shown on the right (not to scale). p1256MCK-CAT (MCK-CAT; 5 μg per flask) and pRSV-CAT (1.8 μg per flask) were used as reporter plasmids. These were transfected either without pEMSV-MyoD (−MD), with pEMSV-MyoD alone (+MD; 800 ng per flask), or with pEMSV-MyoD and different doses of the CMV-cyclin expression constructs (+MD/dose). Total transfected plasmid in each transfection was held constant by the addition of the appropriate vector, with the total plasmid mass transfected in the +MD/4.0 experiments used as standard values. The indicated doses represent amounts of CMV-cyclin plasmid (in micrograms) used to transfect two T25 plates of 10T1/2 cells; consequently, one-half of each value represents the amount transfected per plate (see Materials and Methods). Mean values from duplicate experiments were quantified from thin-layer chromatography plates by phosphorimage analysis. The baseline value for MCK-CAT activity and the 100% value for RSV-CAT activity were derived from the −MD values.

inhibited both p1256MCK-CAT and pRSV-CAT (Fig. 6). CMV-cyclin D1mu inhibited p1256-MCK-CAT and pRSV-CAT equivalently at all doses (Fig. 6). One interpretation of these observations is that binding by pRb (or another protein recognizing this site) negatively regulates inhibition of RSV-CAT expression by cyclin D1 without affecting transcription from p1256MCK-CAT. This may be significant in light of other reports that suggest pRb binding to cyclin D1 is required for cells to assume a flat (growth-suppressed) morphology (16, 17).

CMV-cyclin D1-6 inhibited transcription from pRSV-CAT with a dose dependence similar to that of CMV-cyclin D1; in contrast, inhibition of p1256MCK-CAT by CMV-cyclin D1-6 was not observed (Fig. 6). This result indicates that the acidic C-terminal portion of cyclin D1 is required for inhibition of myogenic bHLH regulators but not for inhibition of RSV-CAT. In addition, it is possible to conclude from these observations that inhibitions of RSV-CAT and of muscle reporter construct expression by ectopic cyclin D1 are mediated by distinct mechanisms of transcriptional inhibition.

DISCUSSION

Inhibition of myogenic bHLH regulator activity by cyclin D1. The functional antagonism between cell proliferation and myogenic bHLH protein activity is evident from the inhibitory effects of oncogenes and growth-promoting agents on muscle-specific gene expression. But this functional antagonism is also mutual: ectopic expression of MyoD can prevent entry into S phase by transformed cells in culture. MyoD mutants lacking the basic region (and therefore DNA-binding activity) but

retaining the HLH domain are active in preventing entry into S phase, indicating that this property is not directly associated with the induction of muscle-specific gene expression (14, 61). Experimental evidence has suggested that cell cycle arrest by the myogenic bHLH regulators results from their interaction with the retinoblastoma protein (pRb [21]). *Rb* was originally identified as a tumor suppressor gene and acts as a negative regulator of cell growth by preventing exit from G_1 (18, 38, 68). Phosphorylation of pRb by G_1 cyclin-cdk complexes renders it inactive and permits cell cycle progression. MyoD binds specifically to unphosphorylated pRb and may thereby stabilize its negative effect on cell cycle progression. Binding to pRb is also thought to positively regulate the activity of myogenic bHLH proteins (21). Structural analyses have indicated that MyoD and cyclin D1 physically interact with a common subdomain on pRb (the pocket [16, 17]). One possible mechanism by which cyclin D1 could inhibit myogenic bHLH regulator activity is by competitively interfering with the interaction between pRb and MyoD. Two results are presented here as evidence that this is not the operant mechanism. (i) Ectopic expression of cyclin D3, which appears to bind pRb with greater efficiency than cyclin D1 (16, 17), does not inhibit myogenic bHLH regulator activity. (ii) Ectopic expression of a cyclin D1 mutant containing an inactive pRb binding site inhibits myogenic bHLH regulator activity with a potency equivalent to that of wild-type cyclin D1.

While this article was in preparation, it was reported that ectopic expression of cyclin D2 or D3, but not cyclin D1, inhibits granulocytic differentiation of 32D myeloid cells (30). From these findings together with the results presented here, it is apparent that different D-type cyclins may negatively regulate gene expression in a cell-type-specific manner. Although elucidation of the mechanism by which cyclin D1 inhibits myogenic bHLH regulator activity awaits further studies, we show here that it does not involve alteration in the DNA-binding properties of the bHLH protein that result from phosphorylation of the basic region by PKC. The C-terminal regions of the three D-type cyclins display significant structural divergence, and it is shown here that the acidic C-terminal region of cyclin D1 is required for inhibition of myogenic bHLH regulator activity. Together, these observations are consistent with the hypothesis that the structural diversity of D-type cyclins is associated with type-specific functions that are relevant only in certain cellular contexts. Antibody microinjection studies have shown that expression of cyclin D1 is necessary for human fibroblasts to enter S phase (3), and rodent fibroblasts expressing ectopic cyclin D1 display a shortened G_1 phase of the cell cycle (55). It will be of interest to determine whether cyclin D1 mutants that are defective in inhibiting myogenic bHLH regulator function are also defective in promoting cell cycle progression.

Role of modulation of cyclin D1 expression in controlling muscle-specific gene expression. Components of serum and embryo extracts such as TGF-β and bFGF inhibit differentiation of skeletal myoblasts in culture (11, 34, 39, 51, 62). Their removal from proliferating myoblast cultures is followed by an interval of 12 to 24 h, after which the cells undergo overt differentiation. Differentiating cells exit the cell cycle, induce transcription of the panel of muscle genes required for contractile activity, and fuse into multinucleated syncytia. Since expression of cyclin D1 has been shown to be positively regulated by some growth factors (reviewed in reference 60), it is possible that inhibition of muscle-specific gene expression by mitogens involves induction of cyclin D1 expression. We show here that induction of myoblast differentiation in low-mitogen medium results in attenuation of cyclin D1 expression. A reduction in the ambient concentration of growth factors does not result in myoblast differentiation until the cells reach early G_1 (reviewed in reference 50). In other cell types, expression of cyclin D1 has been observed to drop sharply in early G_1 in the absence of growth factors (reviewed in reference 60), which may explain the restriction that myoblasts reach this phase in the cell cycle prior to differentiation. Further experiments will be required to detail the kinetics of D-type expression after myoblasts are switched from high- to low-mitogen medium to induce differentiation. In addition, further experiments will be required to characterize the effects of specific growth factors on expression of the cyclin D1 gene in myoblasts.

Although in other cell types, such as peripheral T lymphocytes (1) and some primary fibroblasts (70), cyclin D3 expression is induced by mitogens, in cultured C_2C_{12} myoblasts mitogen withdrawal was associated with accentuated expression of this cyclin. Mitogen withdrawal induces differentiation of C_2C_{12} myoblasts, consistent with a possible regulatory function for cyclin D3 in terminally differentiated myotubes. Although further studies of cyclin D3 protein expression and subcellular localization in muscle are required, the present observations support the notion that cellular context may profoundly influence the function of certain cyclins; indeed, some cyclins, such as D3, may have specific functions in cells that are considered to have permanently exited the cell cycle.

Control of myoblast differentiation: a panoply of regulators. Several mechanisms for the inhibition of muscle gene transcription by growth factors have been described. Two mechanisms negatively regulate the DNA-binding activity of MyoD family members in response to growth factors: (i) expression of protein Id, induced by serum factors, results in either sequestering of E2A gene products or the assembly of heterodimers with MyoD family members that are inactive in binding DNA (4, 28); and (ii) PKC activity, induced by bFGF, inhibits the DNA-binding activity of myogenic bHLH regulators by phosphorylating a conserved site in the region of these proteins that contacts DNA (37). A physical association between the myogenic bHLH regulators and the products of some *jun* gene family members has been demonstrated (5, 36); however, the mechanism by which Jun binding negatively regulates myogenic bHLH protein activity has not yet been elucidated. Hypophosphorylated pRb binds MyoD, an interaction that is thought to promote activation of muscle gene transcription (21). We show in this report that cyclin D1 acts as a negative regulator of myogenic bHLH protein activity and present evidence that inhibition by cyclin D1 may be independent of other pathways. Cyclin D1 may inhibit myogenic bHLH protein activity by a mechanism that involves association with and activation of a cdk. A puzzling aspect of this hypothesis is that cdk activities are also positively regulated by other cyclins, which do not appear to inhibit muscle gene transcription. Even cdk4, which so far has been shown to be activated exclusively by D-type cyclins, in experimental systems employs cyclin D3 as efficiently as a regulatory partner as it does cyclin D1 (29, 40). Thus, a simple explanation for the inhibition of muscle gene transcription by cyclin D1 cannot readily be derived from our current understanding of cell cycle regulators and will require further studies.

The existence of multiple and redundant pathways that inhibit myoblast differentiation is not unreasonable, since myoblasts respond to a variety of different regulatory signals in culture and probably during muscle development in vivo. These pathways apparently work in concert with cyclin D1 expression to regulate myoblast differentiation. Further studies will be required to understand the hierarchy of these mechanisms, their specific significance during muscle development in

vivo, and their possible occurrence as mechanisms of regulating differentiation in other cell types.

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