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E2F1 Inhibition of Transcription Activation by Myogenic Basic Helix-Loop-Helix Regulators

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Abstract Cellular transcription factor E2F1 is thought to regulate the expression of genes important for cell cycle progression and cell proliferation. Deregulated E2F1 expression induces S-phase entry in quiescent cells and inhibits myogenic differentiation. We show here that E2F1 inhibits the activation of gene transcription by myogenic basic helix-loop-helix proteins myoD and myogenin. Transfection assay using different deletion constructs indicates that both the DNA binding and the transactivation domains of E2F1 are required for its inhibition of myoD transcription activation. However, the retinoblastoma protein (RB) binding domain is not required. Furthermore, co-transfection with the RB, which inhibits the transcription activity of E2F1, can also repress E2F1 inhibition of myoD transactivation. These results suggest an essential role of E2F1-mediated transcription in its inhibition of myogenesis. © 1996 Wiley-Liss, Inc.

Key words: bHLH, myogenic differentiation, cellular transcription factor E2F1, myoD transactivation

The myogenic basic helix-loop-helix (bHLH) proteins are master regulators of skeletal myogenesis *in vitro* [Olson, 1990; Weintraub et al., 1991] and muscle development during embryogenesis [Hasty et al., 1993; Nabeshima et al., 1993; Olson, 1990; Rudnicki et al., 1993; Weintraub, 1993]. Four members of this myoD family of genes have been isolated so far, which include myoD, myogenin, myf-5, and MRF4/herculin/myf-6 [Braun et al., 1990; Braun et al., 1989; Davis et al., 1987; Edmondson and Olson, 1989; Miner and Wold, 1990; Rhodes and Konieczny, 1989; Wright et al., 1989]. These gene products function as muscle specific transcription activators, preferably as heterodimers with the E2A gene products in vivo [Lassar et al., 1991]. Upon forced expression in non-muscle cells, they can activate the transcription of muscle-specific genes and induce skeletal muscle phenotype [Davis et al., 1987; Weintraub et al., 1989].

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Myogenic bHLH regulators activate muscle gene transcription in cells cultured in low-mitogen medium. This transcription activation is inhibited by high concentration of serum or growth factors [Benezra et al., 1990; Jen et al., 1992]. The expression of several growth-stimulating genes, including c-*ras* [Olson, 1992], c-*myc* [Miner and Wold, 1991; Olson, 1992], c-*jun* [Bengal et al., 1992; Li et al., 1992], cyclin D1 [Rao et al., 1994; Skapek et al., 1995], adenovirus E1A protein [Webster et al., 1988], and SV40 large T antigen [Gu et al., 1993] can also inhibit the transcription activity of the myogenic bHLH factors.

Members of the E2F family of transcription factors are involved in the regulation of cell cycle progression and cell proliferation [Lam and La Thanque, 1994; Nevins, 1992]. First, E2F activates transcription of several genes important for cell proliferation including dihydrofolate reductase, DNA polymerase α, cyclin E, p34cdc2, β-*myb*, and c-*myc*, etc. [Degregori et al., 1995; Helin and Harlow, 1993]. Second, E2F is in complex with many proteins involved in cell cycle progression such as the retinoblastoma protein (RB), p107, cyclin A-cdk2, and cyclin E-cdk2 [Lam and La Thanque, 1994]. E2F appears to be the target for RB which block cell cycle in G1 phase [Sherr, 1994; Weinberg, 1995].

Third, deregulated expression of E2F1, the first member of the E2F family of proteins been cloned, can lead to S-phase entry and subsequently apoptosis in quiescent cells [Cress et al., 1993; Johnson et al., 1993; Qin et al., 1994; Shan and Lee, 1994; Wu and Levine, 1994].

We have previously shown that deregulated E2F1 expression in C2C12 myocytes inhibits *in vitro* myogenic differentiation [Wang et al., 1995]. In the present study, we investigated the effects of E2F1 on the transcription activity of myogenic bHLH proteins myoD and myogenin. We demonstrated that E2F1 can repress the ability of myoD and myogenin to activate transcription from the muscle specific creatine kinase gene (MCK) promoter. By using different deletion mutants of E2F1, it is shown that this inhibition of myoD transcription activity requires both the DNA binding and transactivation domains of E2F1. Since these two domains are required for the transcription activity of E2F1, our results suggest that E2F1 prevents myogenic differentiation through the induction of genes which inhibit the function of myogenic bHLH regulators.

MATERIAL AND METHODS

Plasmids. The reporter plasmid p1256MCK-CAT which contains the muscle specific creatine kinase promoter linked to chloramphenicol acetransferase (CAT) was given by S. Hauschka (University of Washington); pEMSV-myoD is from H. Weintraub (Fred Hutchinson Cancer Research Center); pEMSV-myogenin is from E. Olson (M.D. Anderson Cancer Center); pCMV-E2F1, pCMV-E2F1$_{1-416}$, and pCMV-E2F1$_{1-284}$ are from K. Helin (Denish Cancer Society); pCMV-E2F1$_{D113-120}$ is from D. Cress (Duke University); pCMV-RB is from X. Qin (DANA Farber Cancer Institute).

Cell culture. C3H10T1/2 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). C3H10T1/2-myoD cells (courtesy of H. Weintraub) were cultured in DMEM/20% FBS. Cell differentiation was initiated by transferring the cells to differentiation medium (DMEM with 2% heat-inactivated horse serum).

Cell transfection and CAT assay. C3H10T1/2 or 10T1/2-myoD cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). Each 100 mm petri dish of cells were co-transfected with the recombination of plasmids as indicated in the figure legends. 24 h after transfection, the cells were transferred to differentiation medium (DMEM with 2% heat-inactivated horse serum). After 48 h in differentiation medium, the cells were collected and whole cell lysates were prepared by repeated freeze and thaw. The Chloramphenicol transferase (CAT) and β-gal assays were performed as described [Schneider et al., 1994]. The CAT activity was normalized with β-gal activity from the same sample and expressed as relative CAT activity in the histogram.

Immunoblotting. C3H10T1/2 cells were transfected with 10 μg of different E2F1 constructs. Two days after transfection, cells were collected and lysed by boiling in 2X sample buffer (100 mM Tris pH 6.5, 2% SDS, 10% glycerol, 0.025% phenol blue, 5% β-mercaptoethanol). The cell lysates were separated on 10% SDS-PAGE and the immunoblotting analysis was performed as described [Wang and Nadal-Ginard, 1995] by using the E2F1 antibody KH20, which recognizes the DNA binding region of E2F1, or KH95 which recognizes the C-terminus of E2F1 [Helin et al., 1993a].

RESULTS

E2F1 Inhibits Transcription Activation by myoD and Myogenin

We have previously reported that constitutive E2F1 overexpression in C2C12 myocytes inhibits its myogenic differentiation [Wang et al., 1995]. To further explore the molecular mechanisms by which E2F1 inhibits myogenesis, we investigated the effects of E2F1 on the transcription activity of myogenic basic Helix-Loop-Helix regulators. In these experiments, we used the muscle-specific reporter construct p1256MCK-CAT, which consists of the muscle creatine kinase (MCK) gene enhancer/promoter driving the CAT gene. MCK promoter region contains multiple E-boxes and its transcription can be activated by co-transfected myogenic bHLH proteins in fibroblasts cultured under low serum conditions. 10T1/2 fibroblast cells were transfected with p1256MCK-CAT and either pEMSV-myoD or pEMSV-myogenin. The transfected cells were cultured for 48 h in differentiation medium before determination of CAT activities. Under these conditions, myoD and myogenin can activate the transcription of CAT gene from the MCK promoter. However, when pCMV-E2F1 was included in these co-transfection assays, this transcription activation by myoD and myogenin was inhibited (Fig. 1). Control experiments showed that transcription from RSV promoter (RSV-CAT) was not significantly inhibited by co-transfected pCMV-E2F1 treated in the

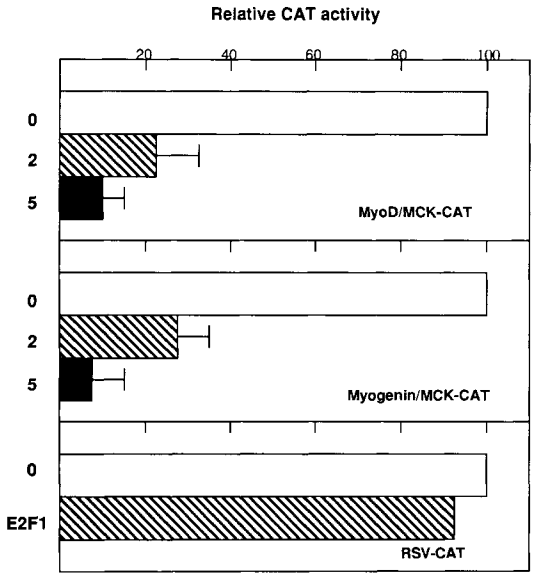

Fig. 1. E2F1 inhibits myoD and myogenin transcription activation. 10T1/2 fibroblasts were co-transfected with 10 μg of the MCK-CAT reporter plasmid, 2 μg of pCMV-β-gal, 5 μg of pEMSV-myoD or pEMSV-myogenin (as indicated), and without (0) or with either 2 μg (2) or 5 μg (5) of pCMV-E2F1. After transfection, the cells were cultured in the differentiation medium for 48 h and then the CAT and β-gal activities of the transfected cultures were measured. The CAT activity from each transfected culture was normalized against the β-gal activity for transfection efficiency and cell numbers. myoD and myogenin can activate transcription from the MCK promoter. Co-transfected pCMV-E2F1 inhibited this transcription activation of myoD and myogenin. In contrast, the normalized CAT activity from RSV-CAT construct did not change significantly in the presence (+E2F1, 2 μg) or absence (0) of co-transfected pCMV-E2F1. The experiments were repeated three times and mean values from the three experiments were expressed as relative CAT activities with standard error bars on the histogram.

same conditions (Fig. 1). These results indicate that E2F1 can inhibit the transcription function of myogenic bHLH regulators.

Both the DNA Binding and Transactivation Domains of E2F1 are Required in its Inhibition of myoD Transactivation

We next tested the ability of different E2F1 mutant constructs to inhibit myoD transactivation. $E2F1_{1-284}$ [Helin et al., 1993b] lacks the C-terminal 153 amino acids which includes both the transactivation and RB binding domains of E2F1. $E2F1_{1-416}$ lacks the C-terminal 21 amino acids which contains part of the RB binding domain [Helin et al., 1993b]. $E2F1_{1-416}$ can still activate transcription from the Adenovirus E2 promoter and this transcription activation is not inhibited by RB. $E2F1_{D113-120}$ [Cress et al., 1993] has an internal deletion in the DNA binding domain and is unable to bind to the E2F consensus DNA sequences (Fig. 2A). When co-transfected with pEMSV-myoD and MCK-CAT into 10T1/2 cells, $E2F1_{1-284}$ and $E2F1_{D113-120}$ failed to inhibit the transcription activity of myoD (Fig. 2C). These results indicate that both the DNA binding and transactivation domains of E2F1 are required for E2F1 inhibition of myoD function. In contrast, $E2F1_{1-416}$ can effectively inhibit the transactivation of myoD indicating that the RB binding domain of E2F1 is not involved in its inhibition of myoD function.

RB Repress E2F1 Inhibition of myoD Function

E2F1 is one target for the cell growth-repression function of RB. Ectopic expression of RB inhibits the transcription activity of E2F1 [Cress et al., 1993; Helin et al., 1993a]. We therefore tested if RB can also inhibit E2F1 function on myogenesis. To facilitate the co-transfection assay, we used the 10T1/2-myoD cells in these experiments. 10T1/2-myoD cells constitutively express an exogenous myoD from RSV promoter and these cells can undergo myogenic differentiation when cultured in low-serum differentiation medium. When MCK-CAT was transiently transfected into these cells, the constitutively expressed myoD can activate transcription of the CAT gene under differentiation conditions (Fig. 3, lane 0). E2F1 and $E2F1_{1-416}$ inhibited this transcription activation of MCK-CAT in 10T1/myoD cells. When pCMV-RB was co-transfected with E2F1 or $E2F1_{1-416}$, it partially restored the transcription of CAT gene in E2F1 transfected cell cultures but not in $E2F1_{1-416}$ transfected cell cultures. Since $E2F1_{1-416}$ does not bind to Rb, these results indicate that RB represses E2F1 inhibition of myogenesis through its physical interaction with E2F1 proteins.

DISCUSSION

The myogenic bHLH regulators can activate transcription from the muscle specific gene promoter, such as the MCK promoter in co-transfected non-muscle cells cultured under low-serum conditions. Factors that inhibit *in vitro* myogenic differentiation, such as high-serum medium and the expression of several growth-stimulating genes, has been shown to inhibit the transcription activity of myoD family proteins. In this study, we demonstrated that the cellular transcription factor E2F1 can also inhibit the transcription activation of myoD and myogenin (Fig. 1). This function of E2F1 requires its DNA

binding and transactivation domains (Fig. 2) and can be repressed by co-transfected RB protein (Fig. 3).

In cultured myogenic cell lines, cell proliferation and myogenic differentiation are mutually exclusive [Nadal-Ginard, 1978]. The myogenic bHLH regulators can activate muscle specific gene transcription and also prevent S-phase entry in transformed cells [Crescenzi et al., 1990; Sorrentino et al., 1990]. Many growth-stimulating genes, including E2F1 as we demonstrated here, can inhibit the transcription activity of myoD. It has been previously shown that the retinoblastoma protein pRB interact and enhance myoD function [Gu et al., 1993]. High level expression of E2F1 proteins may interfere with the interaction between myoD and pRB. However, this may not be the mechanism by which E2F1 inhibit myoD function since $E2F1_{1-416}$,

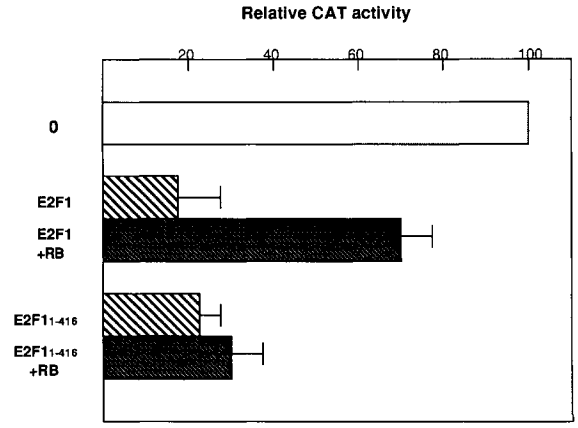

Fig. 3. RB repress E2F1 inhibition of myogenesis. Ten micrograms of MCK-CAT reporter plasmid were transfected into 10T1/2-myoD cells alone (0), with 2 μg of pCMV-E2F1 (E2F1), 2 μg of pCMV-E2F1, and 5 μg of pCMV-RB (E2F1 +RB), 2 μg of $E2F1_{1-416}$ ($E2F1_{1-416}$), or 2 μg of $E2F1_{1-416}$ plus 5 μg of pCMV-RB ($E2F1_{1-416}$ +RB). After the transfected cells were cultured in differentiation medium for 2 days, cells were collected and the relative CAT activities were measured. Mean values from triplicate experiments are presented on the histogram with standard error bars.

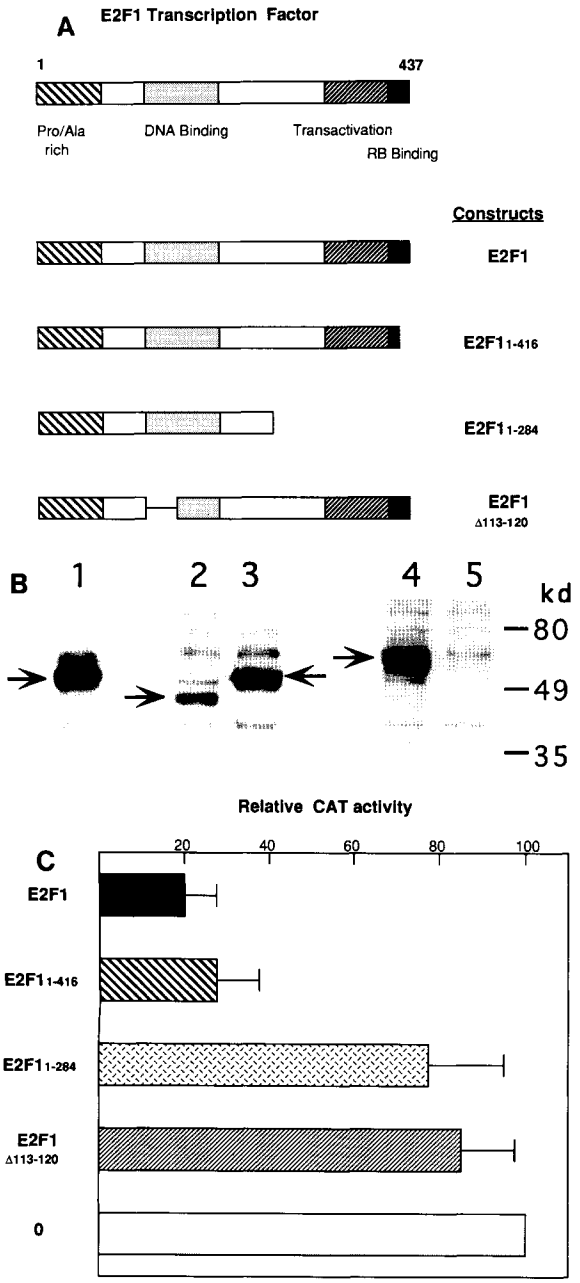

Fig. 2. (A) Diagram of the E2F1 constructs. The various functional domains of E2F1 gene are shown on the upper panel. The different E2F1 constructs used in this study are shown on the lower panels. (B) Transient expression of the different E2F1 constructs in 10T1/2 cells. 10T1/2 fibroblasts were transfected with 10 μg of pCMV-$E2F1_{D113-120}$ (*lane 1*), pCMV-$E2F1_{1-284}$ (*lane 2*), pCMV-$E2F1_{1-416}$ (*lane 3*), pCMV-E2F1 (*lane 4*), or vector alone (*lane 5*). Two days after transfection, cell lysates were collected and subjected to immunoblotting analysis with anti-human E2F1 antibody KH95 (1) or KH20 (2–5). Arrows indicate the ectopically expressed E2F1 proteins in each samples. Molecular weight markers are shown on the right. Note that two bands were detected in the lysates of wild type E2F1 transfected cells (*lane 4*). (C) Both the DNA binding and the transcription activation domains of E2F1 are required for E2F1 inhibition of myoD transcription activation. The various E2F1 constructs shown in (A) were co-transfected with MCK-CAT and pEMSV-myoD into 10T1/2 fibroblasts and the relative CAT activities of each transfected cultures were measured. Only the wild type E2F1 and the $E2F1_{1-416}$ inhibited myoD activation of transcription from MCK promoter. Mean values from triplicate experiments are shown with standard error bars on the histogram. The relative CAT activity from MCK-CAT alone (0) is set as 100% of CAT activity.

which does not interact with pRB, can still inhibit the transcription activity of myoD (Fig. 3).

It has been shown that the adenovirus E1A gene product inhibits myogenin transcription activity by direct physical association with myogenin [Tylor et al., 1993]. It is possible that E2F1 can also associate with myoD and myogenin. However, we have been unable to demonstrate an association between *in vitro* translated E2F1 protein and GST-myoD or GST-myogenin fusion proteins (data not shown). Further study is needed to address if E2F1 inhibition of myogenesis involves a physical association between E2F1 and the myogenic bHLH proteins.

A third possible mechanism exist that E2F1 inhibition of myoD transcription is mediated by genes products that are activated by E2F1. Several lines of evidences support this hypothesis. First, by using different mutants of E2F1, we demonstrated a correlation between the transcription activity of E2F1 and its ability to inhibit myoD function. The two E2F1 mutants that can not activate transcription, $E2F1_{1-284}$ and $E2F1_{D113-120}$, also lost the ability to inhibit myoD function. In contrast, $E2F1_{1-416}$, which can activate the transcription of E2F1-dependent genes but lacks the RB binding domain and is not inhibited by RB, retains the ability to inhibit the transcription activity of myoD and this inhibition is not repressed by co-transfected RB proteins. These data suggest that the transcription activity of E2F1 is involved in its inhibition of myoD function. Second, it has been shown that E2F1 can activate the expression of both DNA synthesis and cell cycle regulatory genes, including c-*myc,* b-*myb,* cyclin A, cyclin E, cyclin D1, cdc2, and cdk2 [Degregori et al., 1995; Singh et al., 1994]. Cyclin D1, c-*myc,* b-*myb* can directly inhibit myoD transcription activation. Although it has not been reported that cyclin A-cdk2 and cyclin E-cdk2 kinases can directly inhibit myoD transactivation, they can induce RB phosphorylation which inactivate this growth suppression protein [Weinberg, 1995]. It has been shown that RB and the RB family protein p107 and p300 are required during *in vitro* myogenic differentiation [Gu et al., 1993; Mymryk et al., 1992; Schneider et al., 1994]. Our previous work also indicated that the down-regulation of cyclin A, cyclin D1, and cdc2 proceed myogenic differentiation [Wang and Nadal-Ginard, 1995]. Therefore, elevated cdk2-cyclin A and cdk2-cyclin E activities induced by E2F1 overexpression may inhibit myoD transcription activity through the inactivation of RB family of proteins.

In summary, we demonstrated that E2F1 can inhibit the transcription activity of myogenic bHLH proteins and this inhibition involves the transcription activity of E2F1. Since E2F1 activate multiple genes important to cell cycle progression, our data support the notion that E2F1 may represent a nodal regulatory protein in coordinating cell proliferation and differentiation during muscle development.

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