Inhibition of Myogenesis by Multiple Cyclin-Cdk Complexes

COORDINATE REGULATION OF MYOGENESIS AND CELL CYCLE ACTIVITY AT THE LEVEL OF E2F

Kun Guo and Kenneth Walsh‡

From the Division of Cardiovascular Research, St. Elizabeth’s Medical Center, Tufts University School of Medicine, Boston, Massachusetts 02135 and Department of Biochemistry, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106

During skeletal myogenesis, cell cycle withdrawal accompanies the expression of the contractile phenotype. Here we show that ectopic expression of each D-type cyclin is sufficient to inhibit the transcriptional activation of the muscle-specific creatine kinase (MCK) gene. In contrast, ectopic expression of cyclin A or cyclin E inhibits MCK expression only when they are co-expressed with their catalytic partner cyclin-dependent kinase 2 (Cdk2). For each of these conditions, myogenic transcriptional inhibition is reversed by the ectopic co-expression of the general Cdk inhibitor p21. Inhibition of MCK expression by cyclins or cyclin-Cdk combinations correlates with E2F activation, suggesting that the inhibition is mediated by the overall Rb-cyclin-dependent cell cycle regulatory molecules on the same MCK transcript.

Irreversible cell cycle withdrawal is a key component of myogenic differentiation, but little is known about the interplay between the myogenic transcription factors and the cell cycle regulatory proteins. Previous studies have shown that the retinoblastoma susceptibility gene product (Rb) has an essential role in maintaining the postmitotic state of differentiated myoblasts (1, 2). Rb is hypophosphorylated upon terminal differentiation and this state of dephosphorylation is maintained when differentiated myotubes are re-exposed to high mitogen media. Hypophosphorylated Rb inactivates the E2F transcription factor which is essential for the expression of genes required for DNA synthesis (3). Myocytes from Rb-deficient mice can differentiate into myotubes, but these myotubes can synthesize DNA upon mitogen stimulation (2). The phosphorylation status of Rb is regulated, at least in part, by the activity of cyclin-dependent kinases including Cdk2 and Cdk4 (4). Recently, the general Cdk inhibitor p21 was shown to be dramatically induced during skeletal muscle differentiation, and a high level of p21 expression is sustained when myotubes are re-exposed to high mitogen media (5–7). p21 is induced early during the differentiation program (8), and its expression is critical for myocyte viability (9). In myotubes, p21 is the predominant inhibitory subunit of the Cdk complex (7, 10). Thus p21 is likely to function to inhibit the phosphorylation of Rb or other Rb family members during myocyte differentiation.

Surprisingly, it has been reported that overexpression of cyclin D1, but not cyclins A, B, D2, D3, or E, can block myogenesis as detected by the transcriptional activation of an MCK-reporter construct in transiently transfected cultures of differentiating C2C12 cells (11). More recently it was reported that the cyclin D1-mediated inhibition of myogenic transcriptional activation could be reversed by the co-expression of the Cdk inhibitors p21 or p16 (12). These data have led to the proposal that cyclin D1 down-regulation may be a nodal point in the coordination of myocyte differentiation and cell cycle activity. Further, it was proposed that myogenic inhibition by cyclin D1 occurs through an Rb-independent pathway because the overexpression of cyclin A or cyclin E, the regulatory subunits of the Cdk2 Rb-kinase, do not inhibit myogenesis (12).

To test the hypothesis that cyclin D1 functions uniquely to coordinate cell cycle with myogenic transcription through an Rb-independent mechanism, we analyzed the effects of additional cell cycle regulatory molecules on the same MCK transcriptional assay that was employed in these previous studies (11, 12). Here we report that in addition to cyclin D1, the overexpression of other D-type cyclins (D2 and D3) or the combinations of Cdk2 and cyclin A or cyclin E also inhibit MCK transcriptional activation, and that this inhibition is reversed by the co-expression of p21. MCK transcription is inversely correlated with expression from an E2F-reporter gene construct, and a hyperactive Rb mutant partially reverses the cyclin-Cdk-mediated inhibition of MCK transcription. Collectively these data indicate that the inhibition of myogenic transcription by cell cycle components can be mediated by a pocket protein/E2F pathway. Myogenic transcription is also inhibited by the ectopic expression of the E2F1 transcription factor. Surprisingly, mutations in the activation, leucine zipper, basic,
and cyclin A-Cdk2 binding domains of E2F1 have no effect on its ability to inhibit myogenic transcription, but this activity was abolished by mutations in the E2F1 helix-loop-helix region. Taken together, these data demonstrate that the inhibition of myogenesis is a general feature of cyclin-Cdk activity and that myogenic differentiation is coordinated with cell cycle activity at the level of E2F.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Plasmids, and Transfections**—C2C12 myocytes and 10T1/2 fibroblasts were maintained in DMEM supplemented with 20% fetal bovine serum (growth medium). Myogenic differentiation was initiated by shifting subconfluent cultures into DMEM supplemented with 2% horse serum (differentiation medium).

The (~650)MCK-Luc plasmid that contains the rabbit MCK promoter and enhancer was described previously (15, 16). All expression plasmids used in these studies were under the control of the CMV promoter/enhancer. The expression plasmids for cyclin D1, D2, D3, A, E, and Cdk2 were from L. Zhu and E. Harlow. The E2F1 expression vectors utilized the CMV promoter/enhancer (15, 16). E2F1(E113) has glutamate substituted for arginine at position 113; E2F1(E120) has glutamate substituted for lysine at position 120; E2F1(E138) has glutamate substituted for phenylalanine at position 138; and E2F1(E177) glutamate substituted for isoleucine at position 177. E2F1(d113–120) was derived by deleting amino acid positions 113 through 120, and E2F1(d87) was derived by deleting amino acids 1 through 87 (15). The deletion mutations E2F1(1–241), E2F1(1–196), and E2F1(1–127) were described in Qin et al. (16). The expression plasmids for p21, pCDNAIII-p21, was from A. Dutta (17). K. Wills provided the expression plasmids for hyperactive and inactive Rb, p53, and p56(H209), respectively. The E2F1x4-E1BTATA-Luc reporter plasmid was constructed by subcloning the Puuv/SacI fragment from (E2F1x4-E1BTATA-CAT (18, 19) into the Smal/SacI site of pGL2-Basic plasmid (Promega Inc.). The plasmid MCK E-box-Luc, that has the luciferase gene downstream from the E1b TATA element and four copies of the MCK enhancer, was provided by A Yee. In all transfection experiments the plasmid pSV2-A-P, that has the alkaline phosphatase gene under the control of the SV40 promoter and enhancer (20), was used to control for differences in transfection efficiencies.

Plasmid transfections were performed by the calcium-phosphate procedure. For transfections in Fig. 1, calcium-DNA precipitates were incubated with cells in growth medium for 12 h, and then cells were switched to DMEM supplemented with 20% fetal bovine serum for 1 day. Cells were either harvested and assayed for luciferase and alkaline phosphatase activities or switched to differentiation medium (DMEM supplemented with 2% horse serum). At indicated times after incubation in low mitogen differentiation medium, cells were harvested and assayed for reporter expression. Luciferase and alkaline phosphatase activities were determined as described previously (21). Luciferase (Promega) and alkaline phosphatase (CSPD chemiluminescent substrate; Trophix) activities were measured with a Berthold Lumat LB9501 luminometer. The amount of DNA was kept constant in each set of transfection experiments by adding the required amount of Rc/CMV plasmid (Promega) as described in Guo et al. (7). All transfection experiments were performed in triplicate. For transient transfections in other figures, transfections were performed as above except that 12 h after transfection, cells were switched to fresh DMEM, 20% fetal bovine serum for 1 day, and then switched to low mitogen differentiation medium for 2 days. Cells were harvested, and luciferase/alkaline phosphatase activities were assayed as described above.

**Immunoblotting**—To induce myogenic differentiation, proliferating C2C12 myoblasts were switched to low mitogen differentiation media (DMEM supplemented with 2% horse serum) for 2 days. Cells were lysed in buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 μg of leupeptin/ml, and 1 mM phenylmethylsulfonyl fluoride) and passed through G21 needles several times. Lysates were cleared of insoluble materials by centrifugation at 4°C and 12,000 × g for 15 min. Protein concentrations of lysates were determined by the Bio-Rad Protein Assay. Fifty μl of cell lysates were loaded into 12% SDS-PAGE gel after boiling in SDS sample loading buffer. Following electrophoresis, proteins were transferred to nylon membranes (Millipore). Membranes were blocked in buffer A (1 × PBS, 0.2% Tween 20, 5% dry milk) overnight at 4°C and then incubated with anti-Cdk4 or anti-Cdk4 antibodies (1:200 dilution in 1 × PBS, 0.2% Tween 20, and 2% dry milk) (Santa Cruz Biotechnology) for 3 h at room temperature. After incubation the membranes were washed three times with buffer B (1 × PBS, 0.2% Tween 20, and 2% dry milk) (10 min each). Then membranes were incubated with secondary antibodies (horse peroxidase-conjugated goat anti-rabbit IgG) for 45 min at room temperature. Membranes were then washed three times with buffer B and three times with buffer C (1 × PBS, 0.2% Tween 20). Membranes were developed by using the enhanced ECL chemiluminescence reagents (Amersham Corp.).

**RESULTS**

**Myogenic Transcriptional Activation Is Inhibited by Multiple Cyclin-Cdk Complexes**—MCK transcriptional activity is enhanced by the co-expression of p21 in 10T1/2 fibroblasts that are transiently transfected with a MyoD expression plasmid (12). To further investigate this property of p21, C2C12 myocytes were transfected with a luciferase reporter plasmid containing the rabbit MCK promoter/enhancer in the presence or absence of a p21-expression plasmid. MCK transcriptional activity was analyzed in replicating myoblast cultures and at different times following the induction of differentiation by myogenin deprivation. As shown in Fig. 1, MCK promoter activity was enhanced by ectopic p21 expression in the high mitogen growth medium (GM) or 1 day following the shift to differentiation medium (DM). However, MCK expression was not enhanced by p21 at the 2-day time point, when the endogenous levels of this protein are high (8, 9).

Various cyclins were compared for their ability to inhibit MCK transcription in differentiating myocytes. As shown in Fig. 2, and consistent with previous reports (11, 12), co-expression of cyclin D1 substantially inhibited MCK promoter activity, but the co-expression of cyclin A or cyclin E had no effect. Further experiments compared the myogenic inhibitory activity of the three D-type cyclins that function as activating subunits for Cdk4 and Cdk6. In contrast with previous reports (11, 12), these analyses revealed an inhibition of MCK transcription by all three D-type cyclins, although cyclin D3 and D2 expression plasmids were less potent inhibitors (Fig. 3A). In each case co-expression of p21 reversed the inhibition of MCK promoter activity by the D-type cyclin (Fig. 3B).
To determine the mechanism by which the D-type cyclins, cyclin A, and cyclin E differentially affects myogenic transcription, Western blot analyses were performed to determine the relative levels of Cdk2 and Cdk4 catalytic subunits in myoblasts and myotubes. During myogenesis the level of the Cdk4 protein (catalytic partner for D-type cyclins) remained constant, but there was a marked decrease in the level of the Cdk2 protein (catalytic partner for cyclins A and E) (Fig. 4A). Thus, we tested whether differences in the expression patterns of Cdk2 and Cdk4 could contribute to the differential effects of their cognate cyclins on myogenic transcription. As shown in Fig. 4B, MCK transcription was inhibited by the expression plasmid combinations of Cdk2 and cyclin A or Cdk2 and cyclin E, but transfections of the Cdk2 plasmid alone had little or no effect on MCK transcription. Furthermore, the inhibition of MCK transcription by the combinations of cyclins A or E and Cdk2 was reversed by the co-expression of p21. These data were corroborated by demonstrating similar regulatory behavior using 10T1/2 cells transfected with a MyoD expression vector and a minimal MCK E-box reporter plasmid (not shown). Collectively, these data provided an initial indication that the inhibition of MCK promoter activity is related to the overall extent Cdk activity within the cell.

Inverse Correlation between MCK Transcription and E2F Activation—Phosphorylation of Rb by the Cdk5 results in the activation of the E2F transcription factor (3). To test whether E2F is activated under the same conditions that inhibit myogenesis, a reporter plasmid for E2F activity, (E2F)x4-E1bTATA-Luc, was co-transfected into C2C12 cells with various combinations of expression plasmids for cyclins, Cdk5 and p21. As shown in Fig. 5, E2F transcriptional activity was stimulated by the ectopic expression of cyclin D1 or the combination of Cdk2 with either cyclin A or cyclin E, and this activation was blocked by the co-expression of p21. Transfections with Cdk2, cyclin A, or cyclin E alone had no effect on E2F activity. These data revealed a good correlation between the inhibition of myogenesis and the activation of the E2F transcription factors. They also suggest that the E2F factors may mediate the inhibition of MCK transcription by the cyclin-Cdk complexes. Therefore, E2F1 was directly tested for its ability to inhibit MCK transcription. MCK promoter activity was repressed by ectopic E2F1 expression, but this repression was not reversed by the co-expression of p21 (Fig. 6).

A Hyperactive Rb Mutant Can Reverse the Cyclin-Cdk- and E2F-mediated Inhibition of MCK Promoter Activity—The potential involvement of Rb in MCK promoter repression by the cyclin-Cdks was investigated using hyperactive Rb mutant which lacks the N-terminal 378 amino acids. This mutant, referred to as p56, repressed E2F transcriptional activity in C2C12 cells (Fig. 7A) and in other cell types. This specificity of this effect was indicated by the inactivity of the p56/H209 variant that has phenylalanine substituted for cysteine at amino acid position 706. This mutation gives rise to an Rb protein that is defective in phosphorylation and oncoprotein binding (22, 23), and it inactivates the p56 Rb construct with regard to its ability to repress transcription from E2F sites (Fig. 7A). The hyperactive form of Rb reversed the inhibition of MCK promoter activity by E2F1, but the p56/H209 variant was ineffective (Fig. 7B). Similarly, p56, but not p56/H209, partially reversed the inhibition of MCK promoter activity by cyclin D1 or by the combination of Cdk2 and cyclin A (Fig. 7C).

2 L. Truong and K. Walsh, unpublished data.
The Helix-Loop-Helix Domain of E2F1 Is Essential for the Inhibition of Myogenic Transcription—E2F1 mutants were analyzed to identify the structural motifs required for the inhibition of myogenic transcription. Initially we tested the E2F1 mutant (1–284) which lacks the C-terminal transcriptional activation domain. This mutant could not activate the expression of an E2F-reporter construct (Fig. 8) or promote S phase entry when overexpressed in serum-deprived cells (16). However, the E2F1(1–284) mutant, like wild-type E2F1, effectively inhibited the ability of MyoD to transactivate a reporter construct containing four E box elements from the MCK enhancer (Fig. 8). Further, this E2F1 mutant which lacks the Rb-binding domain could inhibit myogenic transcriptional activity in the presence of the hyperactive p56 Rb construct (not shown). These data indicate that cell proliferation and myogenic differentiation are coordinated at the level of E2F and that different functional domains within E2F regulate each process.

Additional E2F1 mutants were examined to determine the region required for the inhibition of myogenic transcription. In addition to mutants that lack the transactivation domain, mutant E2F1(1–196), which is missing the leucine (24, 25), and mutant E2F(187), which is missing the cyclin A/Cdk2 binding domain (26), were also effective at inhibiting MyoD transcriptional activity (Fig. 9). However, myogenic transcription was not inhibited by mutant E2F1(1–127), which lacks the transactivation domain, the leucine zipper and the putative helix-loop-helix region (15) (Fig. 9). A series of mutations were analyzed to characterize the involvement of the basic and helix-loop-helix regions in the inhibition of myogenic transcription. The ability of E2F1 to inhibit myogenic transcription was not affected by an 8-amino acid deletion in the basic region or mutations at positions 113 or 120 that change basic amino acids to glutamate residues (Fig. 10). However, the MyoD-inhibitory activity of E2F1 was abolished by mutations that substitute glutamate residues in the helix-loop-helix region (Fig. 10). Similar results were obtained using either 10T1/2 cells transfected with a MyoD expression vector and a minimal MCK E-box reporter plasmid (Figs. 8–10) or using C2C12 cells transfected with the larger MCK enhancer/promoter construct (not shown). Taken together, these data demonstrate that only the helix-loop-helix domain is required for the myogenic inhibitory activity of E2F1.
transcriptional activation, and the studies of Skapek (12) showed that the overexpression of cyclin D1 might be sufficient to block MCK transcription because their catalytic partner, Cdk4, is not down-regulated during myogenesis. Consistent with this hypothesis, the observations that MCK-promoter activity is inhibited, albeit with different efficiencies, by the transfection of these individual plasmids (Figs. 2 and 4), suggests that the differential effects of these cyclins on myogenic transcription might inhibit myogenesis only when they are co-expressed with their catalytic partner Cdk2. On the other hand, the forced expression of cyclins A or E have little or any effect on MCK transcriptional activation (Fig. 2) (11, 12). To investigate the differential effects of these cyclins on myogenic transcriptional activation, the expression patterns of their cognate catalytic subunits were compared. During myogenesis the expression of the Cdk2 protein is markedly down-regulated, but Cdk4 levels do not detectably change (Fig. 4A) (also see Refs. 7, 11, and 12). Thus, we reasoned that cyclin A or cyclin E overexpression might inhibit myogenesis only when they are co-expressed with their catalytic partner Cdk2. On the other hand, overexpression of the D-type cyclins might be sufficient to block MCK transcription because their catalytic partner, Cdk4, is not down-regulated during myogenesis. Consistent with this hypothesis are the observations that MCK-promoter activity is inhibited, albeit with different efficiencies, by the transfection of cyclin D1, D2, or D3 expression vectors (Fig. 3). MCK promoter activity was also inhibited by combinations of expression vectors for cyclin A and Cdk2 or cyclin E and Cdk2, but not by the transfection of these individual plasmids (Figs. 2 and 4B). In all cases, the inhibition of MCK transcription was overcome by the co-expression of p21. Collectively, these data indicate that the inhibitory effects of the cyclin-Cdk complexes are largely mediated by Rb, or a related pocket proteins, and that the E2F transcription factor plays a central role in coordinating myogenic differentiation and cell cycle activity.

Here it is shown that the overexpression of each D-type cyclin is sufficient to inhibit MCK promoter activity. However, the forced expression of cyclins A or E have little if any effect on MCK transcriptional activation (Fig. 2) (11, 12). To investigate the differential effects of these cyclins on myogenic transcriptional activation, the expression patterns of their cognate catalytic subunits were compared. During myogenesis the expression of the Cdk2 protein is markedly down-regulated, but Cdk4 levels do not detectably change (Fig. 4A) (also see Refs. 7, 11, and 12). Thus, we reasoned that cyclin A or cyclin E overexpression might inhibit myogenesis only when they are co-expressed with their catalytic partner Cdk2. On the other hand, overexpression of the D-type cyclins might be sufficient to block MCK transcription because their catalytic partner, Cdk4, is not down-regulated during myogenesis. Consistent with this hypothesis are the observations that MCK-promoter activity is inhibited, albeit with different efficiencies, by the transfection of cyclin D1, D2, or D3 expression vectors (Fig. 3). MCK promoter activity was also inhibited by combinations of expression vectors for cyclin A and Cdk2 or cyclin E and Cdk2, but not by the transfection of these individual plasmids (Figs. 2 and 4B). In all cases, the inhibition of MCK transcription was overcome by the co-expression of p21. Collectively, these data indicate that the inhibitory effects of the cyclin-Cdk complexes are largely mediated by Rb, or a related pocket proteins, and that the E2F transcription factor plays a central role in coordinating myogenic differentiation and cell cycle activity.

**FIG. 7.** A hyperactive mutant of Rb reverses the repression of MCK transcription by E2F1, cyclin D1, or the combination of Cdk2 and cyclin A. A, a hyperactive mutant of Rb (p56), but not an inactive mutant (p56/H209), suppresses the transcriptional activity of E2F1 in differentiating myocytes. Proliferating C2C12 myocytes were co-transfected with the (E2F)x4-E1bTATA-Luc reporter plasmid (1 μg) in the presence or absence of expression plasmids for E2F1 (0.3 μg), p56 Rb (3 μg), or p56/H209 Rb (3 μg). Transfection and promoter activity analysis was performed as described under “Experimental Procedures.” B, a hyperactive mutant of Rb (p56), but not an inactive mutant (p56/H209), reverses the inhibition of MCK transcription by E2F1. Transfections were the same as in A, but the reporter plasmid was MCK-Luc (1 μg). C, a hyperactive mutant of Rb (p56) reverses the inhibition of MCK transcription by cyclin D1 and by the combination of cyclin A and Cdk2. Proliferating C2C12 cells were transfected with the MCK-Luc reporter plasmid (1 μg) in the presence or absence of the indicated expression plasmids for cyclin D1 (1 μg), cyclin A (1 μg), Cdk2 (1 μg), p56 Rb (3 μg), or p56/H209 Rb (3 μg). Transfection and promoter activity analyses are described under “Experimental Procedures.”

**DISCUSSION**

Here we investigated the molecular links between cell cycle activity and myogenic differentiation. These analyses extend the studies of Rao et al. (11), which showed that the overexpression of cyclin D1, but not cyclins A, B, or E, represses MCK transcriptional activation, and the studies of Skapek et al. (12), which showed that the cyclin D1-mediated repression is overcome by the ectopic expression of Cdk inhibitors. Based upon these prior observations it was proposed that cyclin D1 functions uniquely to coordinate cell cycle withdrawal and myogenic differentiation through an Rb-independent mechanism (12). However, this hypothesis is not supported by the data from this current study. Here it is shown that the inhibition of myogenic transcription is a function shared by many cyclins or cyclin-Cdk combinations. Further, these data indicate that the

**FIG. 8.** Transactivation domain of E2F1 is dispensable for myogenic inhibition, but not for the activation of E2F-dependent transcription. Top, schematic representations of wild-type E2F1 (wt) and the mutant E2F1(1–284). TA, transcription domain; LZ, leucine zipper motif; b, basic region; HHL, helix-loop-helix motif; A/K2, cyclin A/Cdk2 binding domain. Bottom, left, E2F reporter plasmid (E2F)x4-E1bTATA-Luc (1 μg) was transfected into 10T1/2 cells with or without the cotransfection of expression plasmids for E2F1(wt) or E2F1(1–284) (0.5 μg). In each transfection, 0.5 μg of pSV2-AP (alkaline phosphatase) plasmid was used as internal control for transfection efficiency. The amount of DNA for each transfection was kept constant by addition of empty pRc/CMV vector. Cells were switched to differentiation media (DMEM supplemented with 2% horse serum) 12 h after transfection, and cells were harvested for luciferase and alkaline phosphatase assays two days later. Right, expression plasmid for MyoD (EMSV-MyoD) (1 μg) and the myogenic reporter plasmid MCK E-box-Luc (1 μg), which has four copies of the high affinity E-box element from the MCK enhancer upstream of the E1b TATA fragment, were co-transfected into 10T1/2 cells, in the presence or absence of expression plasmids for wild-type E2F1(wt) or E2F1(1–284) mutant (0.3 μg). In each transfection, pSV2-AP was used as the internal control for transfection efficiency and pRc/CMV was added to kept the amount of DNA for each transfection constant.
MCK transcriptional inhibition is dependent on the overall cell cycle activity, rather than the action of an individual cell cycle component (i.e. cyclin D1). This hypothesis is consistent with the observation of an inverse correlation between MCK promoter expression and E2F transcriptional activity in cells transfected with different combinations of these cell cycle factors (Fig. 5).

Vectors expressing hyperactive and inactive mutants of Rb were utilized to test the potential role of Rb (or a related pocket protein) as a mediator of myogenic transcriptional inhibition. Ectopic expression of the hyperactive (p56), but not an inactive (p56/H209), Rb mutant partially reversed the MCK transcriptional inhibition that resulted from the overexpression cyclin D1 or the combination of Cdk2 and cyclin A (Fig. 7C). These data further indicate that MCK transcription is inhibited by overall cell cycle activity and that this inhibition is mediated by the Rb/E2F pathway. This notion is further supported by the finding that E2F1 overexpression inhibits MCK transcription (Fig. 6) and overall myogenic differentiation (27). MCK promoter inhibition by E2F1 is reversed by the co-expression of the hyperactive Rb mutant, but not by p21 (Figs. 6 and 7); presumably because E2F1 acts at a step that is downstream from p21 (28).

The data from this study are consistent with the model depicted in Fig. 11. Various cyclins, through interactions with their catalytic subunits, inhibit the transcription of muscle-specific genes via the phosphorylation of Rb or other pocket proteins. This phosphorylation leads to the activation of the E2F family of transcription factors, which induce the expression of essential S-phase genes and also inhibit the expression of muscle-specific genes through an unknown mechanism. The induction of p21 during myogenesis, a broad-specificity Cdk inhibitor, enhances differentiation by blocking the action of multiple cyclin-Cdk complexes. Finally, E2F also activates the expression of cyclin A and cyclin E (29), but this pathway appears to have a minimal effect on MCK transcription because the inhibition of MCK promoter activity by forced E2F1 expression is not reversed by p21.

The data presented here suggest that E2F functions to coordinate the opposing cellular fates of proliferation and differentiation during myogenesis. Support for the hypothesis comes from the finding that different domains within E2F1 function to promote cell proliferation and inhibit myogenic differentiation (Fig. 8). The transcriptional activation, leucine zipper and helix-loop-helix domains are required for the expression of S phase genes and cell cycle progression. However, only the helix-
loop-helix region of E2F1 is essential for the myogenic inhibitory activity, while other regions of the protein are dispensable (Figs. 10 and 11). The inhibition of myogenic transcription by E2F1 overexpression may result from its ability to inactivate E proteins that are essential for myogenic differentiation. Further analyses on the mechanism of E2F-mediated inhibition of myogenic differentiation will be of interest.

Acknowledgments—We thank Drs. K. Wills, L. Zhu, E. Harlow, A. Yee, A. Dutta, and W. G. Kaelin, Jr., for plasmids.

REFERENCES
1. Gu, W., Schneider, J. W., Condorelli, G., and Nadal-Ginard, B. (1993) *Cell* **72**, 309–324
2. Schneider, J. W., Gu, W., Zhu, L., Mahdavi, V., and Nadal-Ginard, B. (1994) *Science* **264**, 1467–1471
3. Nevins, J. R. (1992) *Science* **258**, 424–429
4. Sherr, C. J. (1994) *Cell* **78**, 551–555
5. Parker, S. B., Eichele, G., Zhang, P., Rawls, A., Sands, A. T., Bradley, A., Olson, E. N., Harper, J. W., and Elledge, S. J. (1995) *Science* **267**, 1024–1027
6. Halevy, O., Novitch, B. G., Spicer, D. B., Skapek, S. X., Rhee, J., Hannon, G. J., Beach, D., and Lassar, A. B. (1995) *Science* **267**, 1018–1021
7. Guo, K., Wang, J., Andrés, V., Smith, R. C., and Walsh, K. (1995) *Mol. Cell. Biol.* **15**, 3823–3829
8. Andrés, V., and Walsh, K. (1996) *J. Cell Biol.* **132**, 657–666
9. Wang, J., and Walsh, K. (1996) *Science* **273**, 359–361
10. Wang, J., and Walsh, K. (1996) *Cell Growth Differ.* **7**, 1471–1478
11. Rao, S. S., Chu, C., and Kohitz, D. S. (1994) *Mol. Cell. Biol.* **14**, 5259–5267
12. Skapek, S. X., Rhee, J., Spicer, D. B., and Lassar, A. B. (1995) *Science* **267**, 1022–1024
13. Yi, T. M., Walsh, K., and Schimmel, P. (1991) *Nucleic Acids Res.* **19**, 3027–3033
14. Vincent, C. K., Gualtiero, A., Patel, C. V., and Walsh, K. (1993) *Mol. Cell. Biol.* **13**, 1264–1272
15. Cress, W. D., Johnson, D. G., and Nevins, J. R. (1993) *Mol. Cell. Biol.* **13**, 6314–6325
16. Qin, X. Q., Livingston, D. M., Ewen, M., Sellers, W. R., and Kaelin, W. G., Jr. (1995) *Mol. Cell. Biol.* **15**, 742–755
17. Chen, J., Jackson, P. K., Kirschner, M. W., and Dutta, A. (1995) *Nature* **374**, 386–388
18. Zhu, H., Nguyen, V. T. B., Brown, A. B., Pourhosseini, A., Garcia, A. V., van Bilsen, M., and Chien, K. R. (1995) *Mol. Cell. Biol.* **15**, 4432–4444
19. Zhu, L., van den Heuvel, S., Helin, K., Fattaey, A., Ewen, M., Livingston, D., Dyson, N., and Harlow, E. (1993) *Genes Dev.* **7**, 1111–1125
20. Henthorn, P., Zervos, P., Raducha, M., Harris, H., and Kadesch, T. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 6922–6926
21. Andrés, V., Fisher, S., Wearsch, P., and Walsh, K. (1995) *Mol. Cell. Biol.* **15**, 4272–4281
22. Kaye, F. J., Kratzke, R. A., Gerster, J. L., and Horowitz, J. M. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 6922–6926
23. Kratzke, R. A., Otterson, G. A., Lin, A. Y., Shimizu, E., Alexandrova, N., Zajac-Kaye, M., Horowitz, J. M., and Kaye, F. J. (1992) *J. Biol. Chem.* **267**, 25998–26003
24. Helin, K., Wu, C.-L., Fattaey, A. R., Lees, J. A., Dynlacht, B. D., Ngwu, C., and Harlow, E. (1993) *Genes Dev.* **7**, 1850–1861
25. Bandara, L. R., Buck, V. M., Zamanian, M., Johnston, L. H., and LaThangue, N. B. (1993) *EMBO J.* **12**, 4317–4324
26. Krek, W., Ewen, M. E., Shirodkar, S., Arany, Z., Kaelin, W. G. J., and Livingston, D. M. (1994) *Cell* **78**, 161–172
27. Wang, J., Helin, K., Jin, P., and Nadal-Ginard, B. (1995) *Cell Growth Differ.* **6**, 1299–1306
28. DeGregori, J., Leone, G., Ohtani, K., Miron, A., and Nevins, J. R. (1995)*Genes Dev.* **9**, 2873–2887
29. DeGregori, J., Kowalik, T., and Nevins, J. R. (1995) *Mol. Cell. Biol.* **15**, 4215–4224