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MyoD Stimulates *RB* Promoter Activity via the CREB/p300 Nuclear Transduction Pathway†

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The induction of *RB* gene transcription by MyoD is a key event in the process of skeletal muscle differentiation, because elevated levels of the retinoblastoma protein are essential for myoblast cell cycle arrest as well as for the terminal differentiation and survival of postmitotic myocytes. We previously showed that MyoD stimulates transcription from the *RB* promoter independently of direct binding to promoter sequences. Here we demonstrate that stimulation by MyoD requires a cyclic AMP-responsive element (CRE) in the *RB* promoter, bound by the transcription factor CREB in differentiating myoblasts. We also show that both the CREB protein level and the level of phosphorylation of the CREB protein at Ser-133 rapidly increase at the onset of muscle differentiation and that both remain high throughout the myogenic process. Biochemical and functional evidence indicates that in differentiating myoblasts, MyoD becomes associated with CREB and is targeted to the *RB* promoter CRE in a complex also containing the p300 transcriptional coactivator. The resulting multiprotein complex stimulates transcription from the *RB* promoter. These and other observations strongly suggest that MyoD functions by promoting the efficient recruitment of p300 by promoter-bound, phosphorylated CREB.

Skeletal muscle differentiation is characterized by a coordinated sequence of events that include irreversible exit from the cell cycle and the timely ordered activation of muscle-specific gene expression. This process is regulated by the MyoD family of basic helix-loop-helix (bHLH) transcription factors, including MyoD, Myf5, myogenin, and MRF4 (60). These factors activate transcription by heterodimerizing with ubiquitously expressed E proteins to bind a consensus DNA motif (E box) found in the regulatory region of many muscle-specific genes (25). The myogenic bHLH proteins cooperate with myocyte enhancer factor 2 (MEF2) transcription factors to activate muscle-specific gene transcription (32).

Among the myogenic bHLH factors, MyoD and Myf5 are involved in the determination of skeletal muscle precursors (46) and are expressed in proliferating myoblasts, which must irreversibly exit the cell cycle to activate muscle-specific gene transcription. Differentiation stimuli trigger the MyoD activation required to both promote cell cycle arrest and initiate the transcriptional cascade leading to muscle-specific gene expression (28). These two MyoD functions, although tightly coordinated, are temporally separated and controlled by distinct mechanisms. MyoD can induce growth arrest even in cell types nonpermissive for myogenic differentiation, and MyoD basic region mutants are unable to activate differentiation but can still induce cell cycle arrest (9, 55). The recent finding that MyoD requires SWI/SNF chromatin-remodeling activity for the induction of muscle-specific genes but not for cell cycle arrest adds further support to the notion of distinct mechanisms of action (11).

MyoD-mediated growth arrest relies upon the ability to induce the expression of at least three critical cell cycle regulators: the retinoblastoma growth suppressor, the CDK inhibitor p21, and cyclin D3 (19, 23, 30, 39). These MyoD-activated genes share the properties of being non-muscle-specific genes already expressed in proliferating myoblasts and of having their expression levels raised by MyoD at the onset of differentiation, with a requirement for the p300 transcriptional coactivator but not for new protein synthesis (6).

Very likely, elevated levels of hypophosphorylated retinoblastoma protein (pRB) are required to perform essential functions in differentiating myocytes. pRB promotes myoblast cell cycle arrest and maintains the absence of DNA replication in differentiated myotubes (33, 52). In addition, pRB promotes the expression of late-stage muscle-specific genes and prevents apoptotic cell death during myocyte differentiation (34, 59, 65). The parallel induction of p21 expression and cyclin D3 expression impinges upon the pRB functions in differentiated myocytes, since high levels of these proteins are needed to sustain augmented pRB activity levels: p21 contributes to maintaining pRB in the hypophosphorylated active state, and cyclin D3 is needed by pRB to sequester the proliferative factors CDK4 and PCNA into inactive complexes (6).

In an earlier study, Martelli et al. (30) demonstrated that MyoD stimulates transcription from the *RB* promoter by an E box-independent mechanism. Furthermore, analyses of MyoD mutants showed that, for the induction of *RB* promoter activ-

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ity, the DNA-binding region of MyoD was dispensable, whereas the helix-loop-helix (HLH) region was required; these findings suggested the importance of protein-protein interactions in this mechanism.

The present study identifies a cyclic AMP (cAMP)-responsive element (CRE) as the target of *RB* promoter activation by MyoD. Previous studies showed that the *RB* promoter displays features of a "housekeeping" gene with no typical TATA or CAAT boxes (21, 56) and that both the CRE and an adjacent RBF1/E4TF1-binding site are critical for basal-level promoter activity (17, 48, 51).

We have been able to identify CREB as the main transcription factor recognizing the *RB* promoter CRE in differentiating myoblasts and to determine that the DNA-binding activity of CREB is required by MyoD to enhance transcription from such a promoter. We show that the levels of CREB expression and CREB phosphorylation at Ser-133 are induced at the onset of differentiation and remain high in differentiated myocytes. We provide functional and biochemical evidence that in such cells, MyoD becomes associated with CREB and is targeted to the *RB* promoter CRE in a complex also containing the p300 and P/CAF coactivators. The resulting multiprotein complex appears to stimulate transcription from the *RB* promoter with a requirement for the acetyltransferase (AT) activity of P/CAF but not p300.

MATERIALS AND METHODS

Tissue cultures, transfections, and reporter gene assays. C2.7, a subclone of the C2 mouse myoblast cell line, was obtained from M. Buckingham. C2.7 myoblasts were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 20% fetal bovine serum (growth medium [GM]). To induce terminal differentiation, confluent myoblasts were shifted to DMEM containing 2% equine serum (differentiation medium [DM]). C3H10T1/2 fibroblasts were grown in DMEM supplemented with 20% defined calf serum (HyClone). The C3H/MyoD cell line was previously described (30). Transfections were carried out by using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. For reporter gene assays, cells were seeded at 10^5 per well in six-well dishes and transfected 18 h later. The amount of plasmid used in each transfection assay is indicated in the figure legends. Following transfection, the cells were cultured in GM for another 24 h. Afterward, differentiation was induced by incubation in DM for 48 h. Chloramphenicol acetyltransferase (CAT) assays were performed as previously described (18), as were luciferase assays (13). Equal amounts of protein from cell extracts were used for CAT and luciferase assays. The expression of transfected constructs was analyzed by Western blotting of protein extracts. Wild-type and mutant P/CAF and A-CREB (a dominant-negative inhibitor of CREB) were detected with an antibody against the FLAG epitope (M2; Sigma), p300 (positions 1 to 596) [p300(1–596)] was detected with anti-p300 antibody N-15, and Gal4p300(1514–1922) was detected with anti-Gal4 DNA-binding domain antibody RK5C1 (Santa Cruz Biotechnology). MyoD was detected with anti-MyoD monoclonal antibody 5.8A (kindly provided by P. Houghton).

Plasmid constructs. Many plasmid constructs used in this study were previously described and kindly provided by various laboratories: pEMSV-MyoD and pEMSV-B2ProB3 (10); pcDNA3-FLAG-MyoD (49); CMVβ-p300 (14); pCI-p300, pCI-p300(dl1472–1522), pCI-p300(dl1603–1653), pCX-P/CAF, pCX-P/CAF(dl579–608), pCX-P/CAF(dl609–624), and muscle creatine kinase-luciferase (MCK-LUC) (42); RC/RSV-CREB341 (24); pRC/CMV500A-CREB (1); glutathione *S*-transferase (GST)–p300 (amino acids 436 to 662) [GST-p300(436–662)] (4); pET-CREB327 (16); and pT7-MyoD (57).

pCMV-Gal4p300(1514–1922) was previously described (63). pCMV-p300(1–596) was constructed by cloning the *Bam*HI 5′-terminal fragment of p300 cDNA into the pCDNA3 vector. The pSV0t2-CAT vector was obtained by inserting the polylinker from pCAT-Basic (Promega) into the pSV0t-CAT vector (61).

Six deletion mutants of the human *RB* promoter spanning the promoter sequence from positions −510 to −85 (−510/−85), −370/−85, −228/−85, −176/−85, −508/−176, and −228/−176 (relative to the translation start site) (see Fig. 1A) were obtained by PCR amplification with the HRP-CAT(−510/−85) plasmid (30) as a template. The PCR products were cloned into the pSV0t2-CAT vector.

From the RB-CAT(−228/−176) construct (containing the wild-type sequence), mutants carrying inactivations of either the RBF1, CRE, or E2F sites were obtained by introducing, via PCR amplification, the relevant nucleotide substitutions (Fig. 2, bottom). The effectiveness of such mutations was monitored by gel retardation comparisons with the corresponding wild-type DNA motifs. The same three mutated sequences were cloned into the pt(18)TK-CAT (61) vector to obtain the RB-TK-CAT(−228/−176) mutant derivatives.

Extracts, immunoprecipitations, and Western blotting. Whole-cell extracts of C2.7, CH3/MyoD, or C3H10T1/2 cells, cultured in GM or DM, were prepared in lysis buffer (50 mM Tris [pH 7.5], 250 mM NaCl, 1% Nonidet P-40 [NP-40], 5 mM ATP, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 10 mM NaF) supplemented with a mixture of protease inhibitors (Roche). After 30 min at 4°C, the lysates were centrifuged to remove insoluble components. For immunoprecipitation experiments, 3×10^5 C3H10T[1/2] cells were transfected with expression vectors for MyoD (pcDNA3-FLAG-MyoD) and/or CREB (RC/RSV-CREB341) at 2.5 μg each. Transfected cells were cultured either in GM or in DM for 48 h before being harvested. Cell extracts were prepared in 0.5 ml of immunoprecipitation buffer (50 mM Tris [pH 8], 120 mM NaCl, 0.5% NP-40, 5 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 10 mM NaF) supplemented with Roche protease inhibitors. Lysates were incubated on ice for 30 min, sonicated for 2 min (2-s cycle time), and cleared by centrifugation. A 500-μg sample of each extract was precleared for 6 h with anti-mouse immunoglobulin G covalently linked to agarose beads (Sigma). FLAG-MyoD was immunoprecipitated with anti-FLAG antibody M2 overnight at 4°C. For Western blot analysis, the extracts or the immunoprecipitated products were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by liquid transfer to nitrocellulose membranes (Schleicher & Schuell). Membranes were blocked with 5% nonfat dry milk in phosphate-buffered-saline (PBS) and incubated with primary antibody. Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Filters were then processed for enhanced chemiluminescence detection (Super Signal; Pierce). The following antibodies were used: polyclonal anti-CREB and anti-phospho-CREB(ser133) (NE Biolabs), anti-myogenin IF5D (62), anti-myosin heavy chain MF20 (3), anti-CDK4 C-22 (Santa Cruz Biotechnology), and anti-MyoD (5.8A).

Immunofluorescence. The expression vector carrying A-CREB tagged with FLAG (pRC/CMV500A-CREB) or an empty expression vector was cotransfected with an expression vector carrying enhanced green fluorescent protein (EGFP/C1; Clontech) at a ratio of 9:1 into C2 myoblasts by using the Lipofectamine reagent. The transfected cells were cultured in GM for another 24 h and then induced to differentiate by incubation in DM for 12 h. To maintain proliferation, the cells were sparsely seeded after transfection and cultured in GM for 36 h. Cells were then fixed with 4% paraformaldehyde in PBS for 15 min at 4°C, rinsed with PBS, and incubated in a solution of 50 mM glycine in PBS for 10 min at room temperature. The fixed specimens were permeabilized by treatment with 0.25% Triton X-100 in PBS for 10 min at room temperature and preincubated with 3% bovine serum albumin in PBS to block nonspecific binding. Samples were incubated with anti-FLAG antibody M2 for 1 h at 37°C, washed three times with PBS containing 0.1% NP-40, and incubated with rhodamine-conjugated second-step anti-mouse immunoglobulin antibody (Pierce). Samples were then rinsed several times with NP-40–PBS and stained with Hoechst 33258 (1 μg/ml for 2 min).

DNA-binding assays. For electrophoretic mobility shift assays (EMSAs), whole-cell extracts of differentiated C2.7 myocytes were prepared by a rapid extraction method (37). Briefly, cells were pelleted and resuspended in 1.5 volumes of extraction buffer (20 mM HEPES [pH 7.9], 0.4 M NaCl, 25% glycerol, 1 mM EDTA, 2.5 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], 5 mM ATP, 5 mM $MgCl_2$, 0.1 mM Na_3VO_4, 5 mM β-glycerophosphate) supplemented with Roche protease inhibitors. Cells were kept on ice for 20 min, frozen at −70°C, and thawed on ice. The suspension was then vigorously mixed and cleared by centrifugation. In vitro-translated MyoD and E12 were prepared as previously described (30). Synthetic oligonucleotides (see Fig. 2 and 4) were ^{32}P end labeled with T4 polynucleotide kinase. DNA-binding reactions and electrophoretic conditions were as previously described (30).

For antibody EMSAs, the following antibodies were used: anti-CREB1 X-12, anti-ATF1 F1-1, anti-KID ATF1-1 (25C10G), and anti-CBP A-22 (all from Santa Cruz Biotechnology) and anti-p300 RW102, RW109, RW105, and RW128 (tissue culture supernatants; kindly provided by R. Eckner).

CRE-bound complexes were affinity purified by means of biotinylated oligonucleotides immobilized on streptavidin-conjugated magnetic beads (Dynabeads

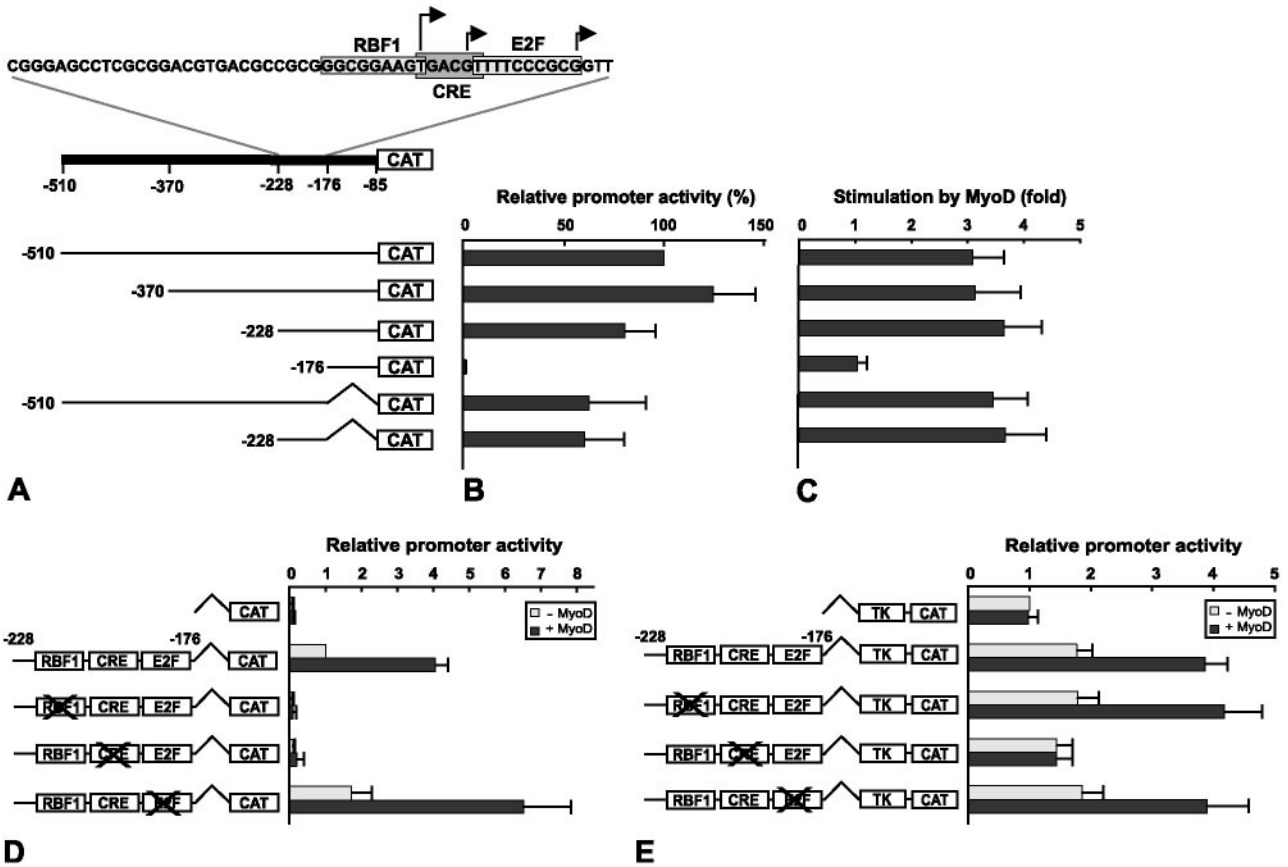


FIG. 1. Identification of the MyoD-responsive element within the *RB* promoter. (A) Schematic diagram of the *RB* promoter-CAT reporter construct [RB-CAT(−510/−85)], with magnification of the −228/−176 nucleotide sequence containing the RBF1, CRE, and E2F DNA elements and the transcription initiation sites (arrows). The promoter deletion mutants are also displayed. (B) C2 myoblasts were transfected with 0.5 μg of the *RB* promoter-CAT reporter constructs shown in panel A. Transfected cells were cultured in DM for 48 h before being harvested for CAT assays as described in Materials and Methods. Values from CAT assays are presented as percentages of the activity obtained with the RB-CAT (−510/−85) construct. (C) C3H10T1/2 fibroblasts were transfected with 0.5 μg of the *RB* promoter-CAT reporter constructs shown in panel A, together with 0.5 μg of either pEMSV-MyoD or the empty expression vector. CAT activity was determined after 48 h of incubation in DM. Bars represent the CAT activity in cells transfected with MyoD divided by that in cells transfected with the empty vector. (D) The pSV0t2-CAT vector, the RB-CAT(−228/−176) construct, or its derivatives carrying mutations inactivating the CRE, RBF1, or E2F site (crossed-out boxes in the drawing) (0.5 μg each) were transfected into C3H10T1/2 fibroblasts, together with either 0.5 μg of pEMSV-MyoD or the empty vector. CAT activity was determined after 48 h of incubation in DM. Values from CAT activity assays are expressed relative to the value obtained with the RB-CAT(−228/−176) construct. (E) Experiments were carried out as described for panel D, except that RB-TK-CAT(−228/−176) and its mutant derivatives were used as promoter-reporter constructs. The CAT activity values obtained with such constructs were divided by the value obtained with the pt(18)TK-CAT vector. The means of at least five independent experiments, each performed in duplicate, are shown. Error bars represent standard deviations.

M-280 streptavidin). High-salt extracts prepared from differentiated C2.7 cells were diluted sixfold in binding reaction buffer (15 mM HEPES [pH 7.9], 40 mM KCl, 5% glycerol, 1 mM EDTA, 0.5 mM DTT) and precleared overnight at 4°C on magnetic beads previously blocked with 1% nonfat milk in PBS. Twenty picomoles of RB(−197/−181) biotinylated oligonucleotide, either wild type or mutated in the CRE, was incubated with 10 μl of blocked magnetic beads in binding reaction buffer for 20 min at room temperature. After repeated washes, the beads were resuspended with 500 μg of precleared cell extract in the presence of 60 μg of poly(dI-dC). After 20 min of incubation at room temperature, protein complexes bound to the magnetic beads were extensively washed, resuspended in SDS sample buffer, and loaded onto SDS-polyacrylamide gels. Immunoblotting was performed with anti-CREB, anti-MyoD (5.8A), anti-p300 (RW128; Upstate Biotechnology), and anti-P/CAF (kindly donated by Y. Nakatani).

Expression and purification of recombinant proteins. GST-p300(436–662) was transformed into *Escherichia coli* BL21, and protein expression was induced at 30°C with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h during the exponential phase of growth of the bacterial culture. The bacteria were harvested and sonicated in a buffer that contained 20 mM Tris (pH 8), 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40 and that was supplemented with 1 mM PMSF and protease inhibitors (Complete; Roche). Following sonication, NP-40 was added to 1% (vol/vol), and the lysates were centrifuged to remove cell debris. The supernatants were mixed with glutathione-Sepharose 4B (Amersham) for 60 min at room temperature. The beads were washed, and bound protein was eluted with 50 mM Tris-HCl (pH 8)–5 mM reduced glutathione–1 mM DTT–1 mM PMSF. The elution step was repeated three times, and the fractions were dialyzed against TM–0.1 M KCl (50 mM Tris-HCl [pH 7.9], 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA [pH 8], 20% glycerol, 0.025% Tween 20, 1 mM DTT, 1 mM PMSF). The bacterial expression plasmid for MyoD, pT7-MyoD, was transformed into *E. coli* BL21(DE3)/pLysS cells, and the protein was induced with 0.4 mM IPTG for 3 h. MyoD protein was purified by the procedure described by Thayer and Weintraub (57). The bacterial expression plasmid for CREB, pET-CREB327, was expressed in *E. coli* BL21(DE3)/pLysS, and the protein was induced with 0.1 mM IPTG for 3 h at 30°C. Cells were harvested, resuspended in 0.2 volume of 10 mM Tris–1 mM EDTA (pH 8.0), and boiled for 8 min as previously described (20). Following centrifugation, the supernatants were subjected to heparin-agarose chromatography and dialyzed against TM–0.1 M KCl. The concentration and purity of each protein were estimated by SDS-PAGE.

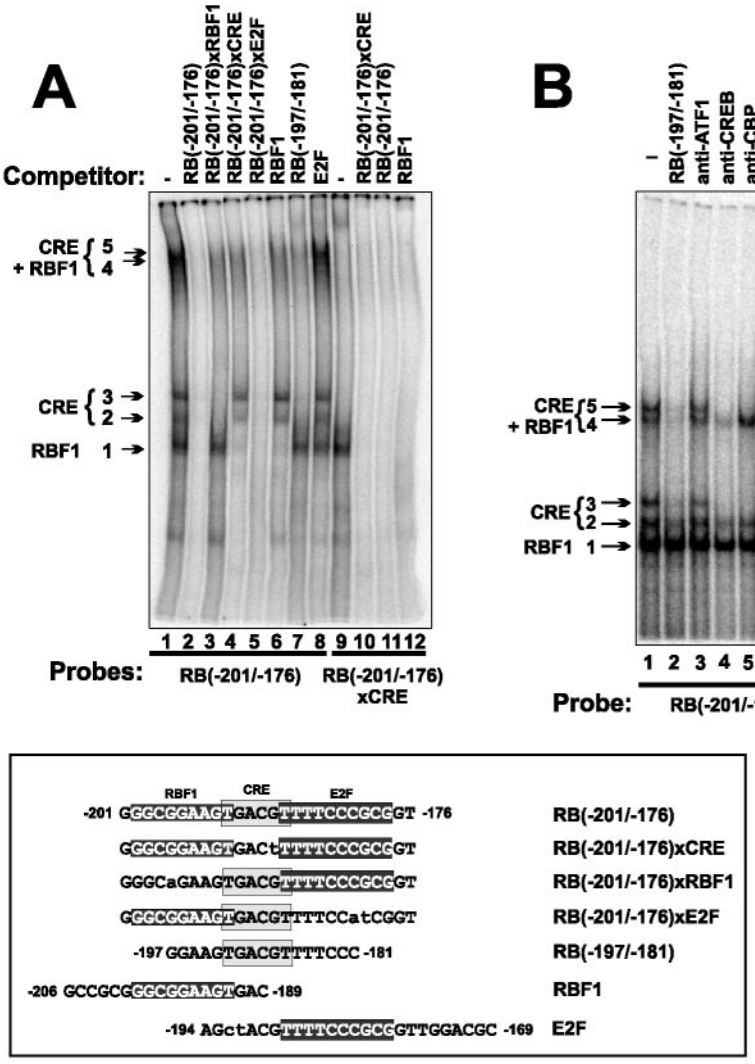


FIG. 2. EMSAs of the *RB* promoter with C2 myotube extracts. (A) Two ^{32}P-labeled oligonucleotides—either RB(−201/−176), with a wild-type sequence (lanes 1 to 8), or RB(−201/−176)xCRE, with a point mutation inactivating the CRE site (lanes 9 to 12), were used in EMSAs with C2 myocyte extracts in the presence of a 100-fold excess of the cold oligonucleotide competitors indicated above the lanes. The arrows indicate the shifted bands generated by nuclear factor binding to the RBF1 and/or the CRE site, as identified through the inhibition of DNA binding by the specific oligonucleotide competitors. (B) The ^{32}P-labeled RB(−201/−176) oligonucleotide was incubated with C2 myocyte extracts and a 100-fold excess of either the cold RB(−197/−181) oligonucleotide (lane 2) or the antibodies indicated above the lanes (lanes 3 to 7). The arrows indicate the shifted bands generated by nuclear factor binding to the RBF1 and/or the CRE site. (C) The ^{32}P-labeled RB(−197/−181) oligonucleotide was incubated with C2 myocyte extracts and the antibodies indicated above the lanes. The arrows indicate the shifted bands generated by CREB binding, as identified through supershifting by the specific antibodies. The sequences of the wild-type and mutant oligonucleotides used in these EMSAs are shown in the box; lowercase characters indicate mutations.

GST-p300, MyoD, and CREB were purified to near homogeneity by these procedures.

Recombinant CREB protein was phosphorylated by using the purified catalytic subunit of protein kinase A (PKA) by incubating 1.6 μM CREB in a reaction mixture containing 200 μM ATP, 10 mM $MgCl_2$, and 10,000 U of PKA (NE Biolabs) in 50 mM Tris-HCl (pH 7.5) for 1 h at 30°C. Successful phosphorylation was monitored by EMSAs, as the phosphorylated CREB protein migrates with a reduced mobility in native gels.

GST pull-down assays. GST pull-down experiments were performed by using 12.5 μl of glutathione-Sepharose beads equilibrated in binding buffer (20 mM HEPES [pH 7.9], 100 mM KCl, 10% glycerol, 0.2% NP-40, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 μM NaF, 10 μM Na_3VO_4) containing 5 mg of bovine serum albumin/ml. Purified GST protein was incubated with the beads for 1 h at 4°C and then washed with binding buffer. The second protein was added to the beads and incubated for 2 h at 4°C. The beads were washed several times as described above, and bound proteins were eluted with SDS sample buffer. The proteins were separated by electrophoresis, transferred to nitrocellulose, and probed with the appropriate antibodies.

RESULTS

Identification of the MyoD-responsive elements in the *RB* promoter. In a previous study (30), it was shown that MyoD stimulates the activity of a human *RB* promoter-reporter construct containing the DNA sequence between nucleotides −510 and −85, relative to the translation start site of the *RB* gene.

To identify the minimal region of the −510/−85 *RB* promoter retaining responsiveness to MyoD, we generated a series

of 5′ and 3′ promoter deletions driving the transcription of a CAT reporter gene (Fig. 1A). The relative activities of these constructs in transiently transfected differentiating C2 myoblasts were measured Fig. 1B). The results indicated that the sequence between nucleotides −228 and −176 was required for promoter activity in differentiated C2 myocytes and that this region alone retained about 60% of the activity of the −510/−85 promoter. We next examined the ability of MyoD to enhance the transcription driven by the *RB* promoter deletion constructs in transiently transfected C3H fibroblasts. The results shown in Fig. 1C indicated that the −228/−176 promoter region was sufficient for transcriptional activation by MyoD.

As previously reported (and illustrated in Fig. 1A), this region of the *RB* promoter contains three functional binding sites: a recognition site for the RBF1/E4TF1 transcription factor (indicated as RBF1), a CRE, and an E2F-binding site (48, 51, 53). These DNA elements are fully conserved in the human and murine *RB* promoters and constitute the core promoter where transcription initiates (17, 64). By using primer extension experiments, we identified three transcription start sites in the −228/−176 *RB* promoter-reporter construct transiently transfected in differentiating C2 cells, and these sites (Fig. 1A) coincided with those identified by the studies cited above. It should also be added that another CRE consensus sequence upstream of the RBF1 site is visible, but no specific binding to it was found by EMSAs with C2 myocyte extracts (data not shown).

To determine which DNA element, among RBF1, CRE, and E2F, is required by MyoD to enhance *RB* promoter activity, we introduced into each element point mutations that prevent binding of the cognate factors. The −228/−176 reporter construct (hereafter referred to as RB-CAT) and its mutant derivatives were transfected either alone or in combination with MyoD into C3H10T1/2 fibroblasts. The results illustrated in Fig. 1D show that the inactivation of either the RBF1 or the CRE site totally abolished *RB* promoter activity. These elements thus appeared to be essential for basal transcription from the *RB* promoter and therefore could not be directly assayed for their responsiveness to MyoD. The E2F site mutation slightly increased the basal activity of the promoter but did not affect its response to MyoD.

In order to establish the role of RBF1 and CRE in MyoD-dependent stimulation of the *RB* promoter, the −228/−176 sequences (either wild type or carrying inactivating mutations) were fused upstream of the TATA box-containing core promoter of the herpes simplex virus thymidine kinase gene. In this context, where the transcription start site was provided by the TATA element, neither the RBF1 nor the E2F site mutations affected responsiveness to MyoD, whereas the CRE mutation completely abolished it (Fig. 1E). This result indicated that the CRE was the target for the MyoD-dependent stimulation of the *RB* promoter.

Analysis of the proteins binding to the CRE site. Because the RBF1 and CRE consensus sequences partially overlap, we tried to determine whether their cognate nuclear factors could simultaneously bind to the promoter and whether such binding was independent or interactive. To address this point, EMSAs were performed with the RB(−201/−176) oligonucleotide probe (carrying the RBF1, CRE, and E2F recognition sites) (Fig. 2). When incubated with extracts prepared from differentiated myocytes, this probe produced a pattern of retarded bands (bands 1 to 5 in Fig. 2A and B); such DNA-protein complexes were specific, since their formation was inhibited by excess cognate competitor (Fig. 2A, lane 2). All of the retarded bands were also competed away by the RB(−201/−176)xE2F oligonucleotide, which carries mutations that prevent E2F binding (Fig. 2A, lane 5). This result indicated that E2F does not contribute to the formation of any of the shifted bands.

Competition by RB(−201/−176)xCRE and RB(−201/−176) xRBF1 oligonucleotides (with mutations in the CRE and RBF1 sites, respectively) did change the pattern of shifted bands, although not in the same fashion. Excess RB(−201/−176)xCRE did not affect the formation of bands 2 and 3 but inhibited that of bands 1, 4, and 5 (Fig. 2A, lane 4); in contrast, excess RB(−201/−176)xRBF1 did not affect the formation of band 1 but inhibited that of bands 2, 3, 4, and 5 (Fig. 2A, lane 3). Thus, band 1 appeared to be generated by protein binding to the RBF1 site, and bands 2 and 3 appeared to be generated by protein binding to the CRE site; the more slowly migrating bands, 4 and 5, were likely generated by concomitant binding to the CRE and RBF1 sites. A similar conclusion was reached by using as unlabeled (cold) competitors a series of oligonucleotides, each containing only the RBF1, CRE, or E2F binding site (Fig. 2A, lanes 6, 7, and 8). Altogether, the results in Fig. 2A indicated that the CRE and RBF1 sites of the *RB* promoter, despite their overlap, allowed simultaneous and independent binding of cognate nuclear factors.

To identify the proteins that bind to the CRE of the *RB* promoter, we performed supershift assays by using the RB (−201/−176) probe and C2 myocyte extracts preincubated with specific antibodies (Fig. 2B). The results indicated that an anti-CREB antibody (unlike an anti-ATF1 antibody) inhibited almost completely the formation of the shifted bands generated by factor binding to the CRE (Fig. 2B, compare lanes 3 and 4); these bands were completely supershifted by an anti-KID antibody, which recognizes both ATF1 and CREB, being raised against a domain conserved in all members of the ATF/CREB family of transcription factors (Fig. 2B, lane 6). These results identify CREB as the main factor binding to the CRE of the *RB* promoter in differentiated C2 cells. Because CREB can recruit the CBP and p300 transcriptional coactivators to target promoters (7, 27), we investigated whether these proteins were also part of the CREB complexes assembled at the CRE of the *RB* promoter. It was found that both an anti-CBP antibody and an anti-p300 antibody altered the formation of the CREB-DNA complexes (Fig. 2B, lanes 5 and 7). Similar analyses were also performed by using as a probe the RB (−197/−181) oligonucleotide (which contains only the CRE); this probe, when incubated with myotube extracts, yielded two complexes (Fig. 2C, lane 1). Both complexes were completely supershifted by the anti-CREB antibody (Fig. 2C, lanes 3 and 4), whereas the addition of the anti-CBP antibody specifically inhibited the formation of the more slowly migrating complex (lane 5). Among the anti-p300 antibodies, only RW128 slightly inhibited the CREB-DNA complexes (Fig. 2C, lanes 6 to 9). These results provided a clue that CBP/p300 might participate in CREB complexes at the CRE site of the *RB* promoter (a question directly addressed by experiments reported below [see Fig. 4]).

MyoD stimulates *RB* promoter activity through CREB. The above results indicated that MyoD stimulates *RB* promoter activity in C2 myocytes by a noncanonical mechanism which does not involve direct MyoD binding to E boxes but instead requires a CRE site occupied by CREB. It could thus be expected that inhibition of the DNA-binding activity of CREB but not MyoD would inhibit the MyoD-mediated transactivation of the *RB* promoter. To verify this issue, we exploited the MyoD mutant B2ProB3, in which an inactivating mutation of the basic region prevents DNA binding (10), and A-CREB which, by heterodimerizing with endogenous CREB (1), selectively inhibits its DNA-binding and transcriptional activities. The results shown in Fig. 3A indicated that the DNA-binding mutant of MyoD could still stimulate the *RB* promoter, albeit with a lower efficiency than wild-type MyoD; this result was likely due to the loss of the self-activating ability of the mutant and the consequent lower level of expression in cells (as directly observed by Western blot analysis of the two proteins, shown in the lower panel). Inclusion of the A-CREB expression construct in the transfection mixtures inhibited the transactivating capacity of both wild-type and mutant MyoD in a dose-dependent manner. In contrast, A-CREB did not alter the ability of MyoD to transactivate the muscle-specific, E box-containing *MCK* promoter (Fig. 3B), whereas the MyoD DNA-binding mutant was completely defective in the transactivation of such a promoter (Fig. 3C). Altogether, these results fully support a mechanism whereby CREB functions as the DNA-recognizing factor of the MyoD-responsive element in the *RB* promoter, whereas the DNA-binding function of MyoD is dispensable.

CREB recruits MyoD, p300, and P/CAF to the CRE site of the *RB* promoter. To elucidate the mechanism by which MyoD stimulates *RB* promoter activity through CREB, we examined whether MyoD participates in CREB complexes at the CRE site of the *RB* promoter. To this end, a biotinylated RB(−197/−181) oligonucleotide immobilized on streptavidin-conjugated beads was incubated with extracts prepared from C2 myocytes; the protein-DNA complexes were then analyzed by Western blotting. The results in Fig. 4A showed that CREB, MyoD, p300, and P/CAF were all affinity purified by the immobilized CRE oligonucleotide, whereas they were not pulled down by a control oligonucleotide containing a mutated CRE site.

The possibility that MyoD by itself could bind to promoter sequences was excluded by analyzing in EMSAs the formation of protein-DNA complexes between in vitro-translated MyoD and the RB(−201/−176) oligonucleotide. An oligonucleotide probe containing the MyoD-binding site from the *MCK* promoter (MCK-R1) (61) was used as a control. As shown in Fig. 4B, MyoD in combination with E12 bound to the *MCK* E box, whereas no interaction of MyoD and/or E12 with the RB (−201/−176) oligonucleotide was detectable.

CREB is phosphorylated and becomes associated with MyoD during myogenic differentiation. One possibility concerning the mechanism allowing the assembly of the multimeric complex at the CRE site of the *RB* promoter is that p300, through its ability to interact directly with CREB, MyoD, and P/CAF, may act as a bridging molecule.

Since it has been well established that the interaction of CREB with p300 requires CREB phosphorylation on serine 133 (38), we first measured the CREB expression level and phosphorylation state during C2 myoblast differentiation. This was done by using, respectively, an anti-CREB antibody and an antibody that specifically recognizes only phosphorylated CREB and phosphorylated ATF1. The results, shown in Fig. 5A, indicated that both the total amount of CREB and its

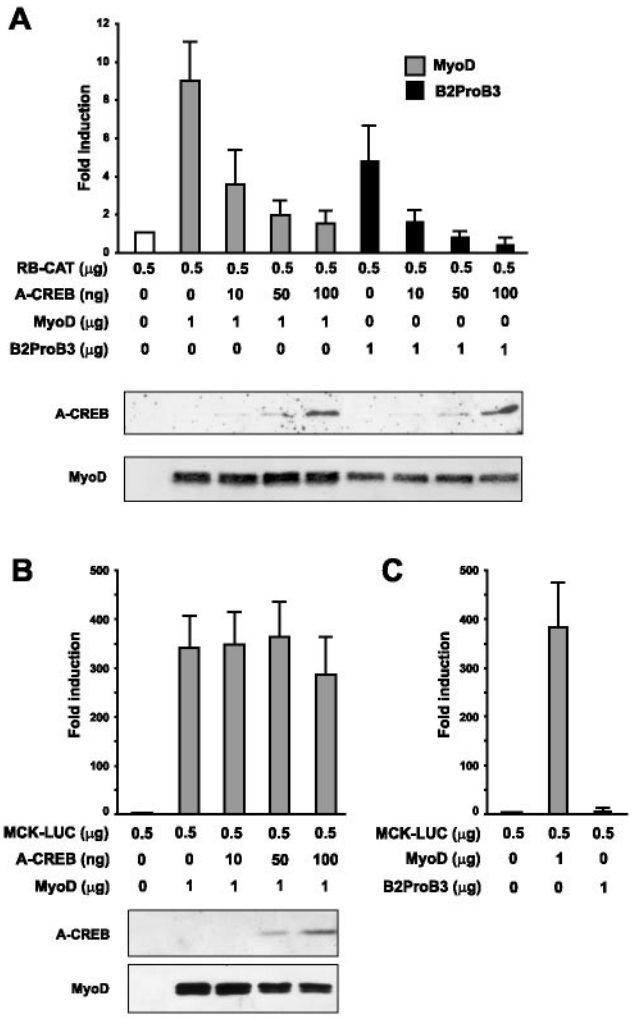


FIG. 3. Effect of a dominant-negative inhibitor of CREB on MyoD-mediated induction of the *RB* and *MCK* promoters. (A) C3H10T1/2 fibroblasts were transfected with the RB-CAT(−228/−176) construct and either pEMSV-MyoD or pEMSV-B2ProB3, along with increasing amounts of an expression construct carrying A-CREB (pRC/CMV500A-CREB). DNA concentrations were kept constant with empty vector DNA. Transfected cells were transferred to DM at 24 h posttransfection and harvested for CAT assays 48 h later. CAT activity values are expressed relative to the CAT activity of cells transfected with the RB-CAT(−228/−176) construct alone. Transfections were carried out in duplicate, and the means of five separate experiments (error bars, standard deviations) are presented. Protein extracts (30 μg) from a representative experiment were analyzed by Western blotting to monitor the levels of expression of MyoD and A-CREB (lower panels). (B) C3H10T1/2 fibroblasts were transfected with the MCK-LUC reporter and pEMSV-MyoD, along with increasing amounts of pRC/CMV500A-CREB. The cells were then processed as described for panel A. (C) C3H10T1/2 fibroblasts were transfected with the MCK-LUC reporter and either pEMSV-MyoD or pEMSV-B2ProB3. The cells were then processed as described for panel A.

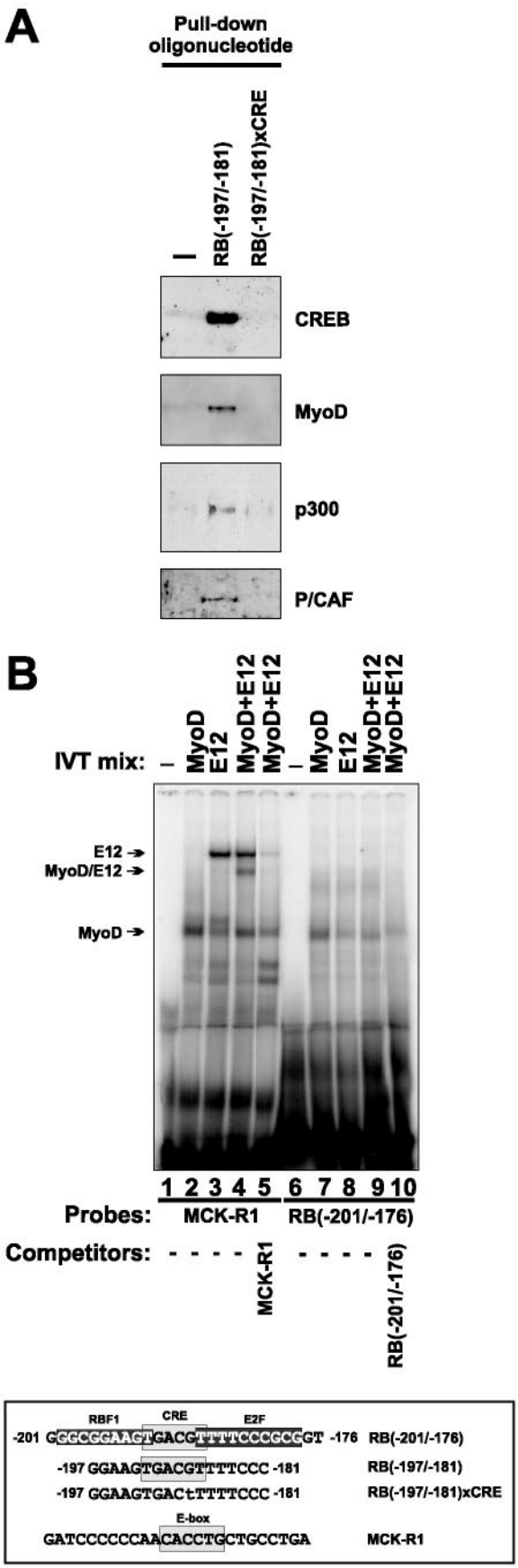


FIG. 4. Localization of MyoD at the CRE site of the *RB* promoter. (A) Protein complexes bound to the CRE site of the *RB* promoter were affinity purified from C2 myocyte extracts by using the RB(−197/−181) biotinylated double-stranded oligonucleotide. The RB(−197/−181)xCRE biotinylated CRE mutant oligonucleotide was used to prepare a parallel control sample. The affinity-purified proteins were analyzed by Western blotting with antibodies specific for CREB, MyoD, p300, and P/CAF. (B) The ^{32}P-labeled, E box-containing MCK-R1 oligonucleotide (lanes 1 to 5) and the RB(−201/−176) oligonucleotide (lanes 6 to 10) were incubated with reticulocyte lysates either unprogrammed (lanes 1 and 6) or programmed with MyoD or E12 mRNA, as indicated above the lanes. The arrows indicate the bands generated by MyoD, E12, and MyoD-E12 dimers, as identified by specific competition with the unlabeled MCK-R1 double-stranded oligonucleotide (lane 5). IVT, in vitro translation. The sequences of the oligonucleotides used in these experiments are shown in the box.

phosphorylation at Ser-133 increased early during the differentiation process.

To determine whether CREB phosphorylation is a specific feature of the differentiation process, we analyzed the expression and phosphorylation state of CREB in C3H10T1/2 fibroblasts shifted to DM for increasing times as well as in parallel cultures of C3H10T1/2 fibroblasts converted to myoblasts by the stable expression of exogenous MyoD (Fig. 5C and B, respectively). It was found that CREB phosphorylation increased in both cell lines as soon as the cells reached confluence and remained at high levels upon the shift to DM. In contrast, the phosphorylation of ATF1 decreased after 24 h in DM. These observations indicated that the phosphorylation of CREB on Ser-133 does not seem to be a peculiar feature of myogenic differentiation but is associated with cell cycle arrest in nonmyogenic cells as well.

If p300 functions as the bridging molecule holding together MyoD and phospho-CREB in the same complex, MyoD would be expected to interact with CREB in a manner dependent on CREB phosphorylation. To address this issue, a FLAG-tagged MyoD expression construct was transfected, either alone or in combination with a CREB expression construct, into C3H10T1/2 fibroblasts. Following transfection, the cells were either kept in GM or shifted to DM for 48 h before being harvested. MyoD was then immunoprecipitated from transfected cells, and the presence of CREB and phospho-CREB in these immunoprecipitates was tested by Western blot analysis with specific antibodies (Fig. 6). The results revealed an association between MyoD and phospho-CREB in cells exposed to DM (Fig. 6, lanes 7 and 8), whereas no association between MyoD and unphosphorylated CREB was observable in cells cultured in GM (lanes 3 and 4). Such a correlation with CREB phosphorylation supports the hypothesis that the interaction between CREB and MyoD in differentiating myoblasts occurs through p300.

MyoD and phospho-CREB can form a ternary complex with the KIX domain of p300. MyoD is known to directly bind to p300 at two distinct sites, one located in the C/H3 region of p300 and the other located in an N-terminal domain comprised within the first 596 residues (15, 49, 63). A recent study more precisely mapped the p300 N-terminal MyoD-interacting domain to a region spanning residues 436 to 662 (45). The minimum phospho-CREB-binding domain of p300, termed KIX, comprises residues 566 to 647 (43). These two binding domains

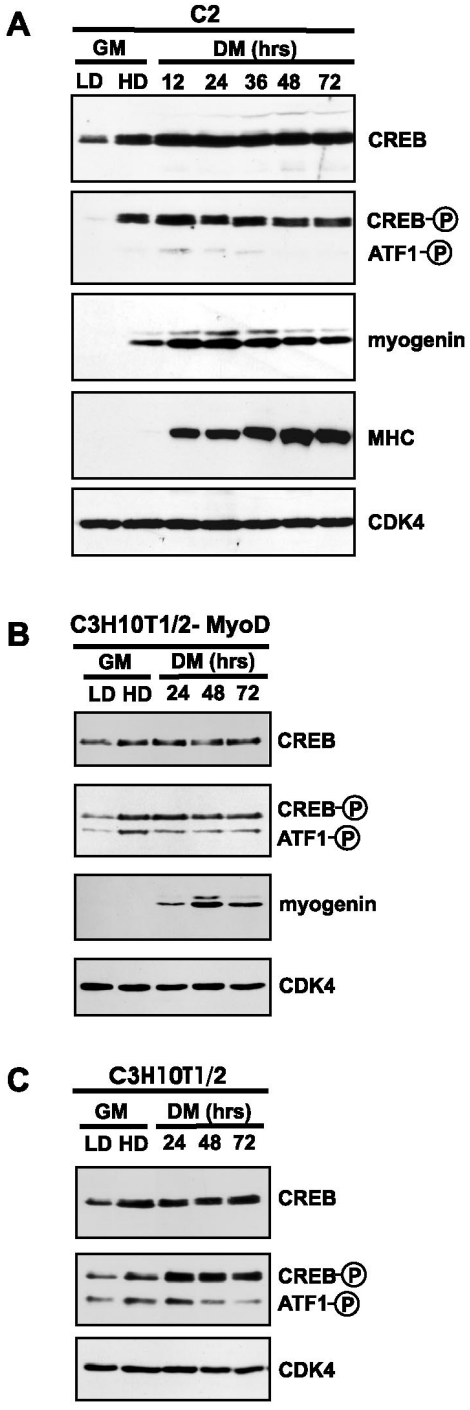


FIG. 5. Analysis of CREB Ser-133 phosphorylation during C2 cell differentiation. (A) Whole-cell extracts prepared either from growing C2 cells (GM) cultured at low or high cell densities (LD or HD) or from C2 cells exposed to DM for the indicated times were analyzed by Western blotting with antibodies specific for CREB or phospho-CREB (Ser-133) (which also recognize the phosphorylated form of ATF1). The timing of differentiation was monitored by analyzing the expression of an early (myogenin) or a late (myosin heavy chain [MHC]) differentiation marker. Equal loading of the extracts was monitored with anti-CDK4. P, phosphorylated. (B) Expression levels and phosphorylation state of CREB during the differentiation of C3H/MyoD cells. (C) Expression levels and phosphorylation state of CREB in C3H10T1/2 fibroblasts cultured in GM versus DM. The experiments shown in panels B and C were performed as described for panel A.

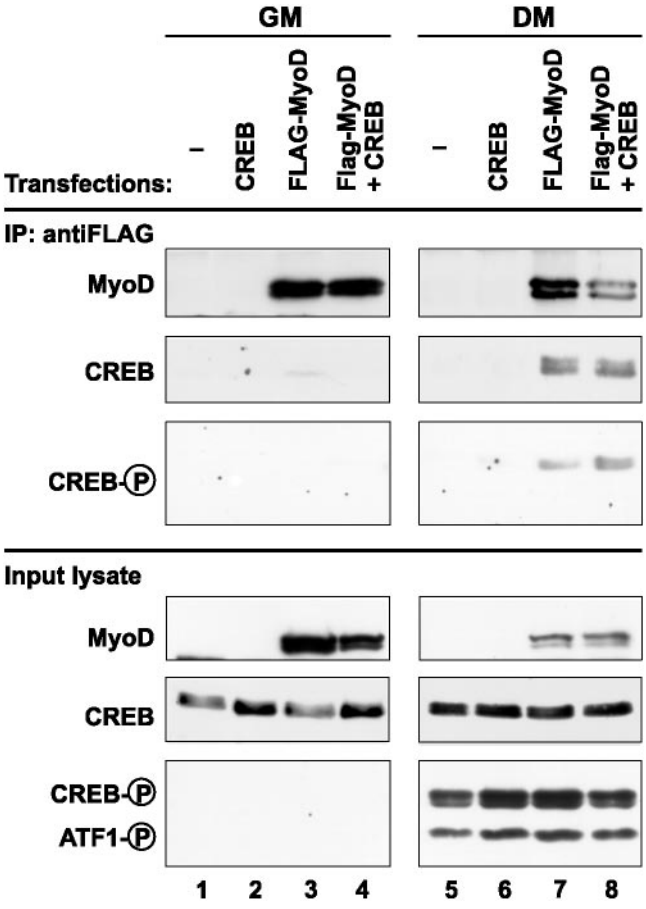


FIG. 6. In vivo association between MyoD and CREB. C3H10T1/2 fibroblasts were transfected with CREB and/or FLAG-tagged MyoD expression constructs as indicated above the lanes. Transfected cells were cultured in either GM or DM for 48 h before being harvested. Aliquots of 500 μg of protein from each lysate were immunoprecipitated (IP) with anti-FLAG antibody M2. The precipitated proteins were fractionated by SDS-PAGE and revealed with antibodies specific for MyoD, CREB, or phospho-CREB (upper panels). P, phosphorylated. Western blot analysis of the input lysates used for immunoprecipitation (20-μg aliquot of each) is also shown (lower panels) (the anti-phospho-CREB antibody also detects the phosphorylated form of ATF1).

thus partially overlap, a situation that could well result in the simultaneous (and potentially cooperative) interaction of MyoD and phospho-CREB with their neighboring sites in p300. To test this hypothesis, we performed GST pull-down assays with the GST-p300(436–662) fusion protein. Purified GST-p300(436–662), bound to glutathione-agarose beads, was incubated with increasing amounts of recombinant MyoD in the presence of either PKA-phosphorylated or unphosphorylated purified CREB (Fig. 7A). The results showed that, in the presence of phosphorylated CREB, the binding of MyoD to GST-p300(436–662) was greatly increased (Fig. 7A, compare lanes 4 to 6 and lanes 8 to 10). As expected (7), only phosphorylated CREB bound GST-KIX. Such a correlation between phospho-CREB– KIX binding and enhancement of the MyoD association clearly supports the hypothesis that phospho-CREB and MyoD can simultaneously form a complex with this domain of p300.

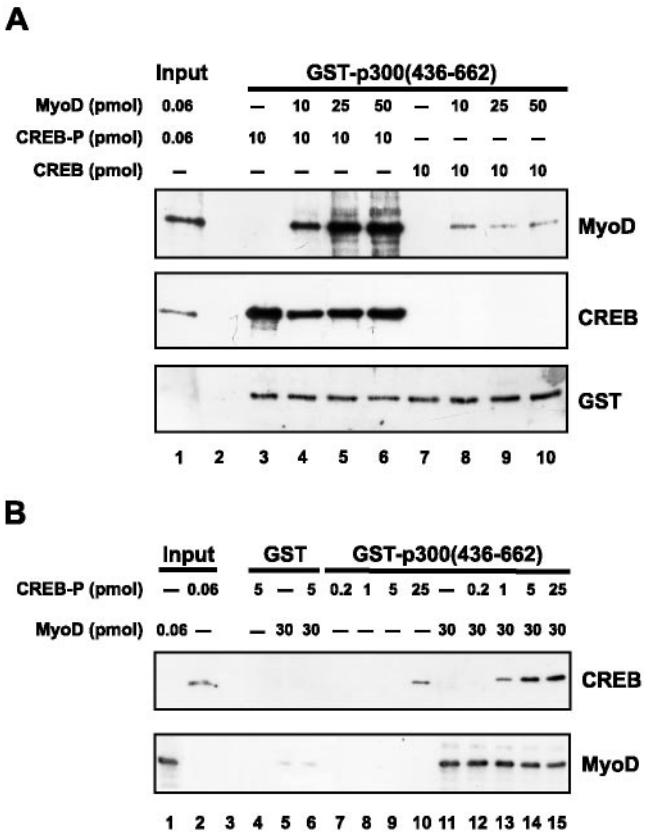


FIG. 7. MyoD and phospho-CREB can cooperatively interact with the KIX domain of p300. (A) Purified CREB, either PKA phosphorylated (CREB-P) or mock phosphorylated, was incubated with GST-p300(436–662) (10 pmol) in the presence of increasing amounts of purified MyoD, as indicated above the lanes. MyoD and CREB binding to GST-p300(436–662) was determined by Western blot analysis with specific antibodies (upper and middle panels). The blot was then reprobed with an anti-GST antibody to visualize GST-p300(436–662) (lower panel). The input lane shows protein samples for quantitative reference. (B) Increasing amounts of purified, PKA-phosphorylated CREB (CREB-P) were incubated with GST-p300(436–662) (1 pmol), either in the absence or in the presence of constant amounts of purified MyoD, as indicated above the lanes. Purified MyoD and CREB-P were also incubated with GST alone (1 pmol), as a control. CREB and MyoD binding to GST-p300(436–662) was determined by Western blot analysis as described above. The input samples for this experiment were placed in separate lanes.

We next asked whether MyoD, in turn, affected the binding of phospho-CREB to KIX. In similar GST pull-down assays, a limiting amount of purified GST-p300(436–662) was bound to glutathione-agarose beads and incubated with increasing amounts of phospho-CREB in the presence or absence of MyoD. As shown in Fig. 7B, the addition of MyoD facilitated phospho-CREB– KIX complex formation (Fig. 7B, compare lanes 7 to 10 and lanes 11 to 15). Altogether, the results in Fig. 7 strongly suggest that MyoD and CREB bind to the p300 KIX domain concurrently and synergistically.

p300 and P/CAF stimulate MyoD-mediated transcription from the *RB* promoter. Since p300 and P/CAF appeared to enter, along with MyoD, into the nucleoprotein complex on the CRE site of the *RB* promoter (Fig. 4A), we examined whether their known coactivator functions contributed to the MyoD-mediated stimulation of the *RB* promoter. For this purpose, we investigated whether increasing the level of available p300 or P/CAF enhanced MyoD-activated or basal transcription from the *RB* promoter. The results presented in Fig. 8A showed that cotransfection of MyoD with either p300 or P/CAF caused a twofold increase in reporter transactivation by MyoD and that such transactivation was further stimulated by cotransfecting p300 and P/CAF together. The overexpression of p300 and/or P/CAF in the absence of MyoD did not influence the basal activity of the *RB* promoter. In the above-described coactivation assay, the MyoD DNA-binding mutant B2ProB3 was also tested (in lieu of wild-type MyoD). The results indicated that the p300 and P/CAF coactivators could enhance this mutant activity like they did with wild-type MyoD activity (Fig. 8A).

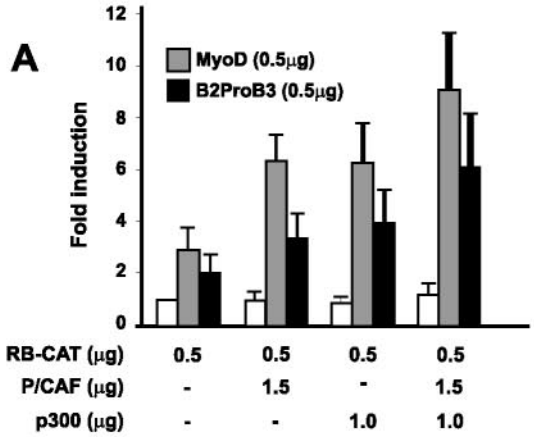
Since both p300 and P/CAF possess intrinsic AT activity (35), we examined whether their AT activity was needed for the observed cooperation with MyoD. To this end, mutants of p300 and P/CAF with deletions in their AT region were used in reporter gene assays. The results shown in Fig. 8B indicated that the AT mutants of p300 were able to enhance the MyoD-mediated transactivation of the *RB* promoter to the same extent as wild-type p300. In contrast, the AT mutants of P/CAF appeared to have lost the ability to stimulate such MyoD activity (Fig. 8C). Western blot determinations of protein levels in transfected cells ensured that the P/CAF mutations had no effect on the expression of this protein (compared to the wild type) or on the expression of MyoD (Fig. 8C, lower panels).

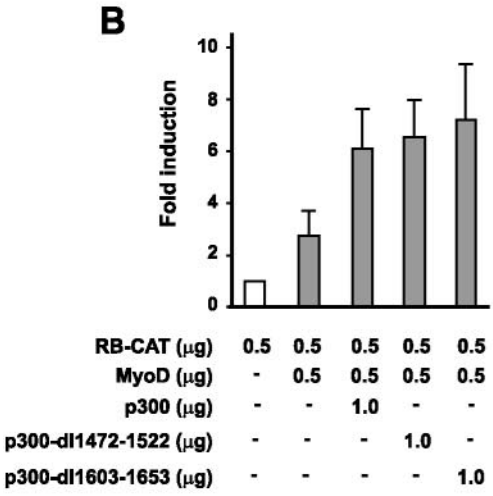
The binding of MyoD to p300 is required for the MyoD-mediated transactivation of the *RB* promoter. The above results indicated that p300 overexpression enhanced the MyoD-mediated transactivation of the *RB* promoter. If such an effect depended on p300 recruitment via a physical interaction with MyoD, then blocking of this interaction should squelch the ability of MyoD to stimulate the *RB* promoter.

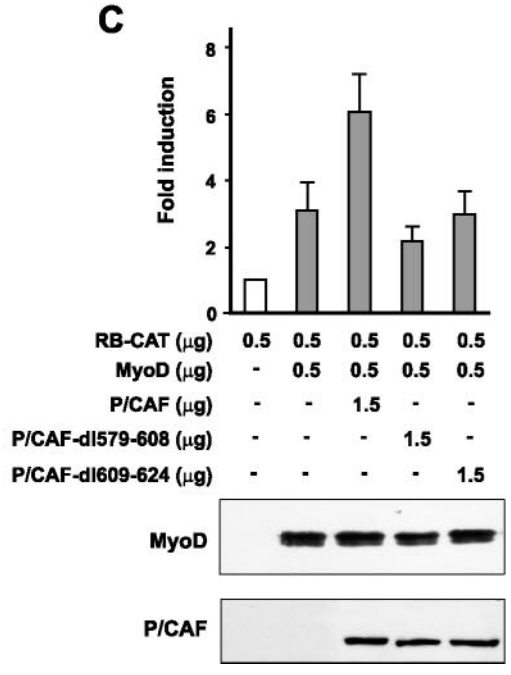
As already mentioned, p300 is known to bind MyoD at two distinct sites: the first in the N-terminal region and the second in the C/H3 region (15, 49, 63). We reasoned that truncated forms of p300 containing such MyoD-binding sites might act as competitive inhibitors of MyoD transactivation by occupying the p300-binding sites on MyoD. We analyzed two constructs encoding truncated forms of p300, p300(1–596) and p300 (1514–1922), neither of which contained the CREB-binding domain of p300 (KIX domain: amino acids 566 to 647) (43), to leave unperturbed the interaction between p300 and CREB.

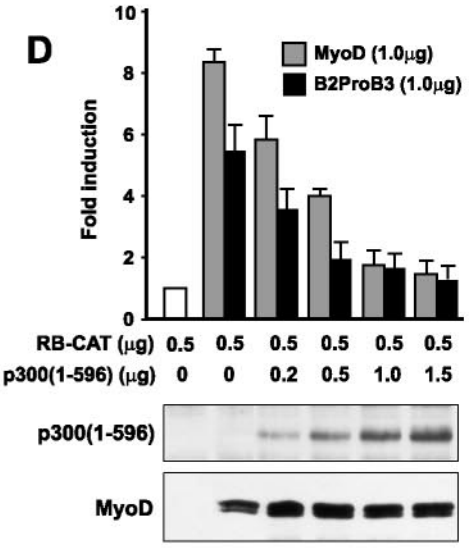
The RB-CAT reporter was cotransfected into C3H10T1/2 fibroblasts with the MyoD expression plasmids and either one of the truncated forms of p300 (Fig. 8D and E). The results showed that increasing amounts of p300(1–596) or p300(1514–1922) squelched in a dose-dependent fashion the ability of MyoD to stimulate the activity of the *RB* promoter. Similar results were obtained with the MyoD DNA-binding mutant B2ProB3. That the truncated forms of p300 had no effect on MyoD expression was determined by Western blotting (Fig. 8D and E, lower panels).

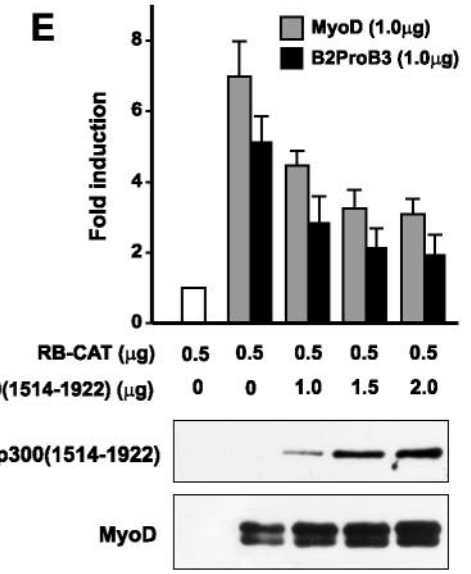
The specific inhibition of *RB* promoter stimulation by MyoD upon cotransfection of the truncated forms of p300 supports the hypothesis that they exert dominant-negative activity, competing with endogenous p300 for association with MyoD on the *RB* promoter.


A
MyoD (0.5μg)
B2ProB3 (0.5μg)
Fold induction
RB-CAT (μg) 0.5 0.5 0.5 0.5
P/CAF (μg) - 1.5 - 1.5
p300 (μg) - - 1.0 1.0


B
Fold induction
RB-CAT (μg) 0.5 0.5 0.5 0.5 0.5
MyoD (μg) - 0.5 0.5 0.5 0.5
p300 (μg) - - 1.0 - -
p300-dI1472-1522 (μg) - - - 1.0 -
p300-dI1603-1653 (μg) - - - - 1.0


C
Fold induction
RB-CAT (μg) 0.5 0.5 0.5 0.5 0.5
MyoD (μg) - 0.5 0.5 0.5 0.5
P/CAF (μg) - - 1.5 - -
P/CAF-dI579-608 (μg) - - - 1.5 -
P/CAF-dI609-624 (μg) - - - - 1.5
MyoD
P/CAF


D
MyoD (1.0μg)
B2ProB3 (1.0μg)
Fold induction
RB-CAT (μg) 0.5 0.5 0.5 0.5 0.5 0.5
p300(1-596) (μg) 0 0 0.2 0.5 1.0 1.5
p300(1-596)
MyoD


E
MyoD (1.0μg)
B2ProB3 (1.0μg)
Fold induction
RB-CAT (μg) 0.5 0.5 0.5 0.5 0.5
p300(1514-1922) (μg) 0 0 1.0 1.5 2.0
p300(1514-1922)
MyoD

Inactivation of CREB induces apoptosis in C2 muscle cells. The results so far described reveal an important role of CREB in the MyoD-mediated induction of *RB* gene expression, an event that occurs in the early phase of terminal muscle differentiation. In view of the essential roles that pRB plays in growth arrest and in the differentiation and survival of skeletal muscle cells, it was interesting to investigate whether inhibition of CREB functioning would affect the differentiation process. Our initial approach was to transfect C2 myoblasts with A-CREB in order to monitor by indirect immunofluorescence the expression in cells of FLAG-tagged A-CREB and of the myosin heavy chain (as a marker of terminal differentiation). It was soon clear, however, that by the time the terminal differentiation of these cells could be observed (i.e., 48 h in DM), no A-CREB-expressing cells were detectable.

Since CREB had been reported to act as a survival factor preventing apoptosis in a number of differentiating cell types, including neurons and adipocytes (44, 58), we reasoned that the cells which expressed A-CREB and in which CREB was thus inhibited might be counterselected at the earlier stages of differentiation. In order to test this hypothesis, we cotransfected C2 myoblasts with expression vectors encoding both A-CREB and GFP (or GFP alone, as a control). These cultures were kept in growth medium for about 24 h, to allow confluence to be reached, and then were exposed to DM for 12 h before being harvested. Under such conditions, the early markers of differentiation are already expressed (see, for instance, Fig. 5). The cells were then stained with anti-FLAG antibody to detect A-CREB and with Hoechst 33342 to detect, if present, the classical apoptotic condensation of nuclei. The results of this experiment are shown in Fig. 9. Whereas the cells transfected with GFP alone displayed a normal nuclear morphology, all of the cells that expressed A-CREB also showed brightly Hoechst-stained nuclear condensation, a typical mark of apoptosis. Interestingly, cells expressing A-CREB and kept under growing conditions (i.e., sparsely seeded in GM) did not exhibit similar condensation. Tentatively (given the complex metabolic differences occurring in cells under the two conditions), these results suggest that CREB functions could be particularly required at the onset of myoblast differentiation.

DISCUSSION

We have here characterized the molecular mechanism by which the muscle-specific transcription factor MyoD activates transcription from the *RB* gene promoter. Our data reveal a novel mechanism of MyoD-dependent transactivation of target genes, linking the activity of MyoD to that of the ubiquitous transcription factor CREB.

In a previous study it was shown that MyoD stimulates *RB* promoter activity in differentiating C2 myocytes by a noncanonical mechanism, not involving MyoD binding to E boxes (30). We have now identified, in the *RB* promoter, a nonpalindromic CRE as the specific target for transcriptional stimulation by MyoD. This site, which lies in the "core" region of the *RB* promoter, is also required for the basal promoter activity in the absence of MyoD. A number of cellular and viral gene promoters are regulated, like *RB*, via nonpalindromic CREs and such sites have been shown to recognize the cognate ATF/CREB family of transcription factors, albeit with a lower affinity than the full CRE palindrome (8).

A previous study also indicated the CRE as a site needed for *RB* promoter induction in differentiating C2 myoblasts (36). In such study, ATF1 was identified as the transcription factor binding the CRE in C2 myoblasts, whereas no factor binding this site was detected in differentiated C2 myotubes. In view of the downregulation of ATF1 expression observed upon differentiation, it was suggested that *RB* promoter activity in differentiating myocytes was stimulated by the removal of a repressive effect exerted by ATF1.

We have now been able to identify CREB as the activating transcription factor binding the CRE in differentiated muscle cells, and to show that a specific inhibition of the DNA-binding activity of CREB prevents MyoD from transactivating the *RB* promoter. In contrast, a MyoD DNA-binding mutant was still able to stimulate the *RB* promoter activity, consistently with the absence of the E box. These results indicate that CREB functions as the DNA-recognizing factor for the MyoD-responsive element in the *RB* gene promoter.

CREB is known to activate the transcription of target genes through the recruitment of the p300/CBP coactivators (2, 24, 27). The direct binding of CREB to a domain of p300/CBP, termed KIX, requires the phosphorylation of CREB at Ser-133 (38). In addition to the cAMP-activated protein kinase A, a variety of other signal-activated cellular kinases can promote the phosphorylation of CREB at Ser-133 (54). The Ser-133 phosphorylation of CREB in response to non-cAMP signals, however, is not enough for efficient recruitment of p300/CBP; a second event is required, possibly a positive regulation by cellular cofactors (31).

FIG. 8. Requirements for p300 and P/CAF in MyoD-dependent stimulation of the *RB* promoter. C3H10T1/2 fibroblasts were transfected with the RB-CAT(−228/−176) construct and either pEMSV-MyoD or pEMSV-B2ProB3, along with expression plasmids for p300 and/or P/CAF. Cells were transferred to DM at 24 h posttransfection and harvested for CAT assays after 48 h. (B) The RB-CAT(−228/−176) construct was cotransfected in C3H10T1/2 fibroblasts along with expression vectors for MyoD and p300 or its enzymatically defective mutant derivatives. Transfected cells were then processed as described for panel A. (C) Experiments were performed as described for panel B, except that P/CAF or its enzymatically defective mutant derivatives were used. In a representative experiment, 30 μg of total cell extract was analyzed by Western blotting to monitor the levels of expression of the transfected DNAs (lower panels). (D) The RB-CAT(−228/−176) construct was cotransfected in C3H10T1/2 fibroblasts with either pEMSV-MyoD or pEMSV-B3ProB3, along with increasing amounts of dominant-negative p300(1–596). DNA concentrations were kept constant with empty vector DNA. Transfected cells were processed as described for panel A. In a representative experiment, 30 μg of total cell extract was analyzed by Western blotting to monitor the levels of expression of MyoD and p300(1–596) (lower panels). (E) Experiments were performed as described for panel D, except that p300(1514–1922) was used as dominant-negative p300. In all of these experiments, the values obtained from CAT activity assays are expressed as the fold increase in CAT activity relative to that in cells transfected with the RB-CAT(−228/−176) construct alone. Values are the means of at least five independent experiments, each performed in duplicate; error bars represent standard deviations.

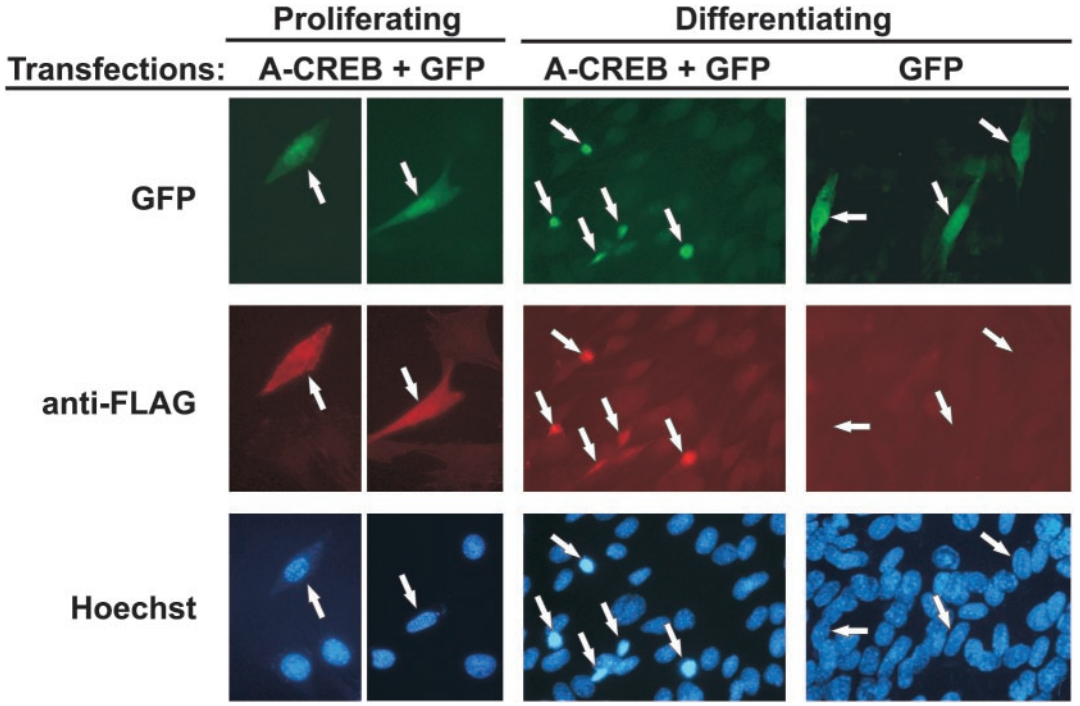


FIG. 9. A-CREB expression induces apoptosis in differentiating myoblasts. C2 myoblasts were transfected with plasmids carrying either dominant-negative A-CREB (FLAG tagged) and GFP or GFP alone. After transfection, the cells were either kept proliferating or induced to differentiate as described in Materials and Methods. The cells were finally stained with an anti-FLAG antibody for A-CREB (red) and counterstained with Hoechst 33342 to reveal nuclear morphology (blue). GFP expression was directly visualized by microscopic inspection under a specific filter (green). Arrows, transfected cells.

The present data show that the total amount of CREB protein and its phosphorylation at Ser-133 rapidly increase at an early stage of C2 muscle cells differentiation and that both remain at high levels throughout the differentiation process. The induction of CREB Ser-133 phosphorylation is not a specific feature of the muscle differentiation program, but occurs upon cell cycle arrest in nonmyogenic cells as well. This observation agrees with the results of another study showing the cell cycle dependence of CREB phosphorylation, with Ser-133 being phosphorylated in G_0 and G_1 phases but not in S and G_2 phases (12).

Coimmunoprecipitation experiments first revealed that an interaction existed between MyoD and phospho-CREB, in differentiating myocytes, whereas no such association was found between MyoD and the unphosphorylated CREB present in growing myoblasts. That the above interaction correlated with CREB phosphorylation was a clue suggesting that MyoD and CREB might interact through p300. MyoD is known to directly bind p300 at two sites, one in the C-terminal C/H3 region, the other in the N-terminal part of p300 (15, 49, 63), recently mapped to a region spanning residues 436 to 662 (45). This region contains the KIX domain that harbors the phospho-CREB binding site (43). We thus tested the possibility that MyoD and phospho-CREB might concomitantly bind neighboring sites in the KIX domain. The results obtained showed that MyoD bound the KIX domain more efficiently in the presence of phosphorylated than of unphosphorylated CREB, and that the MyoD binding, in turn, favored the phospho-CREB-KIX complex formation. The conclusions from these results are that not only MyoD and phospho-CREB can interact simultaneously with the KIX domain of p300, but also that they mutually help their recruitment to this region of the coactivator.

The present work also provides evidence that, in differentiated myocytes, CREB, MyoD and p300 assemble into a multiprotein complex on the CRE of the *RB* promoter, a complex that also contains the p300-associated AT P/CAF. Previous studies have shown that a multimeric complex formed by MyoD, P/CAF, and p300/CBP is recruited on the E boxes of muscle-specific gene promoters to facilitate transcription (42). Our data indicate that the coactivator complex formed by p300, P/CAF, and MyoD also is targeted, through CREB, to the CRE site of the *RB* promoter.

The functional relevance of these molecular interactions was demonstrated by transient transfection experiments showing that an increase in the amount of available p300 or P/CAF stimulated the MyoD-dependent activation of the *RB* promoter and that the simultaneous transfection of both coactivators enhanced transcription above the level seen with either one alone. In contrast, overexpressing p300 and P/CAF in the absence of MyoD had no such effect. We showed also that an overexpression of p300-derived polypeptides carrying MyoD binding sites inhibited the MyoD-dependent stimulation of the *RB* promoter, by competing with endogenous p300 for association with MyoD.

Altogether, the above results strongly suggest that MyoD stimulates *RB* transcription by facilitating the recruitment of p300 and P/CAF on the promoter-bound phospho-CREB. We

found, in addition, that only the AT activity of P/CAF and not p300 is needed by MyoD to transactivate the *RB* promoter. This result suggests that while P/CAF provides the complex assembled at the CRE site with AT activity, p300 may contribute to *RB* promoter activation by recruiting the basal transcription machinery through its N- and C-terminal transactivating domains (63). Which factor might be the target of the P/CAF AT activity in the MyoD-dependent activation of the *RB* promoter is not yet known. However, there are reasons to believe (though direct proof is still missing) that the acetylation of MyoD itself may be functionally critical. Previous studies have shown that MyoD acetylation by either p300/CBP or P/CAF results in an activation of the MyoD transcriptional activity (29, 40, 50). It has also been recently shown that acetylation increases the affinity of MyoD for CBP/p300, with more efficient recruitment of these coactivators to the muscle-specific gene promoters (41). A stronger interaction between acetylated MyoD and p300 could help in recruiting and stabilizing the above coactivator complex on the DNA-bound CREB.

High levels of pRb are needed for both the growth arrest and differentiation of muscle cells, and for their survival (34, 59, 65). Hence the possibility existed that an inhibition of CREB activity might adversely affect the myogenic program. When such possibility was experimentally tested, it was found that the ectopic expression in myoblasts of A-CREB was indeed incompatible with their terminal differentiation; the cells became apoptotic in the early phase of differentiation. The ability of A-CREB to induce apoptosis in differentiating myoblasts is consistent with previously described roles of CREB in the survival of other cell types and with CREB regulatory links with survival-associated factors and genes (22, 26, 44, 58). Also, the scenario presented by knockout models, albeit complicated by functional compensations, confirms the importance of the CREB family of transcription factors in the maintenance of cell viability in vivo (5, 47).

We speculate that *RB* is one of the CREB target genes participating in the maintenance of cell survival.

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