

## Molecular Mechanisms of Myogenic Coactivation by p300: Direct Interaction with the Activation Domain of MyoD and with the MADS Box of MEF2C

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**By searching for molecules that assist MyoD in converting fibroblasts to muscle cells, we have found that p300 and CBP, two related molecules that act as transcriptional adapters, coactivate the myogenic basic-helix-loop-helix (bHLH) proteins. Coactivation by p300 involves novel physical interactions between p300 and the amino-terminal activation domain of MyoD. In particular, disruption of the FYD domain, a group of three amino acids conserved in the activation domains of other myogenic bHLH proteins, drastically diminishes the transactivation potential of MyoD and abolishes both p300-mediated coactivation and the physical interaction between MyoD and p300. Two domains of p300, at its amino and carboxy terminals, independently function to both mediate coactivation and physically interact with MyoD. A truncated segment of p300, unable to bind MyoD, acts as a dominant negative mutation and abrogates both myogenic conversion and transactivation by MyoD, suggesting that endogenous p300 is a required coactivator for MyoD function. The p300 dominant negative peptide forms multimers with intact p300. p300 and CBP serve as coactivators of another class of transcriptional activators critical for myogenesis, myocyte enhancer factor 2 (MEF2). In fact, transactivation mediated by the MEF2C protein is potentiated by the two coactivators, and this phenomenon is associated with the ability of p300 to interact with the MADS domain of MEF2C. Our results suggest that p300 and CBP may positively influence myogenesis by reinforcing the transcriptional autoregulatory loop established between the myogenic bHLH and the MEF2 factors.**

The myogenic basic helix-loop-helix (bHLH) proteins can confer the myogenic phenotype to otherwise committed cell types (15, 30, 45, 62, 77, 82) and are essential for the proper development of skeletal muscles (11, 13, 38, 60, 65, 68, 69, 88). They activate muscle gene transcription by pairing with ubiquitously expressed E proteins (17, 46) via the HLH domain and interact with the E box, a specific DNA sequence (CANNTG) (58, 59), that functions as an operative binding site in a large number of transcription regulatory regions. Interaction with the E-box DNA by the heterodimeric complex is mediated by the basic regions of the myogenic bHLH and E proteins (14, 23) and is necessary but not sufficient for transcriptional activation (9, 79). This indicates that besides DNA binding, additional steps are required to activate transcription.

E-box sites are expected in the genome at random approximately every 256 bp, but myogenic bHLH factors solely transactivate muscle-specific genes. Tissue-specific gene activation by the myogenic bHLH proteins is achieved through at least two mechanisms. First, not all E boxes are equivalent (10). The particular two nucleotides flanking each side of an E box have been shown to repress activation of an immunoglobulin enhancer in muscle cells by a myogenic bHLH protein (80), and E-box activity depends upon its context even in muscle genes (39, 86). Second, two amino acids, alanine 114 and threonine 115, located in the basic region of each myogenic bHLH protein are required for muscle gene transcription (14, 23). Swapping experiments have revealed that the nonmyogenic bHLH

E proteins can be rendered competent to activate myogenesis by simply inserting correctly oriented alanine and threonine in their basic regions (24, 79). For this reason, the alanine and threonine residues are often referred to as the “myogenic code.”

Several lines of evidence are consistent with the notion that bHLH proteins bearing the myogenic code, when bound to the E box, involve a cofactor for myogenesis and muscle gene expression (14, 23, 79). One class of candidates for such coregulatory activities are members of the myocyte enhancer factor 2 (MEF2) family (31, 84). These are transcriptional activators that recognize specific DNA elements (MEF2 sites) found in regions controlling expression of several muscle-specific genes. MEF2 collaborates with the myogenic bHLH proteins to establish a mutually reinforcing transcriptional circuitry (63). Alanine 114 and threonine 115 of MyoD are necessary to physically interact and synergize with MEF2. In fact, after transfection, coexpression of myogenic bHLH and MEF2C augments the efficiency of myogenic conversion of fibroblasts (56). A second line of evidence implicating a coregulator in myogenic transcription involves the steps required in order for the amino-terminal activation domain of MyoD to function. Mutation analysis has delimited this domain to the first 53 amino acids (79). This acid-rich domain is inactive when present in a bHLH molecule bound to a myogenic E box but which lacks the myogenic code residues (23). However, when tested in a DNA binding-independent assay, the N-terminal region of MyoD can activate transcription. Furthermore, when examined as an isolated truncated peptide, the activation domain increases transcription 25- to 50-fold above the level observed with the entire molecule, suggesting that other domains of MyoD repress it by masking it (79). The function of

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the hypothesized coregulator, sometimes referred to as "recognition factor" (14, 23, 79), would be the unmasking of the MyoD activation domain by mechanisms involving the myogenic code. Exposure of the activation domain of MyoD and subsequent promotion of transcription could be the result of a newly acquired conformation of the MyoD protein imposed by the recognition factor. For instance, the unmasked activation domain could recruit protein(s) able to transmit the activation signals to the basal transcriptional machinery. Evidence is accumulating that many enhancer-binding proteins transmit their activation potential to the basal transcriptional machinery by using such proteins, often referred to as adapters, cofactors, or coactivators (32, 53, 87). We searched for factors that might play the role of MyoD coactivator by testing candidate molecules for their ability to augment transcriptional activation or myogenic conversion by MyoD. We report here that p300 and the related protein CBP, two molecules with the properties expected of transcriptional adapters (26, 44), coactivate myogenic bHLH by a mechanism involving a direct association of p300 with the amino-terminal activation domain of MyoD. These interactions can be detected both *in vitro* and in the cell and are independently mediated by domains located at the N and C termini of the p300 molecule and by the FYD domain, located in MyoD's N-terminal activation domain. In addition we have found that in a parallel manner, p300 and CBP also serve as transcriptional coactivators of MEF2C by a mechanism involving the interaction of p300 with the MADS domain of MEF2C. These results indicate that p300, besides directly coactivating the N-terminal domain of MyoD, may promote myogenic differentiation by reinforcing the positive autoregulatory loop that operates between the myogenic bHLH and MEF2 transactivators. Since a truncated segment of p300, which does not physically interact with MyoD but does multimerize with intact p300, acts as a dominant negative mutant for MyoD activity, p300 appears to be a component required for myogenesis.

#### MATERIALS AND METHODS

**Plasmids.** Many of the plasmid vectors used in this study have been kindly provided by various colleagues. Table 1 lists the plasmids used in this study, their source, and the appropriate references. Construction of new plasmids and critical features not previously stipulated are described in more detail below.

**Mammalian expression vectors.** The expression vectors pcDNA3 p300 S and pcDNA3 p300 A were generated after excising the p300 inserts from pBS p300 *SmaI* and pBS p300 *AhaII* with *HindIII* and subcloning them in *HindIII*-linearized pcDNA3. The orientations of the inserts was determined by restriction enzyme mapping. To generate pcDNA3 p300 1-744, pCMV $\beta$ p300 was digested with *SstI*, treated with T4 DNA polymerase, and digested with *NotI*. A 2.3-kb DNA encoding the first 744 N-terminal amino acids of p300 was gel isolated and inserted in the pcDNA3 vector restricted with *XbaI*, treated with T4 DNA polymerase, and cut with *NotI*. pcDNA3MEF2C was generated by excising the *HindIII/XbaI* fragment from MEF2C cDNA and cloning it in *HindIII/XbaI*-restricted pcDNA3. To generate the Gal-N MD(FP) and Gal-N MD(PPA+P) plasmids, an *NdeI* site encompassing the start codon of MyoD was introduced by PCR mutagenesis with the EMSV-MyoD plasmid as a template. The Gal fusion plasmids were generated by fusing the *NdeI-HindIII* fragments from the modified MyoD vectors in frame at the carboxyl terminus of the Gal4 DNA-binding domain (amino acids 1 to 147) in the vector pGal0. In the Gal-N MD(FP) construct, phenylalanine at amino acid position 29 of MyoD has been replaced by a proline. In the Gal-N MD(PPA+P) construct, phenylalanine at amino acid position 29, tyrosine at position 30, asparagine at position 31, and phenylalanine at position 42 of MyoD have been replaced by two prolines, one alanine, and one proline, respectively. The Gal-N MD mutant constructs were sequenced.

**Prokaryotic expression vectors.** The pGEX2TK-MyoD(JHLH) plasmid was generated by PCR amplification of the regions corresponding to the junction and the HLH region of MyoD (amino acids 123 to 162) and subcloning of the PCR product in the pGEX2TK vector restricted with *BamHI* and *EcoRI*. Various segments of MEF2C were synthesized by PCR, and the ends were outfitted with *BamHI* and *EcoRI* restriction sites and then subcloned into *BamHI*- and *EcoRI*-restricted pGEX2T. The resulting vectors carry DNA encoding full-length MEF2C cDNA (pGEX2T-MEF2C), MEF2C without the MADS domain

(pGEX2T- $\Delta$ MADSMEF2C), and the MADS domain (amino acids 2 to 60 of MEF2C) alone (pGEX2T-MADS).

**In vitro transcription vectors.** Truncated forms of p300 were obtained by transcribing pBS p300 deletion constructs *BgIII*, *SmaI*, and *AhaII* with T3 RNA polymerase. The plasmids coding for MyoD, E12, MyoDE12Basic, MyoDT4Basic, MyoDMycBasic, MyoDMycHelix1, and MyoDMycHelix2 were transcribed with T3 RNA polymerase. Transcription from pRc/CMV-CBP was carried out with T7 DNA polymerase. The pcDNA3 N-MyoD vector was constructed by PCR amplification of the first 100 N-terminal amino acids of MyoD (Met1 to Ala100). The first methionine was preceded by an artificial *BamHI* restriction site and a canonical Kozak sequence. Two artificial methionines were introduced after Ala100 and were followed by a stop codon. An *EcoRI* restriction site was introduced after the stop codon. The PCR products were gel isolated, restricted with *BamHI* and *EcoRI*, and subcloned in the pcDNA3 vector digested with *BamHI* and *EcoRI*. To generate the pcDNA3 C-MyoD vector, the sequences encoding amino acids Ala162 to Leu318 of MyoD were PCR amplified. Two artificial methionines were placed N terminal to MyoD Ala162. To optimize translation, a Kozak sequence was placed 5' to the two codons for the artificial methionines. *BamHI* and *EcoRI* restriction sites located in the top and bottom primers used for PCR amplification facilitated subcloning of the PCR products. The PCR products were digested with *BamHI* and *EcoRI*, isolated by gel electrophoresis, and subcloned in pcDNA3 digested with *BamHI* and *EcoRI*. Temperatures for the PCRs were 95°C for 1 min, 55°C for 2 min, and 75°C for 2 min for a total of 25 cycles. A total of 0.5 U of *Pfu* DNA polymerase (Stratagene) was used for each reaction. Both pcDNA3 N- and C-MyoD constructs were sequenced.

**Northern blot analysis.** C2C12 muscle cells were grown and differentiated as previously described (71). Myoblasts were harvested at approximately 40% confluency. Poly(A)<sup>+</sup> RNA was isolated with an Oligotex mRNA Mini Kit (Qiagen). poly(A)<sup>+</sup> RNA (2  $\mu$ g) was resolved on a 1.2% gel containing formaldehyde and transferred to a nylon membrane (Hybond-N<sup>+</sup>; Amersham). After UV cross-linking, the membrane was prehybridized for 2 h at 65°C. Two DNA probes radiolabelled with [ $\alpha$ -<sup>32</sup>P]dCTP with a random primer DNA-labeling kit (Boehringer Mannheim) and corresponding to the coding regions of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene and to amino acids 1 to 168 of p300 were denatured, added to the nylon membrane, and incubated with gentle rotation at 65°C for 16 h. Membrane washing was performed at 65°C in 1% sodium dodecyl sulfate (SDS)-50 mM NaCl-1 mM EDTA. Autoradiography was conducted at -80°C.

**In vitro transcription-translation.** One microgram of various supercoiled DNA plasmids was transcribed *in vitro* and then translated in the presence of [<sup>35</sup>S]methionine with the TnT coupled reticulocyte lysate system (Promega) according to the manufacturer's suggestions.

**Cell transfection and transcription assays.** Transfections of C2C12 muscle cells, 3T3, and C3H10T1/2 mouse embryo fibroblasts were performed according to the calcium phosphate precipitation protocol as described previously (71). Two micrograms of reporter constructs was coprecipitated with 2  $\mu$ g of activator plasmids (Gal fusion, MyoD series, MyoD~E47, or myogenin) and 4  $\mu$ g of coactivator plasmids (p300, CBP, or TAFII250). A total of 0.5  $\mu$ g of the CMV-*lacZ* plasmid was included in each transfection. C2C12 cells were cultured under growth conditions (Dulbecco's modified Eagle's medium [DMEM] supplemented with 20% fetal bovine serum) and differentiated by exposing them to DMEM containing 2% horse serum. Six micrograms of p300 expression vector was transfected in c2C12 cells. 3T3-transfected cells were cultured in either DMEM supplemented with 10% calf serum or in DMEM containing 2% horse serum. Transactivation of the reporter genes was evaluated by either chloramphenicol acetyltransferase (CAT) or luciferase assay as previously described. The CAT and luciferase activities were corrected for the activation of the reporter constructs obtained in the presence of the various expression vector backbones. The quantitative  $\beta$ -galactosidase assay was performed as suggested previously (4) with the chlorophenol red- $\beta$ -D-galactopyranoside (CPRG; Boehringer Mannheim) substrate. The values obtained for the CAT assays were corrected for transfection efficiency with the  $\beta$ -galactosidase assay. Transfection experiments were done in duplicate and repeated several times. CAT measurements were performed by exposing the thin-layer chromatography sheets to a Multi Scan Ambis System.

**In vitro protein-protein interaction assays.** Expression and purification of glutathione S-transferase (GST) fusion polypeptides were performed as described previously (4). The GST fusion proteins were analyzed on an SDS-polyacrylamide gel for integrity and to normalize the amount of each protein. To perform a protein-protein interaction assay, glutathione beads (Sigma) coated with the GST fusion protein (approximately 2 to 3  $\mu$ g) were reacted with 5  $\mu$ l of [<sup>35</sup>S]methionine-labeled translation product in 200  $\mu$ l of binding buffer containing 100 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% Nonidet P-40 detergent, and 5  $\mu$ g of ethidium bromide. The reaction was allowed to proceed for 1 h at 4°C with gentle rocking, after which the glutathione beads were collected by brief centrifugation and subjected to five rounds of washing with 1 ml of binding buffer per wash. The beads were resuspended in 20  $\mu$ l of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and boiled for 5 min. Eluted proteins were resolved by SDS-PAGE; the gel was treated with Enlightening (DuPont), dried, and exposed for autoradiography.

TABLE 1. DNA constructs used in this study

Construct or vector	Characteristics <sup>a</sup>	Source
<b>Reporter constructs</b>		
MCK-CAT	MCK enhancer fused to CAT gene	S. Hauschka
HCA-CAT	HCA promoter fused to CAT gene	Our laboratory
4RE-CAT	Four copies of right E box from MCK fused to CAT gene	H. Weintraub
2XA/Temb-CAT	Two copies of MEF2 binding site from embryonic MHC	Y.-T. Yu (Vanderbilt University)
G5E1bCAT	5XGal4 binding site fused to CAT gene	E. Olson
p21-luciferase	p21 enhancer fused to luciferase gene	B. Vogelstein
CMV- <i>lacZ</i>	<i>lacZ</i> cDNA	
<b>Vectors</b>		
<b>Mammalian expression</b>		
EMSV-MyoD	MyoD cDNA	A. Lassar and H. Weintraub
FLAG-MyoD	FLAG-tagged MyoD	Y. Hamamori (University of Southern California)
EMSV-MyoD $\Delta$ N	Activation domain of MyoD is deleted	H. Weintraub
EMSV-MyoD $\Delta$ NVP16	Activation domain of VP16 replaces the activation domain of MyoD	H. Weintraub
SV-E2-5	E2-5 cDNA	T. Kadesch
EMSV-E12(ATK)	Myogenic code of MyoD is inserted in the E12 molecule	H. Weintraub
pECE-MyoD~E47	MyoD is linked to E47 via a polypeptide tether	B. Wold
Gal-MD	MyoD fused to DBD of Gal4	S. Tapscott
Gal-MyoD $\Delta$ HLH	As Gal-MyoD; HLH region of MyoD is deleted	S. Tapscott
Gal-MyoD $\Delta$ Basic	As Gal-MyoD; basic region of MyoD is deleted	S. Tapscott
Gal-NMyoD	First 53 N-terminal amino acids of MyoD fused to the DBD of Gal4	S. Tapscott
Gal-MyoD bHLH	BHLH region of MyoD is fused to the DBD of Gal4	Y. Hamamori (University of Southern California)
Gal-myc	myc cDNA encoding amino acids 262–439 fused to Gal4 DBD	G. Tomaselli
Gal-p53	Human p53 cDNA fused to Gal4 DBD	G. Lozano
Gal-Sp1	C terminus of Sp1 fused to Gal4 DBD	G. Gill and R. Tjian
Gal-VP16	Activation domain of VP16 fused to Gal4 DBD	S. Tapscott
CMV $\beta$ p300	p300 cDNA	R. Eckner and D. Livingston
CMV $\beta$ p300-HA	p300 tagged with HA peptide	R. Eckner and D. Livingston
CMV $\beta$ p300 $\Delta$ 30	p300 cDNA with a deletion of the E1A binding site	R. Eckner and D. Livingston
pRc/CMVCBP	CBP cDNA	R. Goodman
CMVTAFII250	TAFII250 cDNA	R. Tjian
NVP16p300	N-terminal region of p300 fused to VP16	R. Eckner and D. Livingston
CVP16p300	C-terminal region of p300 fused to VP16	R. Eckner and D. Livingston
pcDNA3	Cloning vector	Invitrogen
CMVMEF2C	MEF2C cDNA	J. Martin and E. Olson
<b>Prokaryotic expression</b>		
pGEX-MyoD	MyoD fused to GST	A. Lassar and H. Weintraub
pGEXp300 1–596	N-terminal 596 amino acids of p300 fused to GST	R. Eckner
pGEXp300 744–1571	Amino acids 744–1571 of p300 fused to GST	R. Eckner
pGEXp300 1572–2370	Amino acids 1572–2370 of p300 fused to GST	R. Eckner
pGEXE12	Amino acids 158–439 of E12 fused to GST	Our laboratory
pGEX2T	Cloning vector	Pharmacia
<b>In vitro transcription</b>		
E12	E12 cDNA	C. Murre and D. Baltimore
MyoDE12Basic	MyoD with the E12 basic region	R. Davis and H. Weintraub
MyoDT4Basic	MyoD with the achaete-scute basic region	R. Davis and H. Weintraub
MyoDMycBasic	MyoD with the Myc basic region	R. Davis and H. Weintraub
MyoDMycHelix1	MyoD with Myc helix 1	R. Davis and H. Weintraub
MyoDMycHelix2	MyoD with Myc helix 2	R. Davis and H. Weintraub
pBSp300BglIII	N-nested deletion of p300	R. Eckner and D. Livingston
pBSp300SmaI	N-nested deletion of p300	R. Eckner and D. Livingston
pBSp300AhaII	N-nested deletion of p300	R. Eckner and D. Livingston

<sup>a</sup> DBD, DNA-binding domain.

**Immunoprecipitations.** Plates of 70 to 80% confluent COS cells were transfected with 10  $\mu$ g of FLAG-MyoD and HA-p300 vectors per plate by the calcium-phosphate protocol and incubated in the presence of DNA for 20 h in DMEM supplemented with 10% fetal calf serum at 37°C in 3.5% CO<sub>2</sub>. After DNA removal, cells were incubated for an additional 48 h in 10% CO<sub>2</sub>. For metabolic labeling, the cells were washed twice with phosphate-buffered saline (PBS) and incubated for 30 min in 10 ml of methionine-free DMEM supplemented with 5% dialyzed fetal calf serum. The medium was decanted from the dish, 3 ml of prewarmed methionine-free DMEM–5% dialyzed fetal calf serum

containing 0.37 mCi (>1,000 Ci/mmol) of L-[<sup>35</sup>S]methionine (Amersham) was added to the cells, and the incubation continued for 3 h. The medium was aspirated, and the cells were briefly washed four times with ice-cold PBS. PBS supplemented with 10 mM EDTA was added, and the cells were rocked for 10 min at 4°C and harvested. The cells were centrifuged at 4°C, and the pellet was lysed by incubation in 200  $\mu$ l of lysis buffer (20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.6], 20% glycerol, 10 mM NaCl; 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g of pepstatin per ml, 100  $\mu$ g of aprotinin per

ml) for 1 h at 4°C. The lysate was centrifuged, and the supernatant was recovered. An aliquot of the supernatant containing approximately  $3 \times 10^7$  cpm was added to 400  $\mu$ l of radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 0.5% deoxycholate, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g of pepstatin per ml, 100  $\mu$ g of aprotinin per ml). Three micrograms of anti-FLAG M2 antibody (Kodak) or 5  $\mu$ g of antihemagglutinin (anti-HA) antibody 12CA5 (Boehringer Mannheim) was preadsorbed to 30  $\mu$ l of a 50% slurry of protein G-agarose (Sigma) for 3 h and extensively washed. Radiolabeled nuclear extracts resuspended in RIPA buffer were incubated in the presence of either anti-FLAG-protein G-agarose or anti-HA-protein G complexes for 3 h with 25  $\mu$ g of ethidium bromide per ml. The agarose resins were washed four times in RIPA buffer and resuspended in 20  $\mu$ l of SDS buffer. For the double immunoprecipitation experiments, the radiolabeled nuclear extracts were incubated first with anti-HA-protein G-agarose for 3 h. The agarose resin was washed three times in RIPA buffer, and the bound proteins were released by incubating the resin at 90°C for 10 min. The supernatant was recovered after centrifugation and incubated with the anti-FLAG-protein G-agarose complex for an additional 3 h. The resin was collected by centrifugation, extensively washed in RIPA buffer, and resuspended in 20  $\mu$ l of SDS buffer. The samples were boiled and fractionated by SDS-PAGE (7% polyacrylamide).

**Immunocytochemistry.** C3H10T1/2 cells were plated on glass slides (2 by 2 cm) located in 60-mm-diameter culture dishes. Transfections were performed by the calcium phosphate precipitation protocol with 2  $\mu$ g of MyoD and 4  $\mu$ g of CMV $\beta$ p300 expression vector. After 20 h, the precipitate was removed, and the cells were refed with DMEM supplemented with 10% calf serum. Differentiation was induced after 24 h with DMEM containing 2% horse serum and continued for 4 days. For immunostaining, the cells were washed twice with cold PBS and fixed in 2% paraformaldehyde for 15 min. After aspiration of the fixing solution, glycine-PBS was added to a final concentration of 0.1 M for 5 min. Cells were rinsed twice with cold PBS and permeabilized in PBS containing 0.2% Triton X-100 for 20 min. One additional rinsing in cold PBS preceded incubation of the glass slides in blocking solution of PBS containing 5% goat serum and 1% bovine serum albumin for 1 h at room temperature. An antibody directed against the skeletal fast myosin (Sigma; clone MY32) was diluted 1:200 in blocking solution and added to the glass slide at room temperature for 2 h. Cells were rinsed three times in PBS and incubated with a biotinylated secondary antibody (Zymed Laboratories, Inc.) for 15 min at room temperature. This step was followed by incubation with streptavidin-peroxidase complex for 20 min. Peroxidase activity was detected with aminoethylcarbazole as a chromogen (liquid AEC Kit; Zymed Laboratories, Inc.). Slides were then counterstained with hematoxylin.

## RESULTS

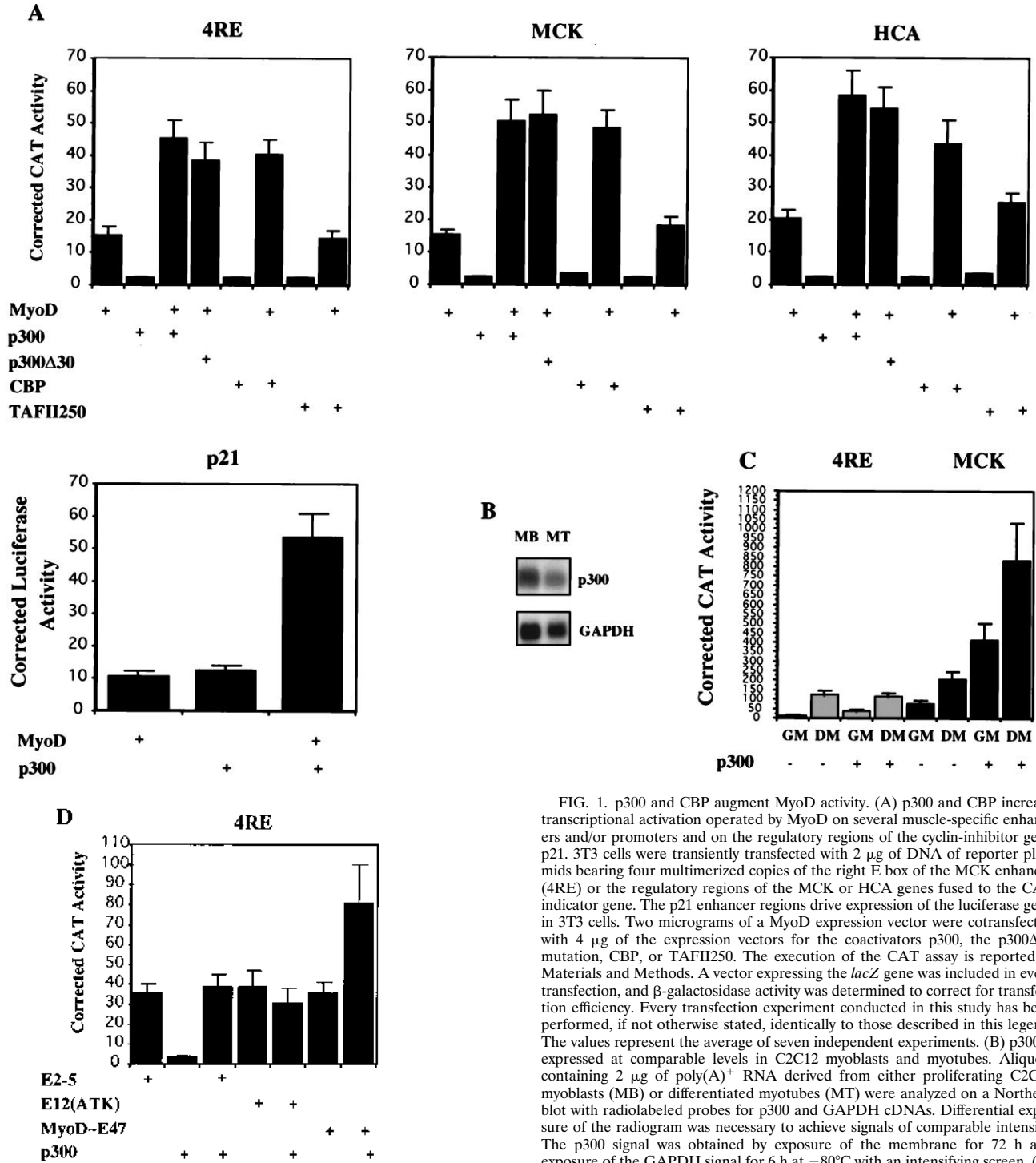
**p300 and CBP are coactivators of myogenic bHLH proteins, and p300 is expressed in muscle cells.** Few molecules have been demonstrated to act as transcriptional coactivators in higher eukaryotes (32). We tested whether three of them, p300, CBP (44), and one of the TATA-binding protein (TBP)-associated factors (TAFs), TAFII250 (81), might enhance MyoD-dependent transactivation. CBP and p300 were chosen because they show significant amino acid sequence homology (2) and because they appear to be capable of coactivating the same targets (3, 41, 50). Furthermore, the suppression of myogenesis by the E1A oncoprotein (76), which is mediated by inactivation of MyoD (12, 16) has more recently been shown to be relieved by p300 (85). TAFII250 was tested because it shares a bromodomain amino acid motif with p300 and CBP. We transiently transfected mouse 3T3 fibroblasts with combinations of a MyoD expression plasmid, an expression vector encoding one of the three coactivators, and one of three reporter CAT vectors carrying muscle-specific regulatory regions: the muscle creatine kinase (MCK) enhancer, the human cardiac  $\alpha$ -actin (HCA) promoter, or an artificial construct composed of four reiterated copies of a MyoD-binding sites (4RE). Cotransfection of MyoD plus either p300 or CBP expression vectors caused a three- to fourfold increase in MyoD transactivation of each of the reporter constructs (Fig. 1A). Analogous results were obtained with mouse embryonic C3H10T1/2 cells (data not shown). However, coexpression of the coactivator TAFII250 had no apparent effect. A p300 expression vector bearing an internal deletion (p300 $\Delta$ 30) (26) that prevents binding to the E1A oncoprotein was also able to increase MyoD-dependent transactivation (see below). When

myogenin was used rather than MyoD, the same results were obtained (data not shown).

To determine whether p300 could assist MyoD in transactivating nonmuscle genes, we investigated the transcriptional activation of p21, a gene encoding an inhibitor of several cyclin-dependent kinases involved in controlling cell cycle exit (29, 34, 37) and terminal differentiation in many cell types, including myogenic cells (33, 35, 64, 73). Regulatory regions of p21 linked to the luciferase reporter gene were introduced in 3T3 cells with the plasmids encoding MyoD and p300 (Fig. 1A). As previously reported (35), MyoD activated the p21 reporter construct. Cotransfection of expression vectors for MyoD and p300 activated the p21 reporter gene synergistically, indicating that p300-mediated coactivation of MyoD is not restricted to transcription associated with muscle-specific genes only but also applies to a broader range of genes associated with the muscle phenotype. Unexpectedly, expression of exogenous p300 alone resulted in reporter activation, suggesting that p300 might coactivate additional endogenous transcriptional activators, such as p53 (29), known to regulate p21 expression.

The mRNA for p300 is expressed in a variety of cells, including those derived from a B-cell lymphoma, embryonic kidney-derived 293 cells (26), and primary keratinocytes (55). To determine whether p300 is also expressed in muscle cells, poly(A)<sup>+</sup> RNA was isolated from nondifferentiated myoblasts as well as from differentiated C2C12 myotubes and analyzed with a p300 radiolabeled probe. A hybridization signal corresponding to the expected transcript size of approximately 10 kb was detected in both myoblast- and myotube-derived RNAs (Fig. 1B). The intensity of the signal did not seem to vary significantly with the differentiation status. The expression of p300 in muscle cells is consistent with its coactivating potential on the myogenic bHLH. It proved necessary to use poly(A)<sup>+</sup> RNA because p300 nonspecifically cross-hybridizes with the 28S rRNA (26). The level of expression of p300 in C2C12 muscle cells is relatively quite low, based on the fact that to obtain hybridization signals of comparable intensity with probes of similar specific radioactivity, it was necessary to expose autoradiograms 12-fold longer (72 h) for p300 than for GAPDH (6 h). To investigate whether p300 may coregulate transcription according to the state of cellular differentiation, the 4RE and MCK reporter constructs were transfected in C2C12 cells either kept under growth conditions or cultured in a medium that allows differentiation (Fig. 1C). Regulatory regions of both the reporter constructs sensed the different culture conditions, and as a result, transcription was increased in the cells cultured in differentiation medium. When a p300 expression vector was cotransfected with 4RE, transcription in C2C12 cells kept in growth medium was increased by approximately fourfold, whereas p300 did not seem to coactivate 4RE in differentiation medium-cultured cells. The MCK reporter behaved somewhat differently in that p300 coactivated under both culture conditions. These results suggest that p300 activity is lower in undifferentiated C2C12 cells and are consistent with a role of p300 in promoting differentiation.

To investigate the impact of p300 on MyoD-dependent myogenic conversion, C3H10T1/2 cells transiently transfected with MyoD and p300 expression vectors were subjected to immunostaining with a monoclonal antibody against a myogenic differentiation marker, the myosin heavy chain (MHC) protein. The culture dishes that received both the p300 and MyoD plasmids exhibited an increased number of cells reacting with the MHC antibody compared with the dishes transfected with the MyoD vector, whereas expression of p300 alone did not affect the phenotype of C3H10T1/2 cells (Fig. 2 and Table 2).



**FIG. 1.** p300 and CBP augment MyoD activity. (A) p300 and CBP increase transcriptional activation operated by MyoD on several muscle-specific enhancers and/or promoters and on the regulatory regions of the cyclin-inhibitor gene p21. 3T3 cells were transiently transfected with 2  $\mu$ g of DNA of reporter plasmids bearing four multimerized copies of the right E box of the MCK enhancer (4RE) or the regulatory regions of the MCK or HCA genes fused to the CAT indicator gene. The p21 enhancer regions drive expression of the luciferase gene in 3T3 cells. Two micrograms of a MyoD expression vector were cotransfected with 4  $\mu$ g of the expression vectors for the coactivators p300, the p300 $\Delta$ 30 mutation, CBP, or TAFII250. The execution of the CAT assay is reported in Materials and Methods. A vector expressing the *lacZ* gene was included in every transfection, and  $\beta$ -galactosidase activity was determined to correct for transfection efficiency. Every transfection experiment conducted in this study has been performed, if not otherwise stated, identically to those described in this legend. The values represent the average of seven independent experiments. (B) p300 is expressed at comparable levels in C2C12 myoblasts and myotubes. Aliquots containing 2  $\mu$ g of poly(A)<sup>+</sup> RNA derived from either proliferating C2C12 myoblasts (MB) or differentiated myotubes (MT) were analyzed on a Northern blot with radiolabeled probes for p300 and GAPDH cDNAs. Differential exposure of the radiogram was necessary to achieve signals of comparable intensity. The p300 signal was obtained by exposure of the membrane for 72 h and exposure of the GAPDH signal for 6 h at  $-80^{\circ}\text{C}$  with an intensifying screen. (C) p300 augments transcription driven by 4RE and MCK enhancers in C2C12 muscle cells cultured under growth conditions and increases MCK enhancer activity in differentiating C2C12 cells. C2C12 cells were transfected with either 4RE-CAT (shaded bars) or MCK-CAT (black bars) reporter constructs with (+) or without (-) an expression vector for p300. After transfections, the cells were cultured in either DMEM supplemented with 20% fetal bovine serum (GM; growth medium) or DMEM containing 2% horse serum (DM; differentiation medium) for 48 h before the CAT assay was performed. The experiment was repeated twice in duplicate with different p300 plasmid preparations. (D) p300 fails to coactivate transactivation by E2-5 and E12 harboring the myogenic amino acids but increases the activity of the MyoD~E47 forced dimer. 3T3 cells were transfected with the 4RE-CAT reporter construct and expression vectors for E2-5, the chimeric E12 containing two myogenic amino acids of MyoD in the basic region [E12(ATK)], and the dominant positive MyoD~E47 forced dimer. The transactivation potential of each construct was determined in either the absence or the presence of a p300 expression vector. The values represent the averages of three independent experiments.

We conclude that p300 and the related CBP protein but not TAFII250 coactivate transcription of at least two myogenic determinant factors (MyoD and myogenin) and that concomitant overexpression of MyoD and p300 leads to a more efficient myogenic conversion of naive fibroblasts.

**p300 coactivation is not directed at either the myogenic code or dimerization properties of MyoD.** The ubiquitously expressed E12 protein can be rendered partially myogenic by substituting asparagines 548 and 549 in its basic region with alanine and threonine to recreate the myogenic code described earlier. This vector, E12(ATK), confers the muscle phenotype to C3H10T1/2 cells, albeit with one-third the efficiency of

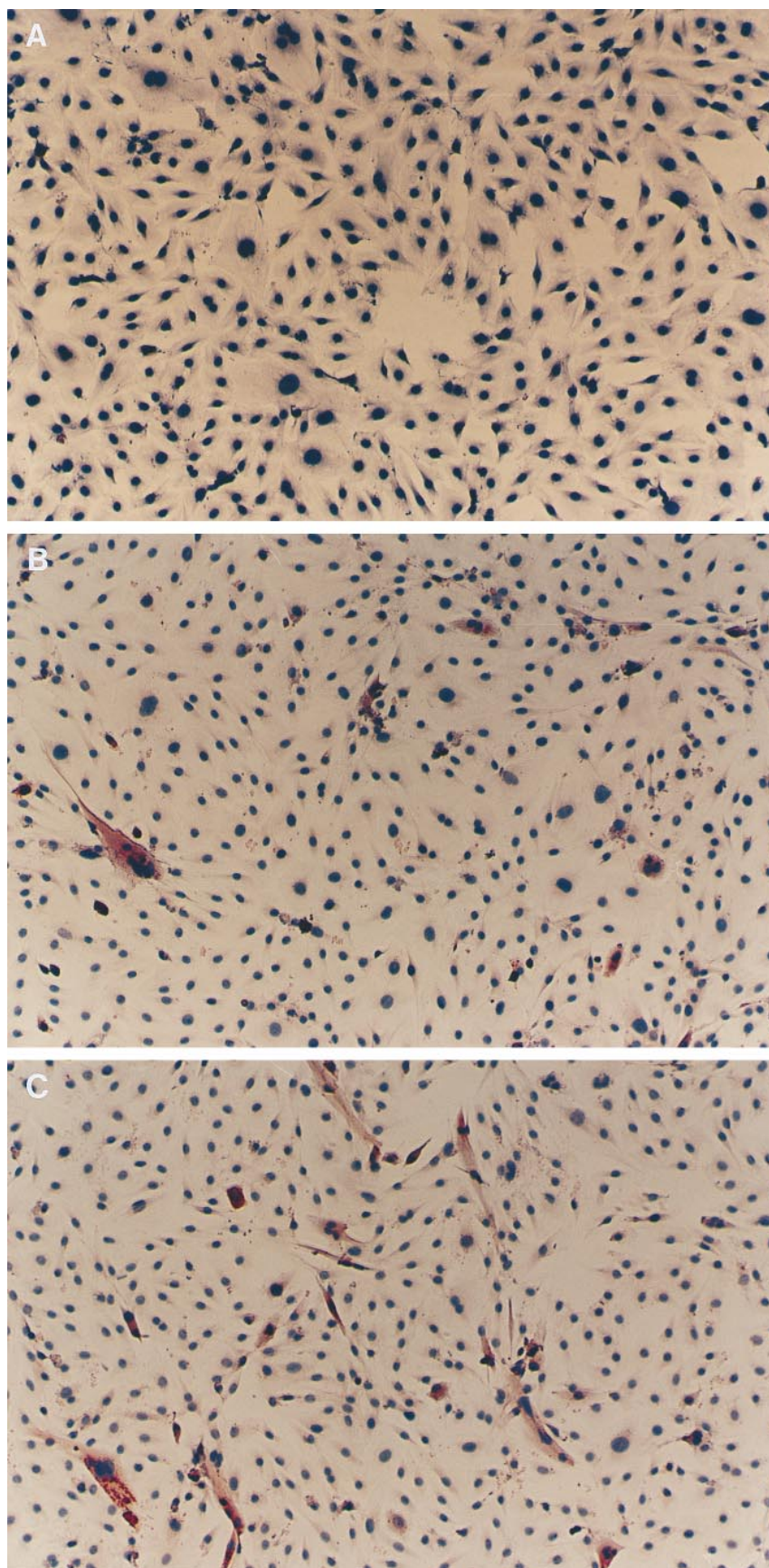


FIG. 2. Expression of exogenous p300 increases the number of C3H10T1/2 cells converted to a myogenic phenotype by MyoD. C3H10T1/2 cells were transiently transfected with expression vectors for p300 (A), MyoD (B), and MyoD and p300 (C) as shown. Myogenic conversion was scored with an MHC monoclonal antibody after 4 days in differentiation medium. Hematoxylin was employed for nuclear staining. The MHC-positive cells are stained red.

TABLE 2. Myogenic conversion of C3H10T1/2 cells operated by MyoD and p300

Field	No. of MHC-positive cells <sup>a</sup>			
	Expt 1		Expt 2	
	MyoD	MyoD + p300	MyoD	MyoD + p300
1	10	23	3	14
2	12	31	8	15
3	19	40	4	14
4	17	42	1	10
5	14	35	2	10
6	17	15	3	11
7	24	30	3	13
8	32	38	5	16
9	12	61	6	14
10	19	44	11	8
11	13	33	5	11
12	9	38	7	7
Total	198	430	58	143

<sup>a</sup> MHC-positive cells were scored by random selection of 12 microscopic fields of cells transiently transfected with MyoD and MyoD plus p300 vectors, respectively. MHC-positive cells were never observed in dishes transfected with the p300 vector alone. Equal numbers of cells were plated in dishes receiving either MyoD alone or in combination with p300. The results of two independent experiments are reported.

MyoD (24). To explore the possibility that p300 might act on the myogenic amino acids to coactivate transcription, the E12(ATK) plasmid and the 4RE reporter construct were introduced in 3T3 mouse fibroblasts with or without a p300 expression vector. The presence of the myogenic code is not sufficient to confer on p300 the ability to coactivate the E proteins (Fig. 1D). E2-5, an E2A gene product, was also insensitive to p300 coactivation (Fig. 1D).

In the MyoD~E47 molecule, forced heterodimerization of MyoD and E proteins is obtained through artificial intramolecular bonding. Compared to MyoD, MyoD~E47 displays increased transactivation, myogenic potential, and resistance to the effect of the HLH negative regulator Id (61). We reasoned that if p300 increased bHLH transcriptional activity by augmenting or stabilizing the dimerization of myogenic and ubiquitous bHLH, the activity of the MyoD~E47-tethered dimer would not be significantly impacted by p300. In the event, p300 increased MyoD~E47 transactivation of 4RE (Fig. 1C) and MCK (not shown) with an efficiency comparable to its effect on MyoD. These results indicate that neither myogenic code recognition nor MyoD heterodimerization is the target of the coactivation of myogenic bHLH by p300.

**The N-terminal activation domain of MyoD mediates the effects of p300.** To understand how p300 causes potentiation of myogenic transcription, we decided to use a series of constructs in which the DNA-binding domain of GAL4 is fused to different regions of MyoD (79). The Gal-MyoD construct expresses full-length MyoD, whereas the Gal-MyoD $\Delta$ Basic and Gal-MyoD $\Delta$ HLH plasmids bear a MyoD cDNA devoid of the regions coding for the basic and HLH regions, respectively. The Gal-NMyoD and Gal-MyoD bHLH constructs express the N-terminal acidic activation domain from amino acid 3 to amino acid 53 of MyoD and the bHLH region of MyoD, respectively. Binding of the various Gal-MyoD proteins does not depend on the ability of MyoD to recognize its DNA target. Different combinations of the Gal-MyoD and p300 expression vectors were transfected in 3T3 cells, and transactivation was determined with a Gal-CAT reporter construct

(Fig. 3A). The results of such transfection experiments indicate (i) that both the basic and the HLH regions of MyoD are dispensable for coactivation; (ii) that the bHLH region of MyoD is insensitive to p300 coactivation; (iii) that p300 coactivation is not directed at the regions required for MyoD to interact with its DNA binding site, the E box; (iv) that p300 coactivation is mediated by the activation domain of MyoD; and (v) that the isolated N-terminal domain of MyoD and full-length MyoD are coactivated by p300 with equal efficiencies. The coactivation efficiency of p300 on the Gal-MyoD constructs was not influenced by the culture conditions of 3T3 cells, since essentially the same results were obtained with media containing 10% fetal bovine or 2% horse serum (data not shown).

**Addition of the VP16 activation domain to an N-terminal-truncated MyoD restores its ability to be coactivated by p300.**

The activation domain of MyoD can be functionally replaced by amino acids 412 to 490 of virion protein 16 (VP16) from herpes simplex virus type 1 (79). This is most likely due to the conservation in both MyoD and VP16 of a stretch of polar amino acids known to be critical for VP16 function (20). To evaluate whether the VP16 activation domain could confer p300 coactivation to a MyoD mutant peptide lacking the activation domain, we made use of expression vectors in which the N-terminal domain of MyoD was either deleted (MyoD $\Delta$ N) (78) or replaced by the VP16 activation domain (MyoD $\Delta$ N-VP16) (79). Truncation of the N-terminal domain enormously reduced the ability of MyoD to activate the 4RE reporter. The residual MyoD transactivation could not be increased by cotransfection with a p300 expression vector. However, attaching the VP16 activation domain to MyoD $\Delta$ N restored the ability of the chimeric molecule to activate transcription with an efficiency even greater than that observed with the full-length MyoD vector and conferred the ability to respond to coactivation by p300 (Fig. 3B). These data confirm the finding shown in Fig. 3A that the N-terminal domain of MyoD is the target of p300 coactivation. Furthermore, they raise the possibility that p300 recognizes common features in the activation domains of VP16 and MyoD.

**p300 coactivates several, but not all transcription factors.**

To address the specificity of p300 coactivation, we investigated whether p300 could augment transcription mediated by several activators. As suggested by the experiments reported in Fig. 3B, the activation domain of VP16 was sensitive to p300 coactivation (Fig. 3C). Similarly, transcription mediated by the proto-oncogene *myc* and the tumor suppressor p53 fused to the Gal4 DNA-binding domain was increased by p300. On the contrary, the activity of the transcription factor Sp1 was not influenced by p300 (Fig. 3C). From these experiments, we conclude that numerous transcription factors whose activation domains are rich in acidic residues are coactivated by p300 and that at least one transcriptional regulator, the glutamine-rich transactivator Sp1, is insensitive to p300.

**Mutation of four conserved amino acids in the activation domain of MyoD impairs transactivation and abrogates coactivation and interaction with p300.**

Weintraub et al. (79) have observed that the activation domain of MyoD contains a conserved stretch of amino acids shown to be important for VP16 function. Within this cluster of conserved amino acids resides an FYD (phenylalanine-tyrosine-aspartic acid) motif at amino acids 29, 30, and 31 of MyoD. The FYD motif is also found in the activation domains of both myogenin (28) and Myf-5 (70), whereas in the activation domain of MRF4 (51, 54, 67), the aspartic acid is replaced by leucine. Furthermore, a phenylalanine residue conserved at position 42 of MyoD and the corresponding position of Myf-5 and myogenin (38a) is located in

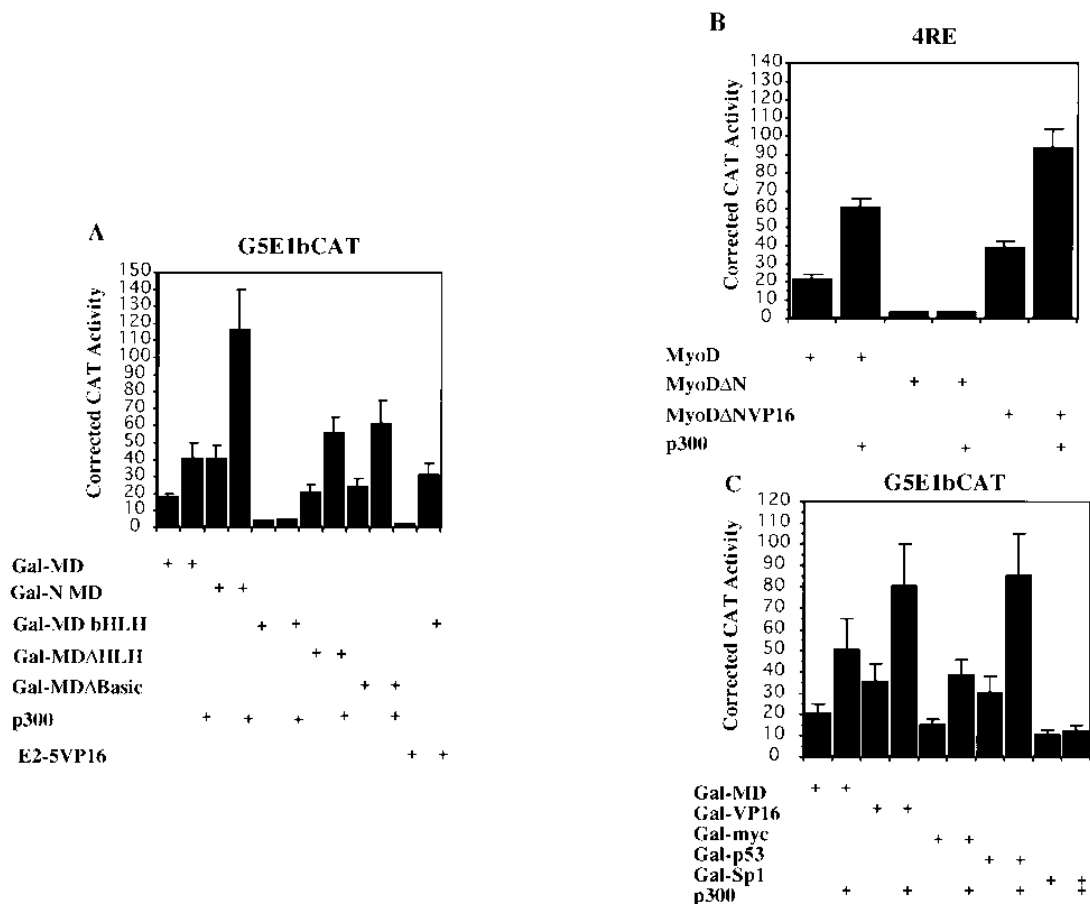


FIG. 3. Amino terminus of MyoD is the target of p300 coactivation. (A) Activation domain of MyoD fused to the Gal4 DNA-binding domain is sufficient for p300 coactivation. Different regions of MyoD linked to the Gal4 DNA-binding domain were assayed for their ability to stimulate transcription in 3T3 cells of the Gal-dependent G5E1bCAT reporter construct with or without the collaboration of p300. Gal-N MD expresses amino acids 3 to 53 of MyoD, and Gal-MyoD bHLH expresses the bHLH region of MyoD. Deletions of the HLH (Gal-MDΔHLH) and Basic (Gal-MDΔBasic) regions of MyoD do not affect p300 coactivation. One microgram of each Gal-MD construct and 2  $\mu$ g of the p300 expression vector were used as indicated. Gal-VP16 is coactivated by p300. Transfection of the Gal-MD constructs generates equivalent levels of fusion proteins in the recipient cells (79). The values represent the average of five independent experiments. Cotransfection of the Gal-MD bHLH and E2-5VP16 constructs causes transcriptional activation, indicating that Gal-MD bHLH is functionally competent. (B) Acidic activator VP16 confers coactivation potential on an otherwise p300-insensitive MyoD mutant. Wild-type (MyoD) or N-terminally truncated (MyoDΔN) MyoD or N-terminally truncated VP16 (MyoDΔNVP16) plus an expression vector for p300 was introduced in 3T3 cells, and transcriptional activation of the 4RE reporter was measured. The values represent the averages of three independent experiments. (C) p300 coactivates several, but not all, activation domains. The DNA-binding domain of Gal4 is fused to MyoD (Gal-MD), VP16 (Gal-VP16), Myc (Gal-myc), p53 (Gal-p53), and Sp1 (Gal-Sp1). Coactivation of p300 on each of the Gal fusion proteins was assayed in the presence of the G5E1bCAT reporter gene in 3T3 cells. The values represent the averages of five independent experiments.

a domain that shows homology with a region of VP16 that is critical for activation (20). The phenylalanine at position 442 of VP16 is critical, since its substitution with a proline (VP16 Phe442→Pro) abolishes transcriptional activation by VP16 (20). We thus created two mutated vectors, first substituting the phenylalanine at position 29 of MyoD with a proline residue to create [(MD-N (FP))] and then substituting all three FYD residues and the phenylalanine at position 42 of MyoD with two prolines, an alanine, and a proline, respectively, to create [MD-N (PPA+P)] (see Materials and Methods). These constructs were fused to the GAL4 DNA-binding domain to generate the constructs Gal-N MD(FP) and Gal-N MD (PPA+P). Transcription promoted by the Gal-N MD(FP) and Gal-N MD(PPA+P) constructs was reduced by approximately 70 and 90%, respectively, compared to that of Gal-N MD (Fig. 4A), demonstrating the critical role played by the wild-type amino acids of the FYD motif in creating a fully functional activation domain. More interestingly, Gal-N MD(FP) but not Gal-N MD(PPA+P) was coactivated by p300 (Fig. 4A).

To determine whether the lack of coactivation of Gal-N MD(PPA+P) was paralleled by an absence of physical interaction with p300, the constructs were assayed in a mammalian two-hybrid system. Contrary to the synergism observed between VP16p300 and Gal-N MD, VP16p300 constructs failed to promote transcription in association with the Gal-N MD(PPA+P) plasmid (Fig. 4B), indicating an absence of interaction between the p300 and N MD(PPA+P) molecules. The efficiency with which VP16p300 augments Gal-N MD transactivation is greater than that engendered by the p300 vector alone (compare Fig. 4A and B). This suggests that VP16 is an active component in stimulating transcription and is a further indication of interactions between the two chimeric proteins. A plasmid expressing the VP16 protein alone does not influence transactivation of the Gal-N MD construct (data not shown). These results indicate that the FYD motif, and perhaps phenylalanine at position 42, is a critical module for transcriptional activation by the myogenic bHLH proteins and for their coactivation by p300.



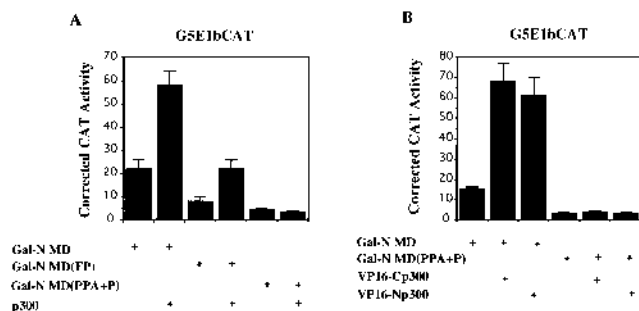


FIG. 4. Identification of the FYD amino acid motif and phenylalanine 42 in the MyoD activation domain as the target for p300 coactivation. (A) Substitution of four amino acids in the activation domain of MyoD abrogates its ability to transactivate, to respond to p300 coactivation, and to interact with p300. Gal-N MD, Gal-N MD(FP), and Gal-N MD(PPA+P) were compared for their abilities to promote transcription and to be coactivated by p300 in 3T3 cells. The Gal-N MD constructs express 3 to 53 N-terminal amino acids of MyoD fused to the GAL4 DNA-binding domain. For details regarding the mutations introduced in the Gal-N MD(FP) and in the Gal-N MD(PPA+P) constructs, see Materials and Methods. The Gal-N MD constructs were used at 1  $\mu$ g per transfection, and the p300 construct was used at 2  $\mu$ g per transfection. The reporter G5E1bCAT was used at 2  $\mu$ g per transfection. The values represent the averages of five independent experiments. (B) Gal-N MD but not Gal-N MD(PPA+P) is recognized by VP16p300. A mammalian two-hybrid system assay was conducted with 3T3 cells with 2  $\mu$ g of the Gal-N MD or mutated Gal-N MD(PPA+P) constructs and 2  $\mu$ g of the C-terminal or N-terminal domain of p300 appended to VP16 (VP16-Cp300 and VP16-Np300, respectively). The values represent the averages of five independent experiments.

**p300 and CBP can associate with MyoD in vitro.** How does p300 coactivate the activation domain of MyoD? The demonstration of interactions between VP16p300 and Gal-N-MD in the mammalian two-hybrid system (Fig. 4) strongly suggests a direct interaction of p300 with the N-terminal domain of MyoD but does not eliminate the possibility of an indirect mechanism in which p300 either activates additional, unknown factors or pairs with MyoD via a third protein.

To test whether or not p300 can directly interact with MyoD, various segments of p300 were radiolabeled in vitro and allowed to react with agarose beads coated with GST-MyoD fusion protein as described in Materials and Methods. The results of these experiments are presented in Fig. 5 and show that GST-MyoD specifically interacts with p300. To delineate the regions of p300 involved in the interaction, we used a combination of nested N-terminal deletions of in vitro radiolabeled p300 as well as various segments of p300 fused to GST. Maps of the polypeptide domains used are presented in Fig. 5A and B. Agarose-immobilized GST-MyoD protein was able to bind polypeptides expressed from the nested amino-terminal deletions of types B and S but not of deletion type A. Thus, the p300 amino acids between amino acids 1572 and 1869 interact with MyoD. This region spans a domain also known to be necessary for p300 to recognize the E1A protein; deletion of amino acids 1737 to 1809 (p300 $\Delta$ 30) impairs the ability of p300 to interact with E1A (26). Nevertheless, the p300 $\Delta$ 30 expression vector can still coactivate MyoD as we showed earlier (Fig. 1), further restraining the sites of interaction between this region of p300 and MyoD to amino acids 1572 to 1736 and/or 1810 to 1869. We also scanned the p300 protein for interaction with MyoD by using equimolar amounts of three GST-p300 fusion proteins encoding amino acids 1 to 596, 744 to 1571, and 1572 to 2370 and designated as left (L), central (C), and right (R), respectively (Fig. 5B). In keeping with the results shown in Fig. 5A, the GST-p300 polypeptide R from amino acids 1572 to 2370 binds in vitro radiolabeled MyoD. Unexpectedly, the GST-p300 polypeptide L from 1 to 596 also interacted with

MyoD, whereas the GST-p300 polypeptide C from 744 to 1572 failed to do so. These data indicate that the L and R regions of p300 located at the N and C termini of the molecule have the ability to bind independently to MyoD in vitro, suggesting that multiple interfaces are involved in this protein-protein interaction. The L and R regions of p300 that can bind MyoD in these in vitro assays contain a very high degree of amino acid identity with the corresponding domains of CBP (2). Furthermore, CBP coactivates MyoD (Fig. 1). GST-MyoD appears to bind to bacterially synthesized <sup>35</sup>S-labeled CBP (Fig. 5C), suggesting that the structural and functional similarities of CBP to p300 include the ability to interact directly with MyoD. We wanted to investigate whether p300 could also interact with the E proteins, which are required for functional activity of MyoD in vivo (46). The experiments shown in Fig. 5H indicate that indeed p300 recognizes the E12 protein.

**N-terminal region of MyoD is contacted by p300.** Our functional analysis of the MyoD domains capable of responding to coactivation by p300 (see Fig. 3 and 4) demonstrated that the amino terminus was both sufficient and required and that the basic and HLH domains were dispensable. We used various segments of the MyoD polypeptide to test whether there was a correspondence between the domains of MyoD responsible for binding with p300 and the domains required for coactivation. We first tested whether the integrity of helix 1 and helix 2 of MyoD is necessary for p300 binding. Domain replacement mutants of MyoD in which helix 1 or 2 is replaced with the corresponding regions of Myc (MyoDMycHelix1 and MyoDMycHelix2) are incapable of binding to DNA or of heterodimerization with E12 (23). The results of experiments presented in Fig. 5D indicate that both the MyoDMycHelix1 and the MyoDMycHelix2 polypeptides retain the ability to bind to p300. This set of observations suggests that regions of MyoD other than H1 or H2 interact with p300. To confirm this conclusion, we tested a GST fusion polypeptide containing just the HLH region of MyoD plus four adjacent amino acids (the junction) and found that it failed to bind radiolabeled p300 (Fig. 5E).

We next performed experiments to test whether the MyoD basic region is required for p300 binding. MyoD replacement proteins in which the basic region of MyoD was replaced by the corresponding regions of other bHLH proteins, including E12 (MyoD-E12Basic), Myc (MD-Myc Basic), and T4 achaete-scute (MyoDT4Basic) (23), were radiolabeled and tested for their ability to bind to p300. The three chimeric proteins are efficiently retained by both the GST-p300 L and R domain proteins (Fig. 5F) but not on the GST-p300 C domain (data not shown). The L and R domains bound the chimeric bHLH proteins with an affinity comparable to that of the MyoD wild type. These observations suggest that the basic region of MyoD is dispensable for p300 recognition.

To analyze the p300 binding behavior of the C- and N-terminal regions of MyoD, the first N-terminal 100 amino acids and the C-terminal amino acids 162 to 318 of MyoD were translated and radiolabeled in vitro and tested for their ability to bind either the GST-p300 L or GST-p300 R polypeptide. The data in Fig. 5G show that the N-terminal region of MyoD was retained by both of the agarose-immobilized GST-p300 fusion proteins, whereas the C-terminal domain was not. We conclude that the N-terminal region of MyoD contains amino acid domains responsible both for physical binding and functional coactivation by p300. To our knowledge, p300 is the first cellular protein that has been shown to interact with the N-terminal region of MyoD.

**MyoD associates with p300 in cells.** To test whether exogenously expressed MyoD and p300 associate in cells, COS cells were transiently cotransfected with a FLAG-tagged MyoD ex-

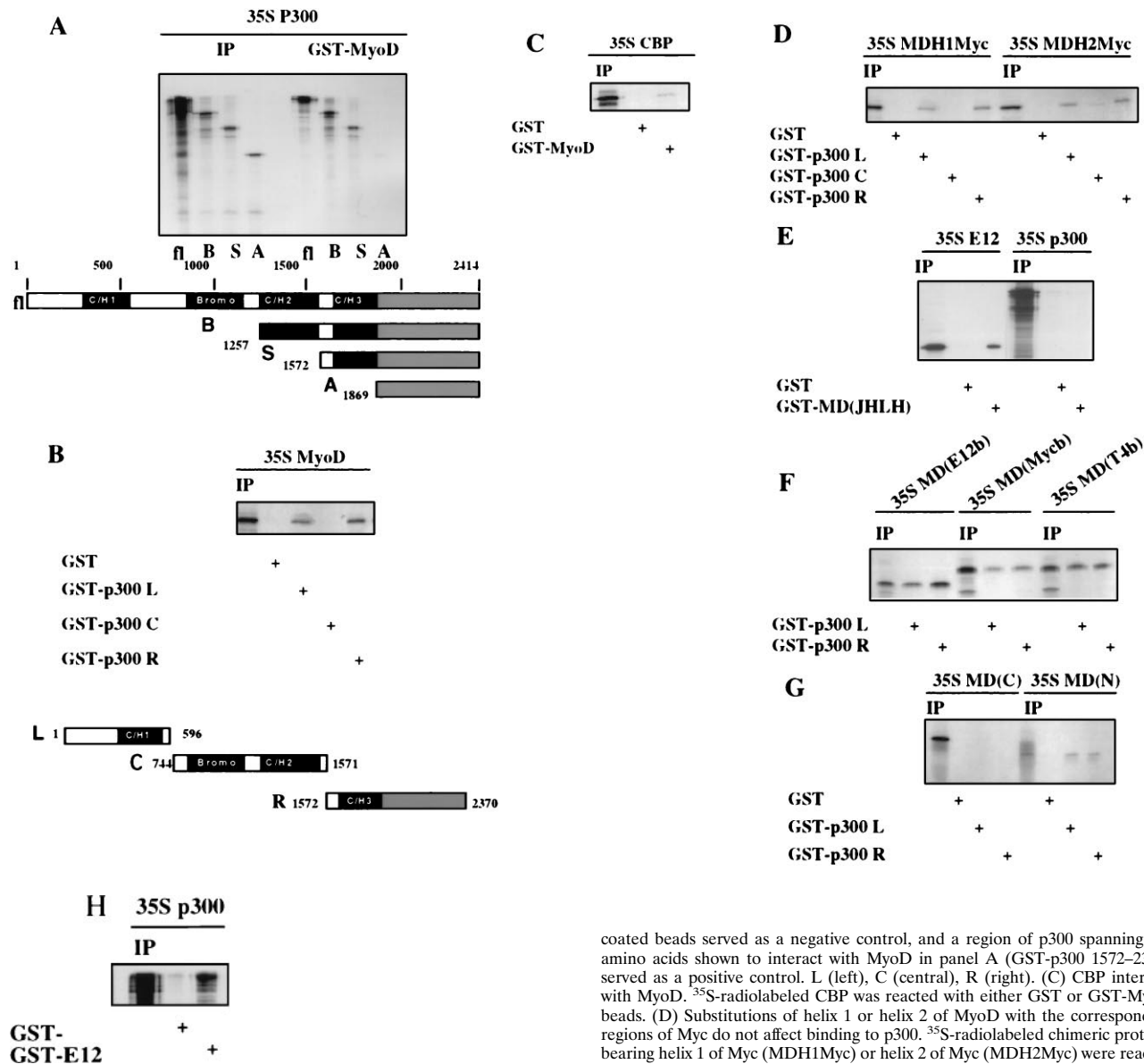


FIG. 5. In vitro assays define p300 and MyoD polypeptide domains involved in direct interactions. In vitro-synthesized and  $^{35}\text{S}$ -radiolabeled polypeptides derived from segments of p300 or MyoD were mixed with agarose beads coated with GST fusion polypeptides. The specific polypeptide segments used are designated in the panels. After incubation, the beads were repeatedly washed, and the bound peptides were eluted by boiling. The eluates and the input proteins (IP) were analyzed by SDS-PAGE. In each reaction mixture, the input protein was estimated to be approximately 10% of the total protein. The diagrams (not to scale) illustrate the segments of p300 that were radiolabeled (A) or included in GST fusion moieties (B, D, F, and G). The numbers indicate the amino acid residues of p300, while the designations C/H1, Bromo, C/H2, and C/H3 refer to the bromodomain and three cysteine/histidine-rich regions (26). The source of the radiolabeled input proteins is indicated above each panel, and the presence of GST fusion peptides in each reaction mixture is indicated by the letter designations defined in the schematic maps or by a + below the lanes for the corresponding reactions. (A) A segment of p300 between amino acids 1572 and 1869 binds to MyoD. Beads coated with GST alone did not bind input proteins (not shown). The restriction enzymes employed to generate 5' DNA deletions encoding the truncated p300 proteins were as follows: B, *Bgl*I; S, *Sma*I; and A, *Aha*II. fl, full-length. (B) Amino and carboxyl termini of p300 independently bind to MyoD. GST-p300 fusion proteins schematically illustrated were mixed with  $^{35}\text{S}$ -labeled MyoD protein, washed, eluted, and analyzed by SDS-PAGE. GST-

coated beads served as a negative control, and a region of p300 spanning the amino acids shown to interact with MyoD in panel A (GST-p300 1572–2370) served as a positive control. L (left), C (central), R (right). (C) CBP interacts with MyoD.  $^{35}\text{S}$ -radiolabeled CBP was reacted with either GST or GST-MyoD beads. (D) Substitutions of helix 1 or helix 2 of MyoD with the corresponding regions of Myc do not affect binding to p300.  $^{35}\text{S}$ -radiolabeled chimeric proteins bearing helix 1 of Myc (MDH1Myc) or helix 2 of Myc (MDH2Myc) were reacted with agarose beads coated with GST alone or the indicated GST-p300 fusion proteins. Like MyoD wild-type protein, the MDMyC proteins are retained by both the L and R regions of p300 (corresponding to amino acids 1 to 596 and 1572 to 2370, respectively). The p300 M polypeptide (744 to 1571) does not bind the MDMyC chimeric polypeptides. (E) A region corresponding to the junction and HLH domains of MyoD does not recognize p300. In vitro-translated radiolabeled E12 and p300 proteins were tested for their ability to bind with the GST-MD(JHLH) fusion protein. The interacting polypeptides were resolved by SDS-PAGE. p300 does not bind to GST-MD(JHLH). The binding of the radiolabeled E12 protein, however, demonstrates the ability of the GST-MD(JHLH) molecule to mediate protein-protein interactions. (F) Replacement of the basic region of MyoD with other basic domains of nonmyogenic bHLH proteins preserves the interaction of the chimeric molecules with p300. The MD(E12b), MD(mycb), and MD(T4b) constructs are MyoD-based proteins with the basic domain replaced by the corresponding domain of E12, Myc, and *Drosophila* T4 achaete-scute, respectively. (G) The N-terminal region of MyoD, but not the carboxyl domain, interacts with p300. The regions corresponding to the first N-terminal 100 amino acids [MD(N)] and the last C-terminal 156 amino acids [MD(C)] of MyoD were translated in vitro in the presence of [ $^{35}\text{S}$ ]methionine, mixed with the indicated GST-p300 proteins, and, after incubation, washing, and elution, resolved by SDS-PAGE. (H) The E2A gene product E12 interacts with p300. In vitro  $^{35}\text{S}$ -radiolabeled p300 was incubated in the presence of GST- or GST-E12-coated agarose beads and, after washing and elution, resolved by SDS-PAGE.

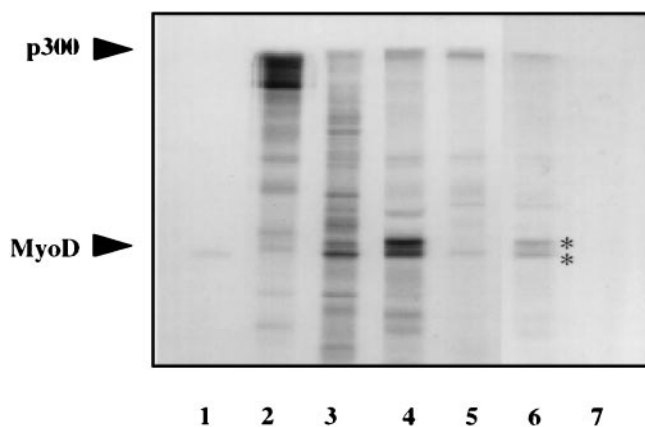


FIG. 6. MyoD is associated with p300 in nuclear extracts derived from COS cells cotransfected with MyoD and p300 expression vectors. SDS-PAGE analysis of [<sup>35</sup>S]methionine-labeled nuclear extracts derived from COS cells transiently transfected with vectors expressing FLAG-tagged MyoD and HA-tagged p300. Lanes: 1, in vitro transcribed and/or translated MyoD; 2, in vitro transcribed and/or translated p300; 3, an aliquot of radiolabeled COS extract (approximately 10<sup>5</sup> cpm) before immunoprecipitation; 4, radiolabeled COS extracts subjected to immunoprecipitation with an anti-FLAG-protein G-agarose complex; 5, radiolabeled COS extracts subjected to immunoprecipitation with an anti-HA-protein G-agarose complex; 6, radiolabeled COS extracts subjected to double immunoprecipitation, first with an anti-HA and then with an anti-FLAG-protein G complex; 7, radiolabeled COS extracts subjected to double immunoprecipitation, first with a preimmune serum-protein G-agarose and then with an anti-FLAG-protein G complex. Omission of the p300-HA plasmid from the transfection buffer resulted in the inability to immunoprecipitate MyoD from radiolabeled COS extracts with the anti-HA and anti-FLAG antibodies sequentially (data not shown). The vector employed to express the HA-tagged p300 does not contain a simian virus 40 origin of replication, and this might explain the low levels of p300 produced in COS cells. The asterisks indicate two bands corresponding to hypo- (fast-migrating) and hyper- (slow-migrating) phosphorylated forms of MyoD. Arrowheads indicate the location in the gel of p300 and MyoD.

pression vector and a p300 HA-tagged vector. The transfected cells were metabolically radiolabeled with [<sup>35</sup>S]methionine, and the nuclear extracts derived from them were subjected to a series of single or double immunoprecipitations with antibodies directed against the FLAG and the HA peptides under highly stringent conditions (RIPA buffer). The results reported in Fig. 6 indicate that exogenous p300 immunoprecipitated from nuclear extracts of the doubly transfected COS cells carries with it exogenous MyoD. The MyoD doublet represents hypo- (fast-migrating) and hyper- (slow-migrating) phosphorylated forms of the protein (46).

**Both C and N termini of p300 bind and coactivate the N-terminal domain of MyoD.** The two domains of p300 at the N and C termini that interact with MyoD in vitro contain known activation domains (85). We determined whether these regions of p300 alone can coactivate MyoD by transfecting NIH 3T3 cells with expression vectors coding for MyoD as well as for p300 or the first N-terminal 744 amino acids (p300-N) or for amino acids 871 to 2377 of p300 (p300-C). The results shown in Fig. 7A indicate that both the N- and C-terminal domains of p300 can coactivate MyoD. The C terminus of p300 was able to coactivate at a level equal to that induced by the entire p300 molecule; the p300 N-terminal construct, although active, was less efficient. We next proceeded to determine whether the isolated N-terminal region of MyoD can respond to these C- or N-terminal domains of p300. In fact, the Gal-NMD construct was also coactivated by both of these p300 domains (Fig. 7B). As observed for the experiments conducted with MyoD (Fig. 6A), the coactivation potential of the C terminus was equal to that of p300, but the N-terminal domain

was less active. Thus, the N-terminal activation of MyoD appears to be a binding target of both the N and C termini of p300, and both interactions are associated with coactivation of transcription.

**p300 augments MEF2-dependent transactivation and interacts with the MADS domain of MEF2C.** MEF2C and the myogenic bHLH can activate transcription either by using their own activation domains or by tethering on the DNA and using each others' activation domains (56). We wanted to test the hypothesis that the myogenic bHLH and MEF2 members have a common coactivator, namely p300. To this end, a reporter construct bearing two copies of a MEF2-binding site derived from the rat embryonic MHC promoter (2XA/Temb) was transfected in 3T3 cells with an expression vector for MEF2C in the absence or presence of a p300 expression vector. MEF2C activated the reporter construct, and p300 further increased MEF2C transactivation by an additional threefold (Fig. 8A). To determine whether the synergism observed between MEF2C and p300 was paralleled by a direct interaction of the two proteins, an in vitro protein-protein interaction assay was performed with radiolabeled p300 and various GST-MEF2C fusion proteins. The results of this experiment demonstrated in Fig. 8B indicate that p300 is recognized by MEF2C and that the MADS domain of MEF2C is necessary and sufficient to mediate this recognition. As shown for MyoD (Fig. 1), CBP was also able to substitute for p300 in coactivating MEF2C (Fig. 8A). These results support the notion that the interactions of p300 (and CBP) with the two families of myogenic transcriptional activators may be similar.

**A truncated form of p300 acts as a negative regulator of myogenesis.** Since the p300 amino-terminal deletion construct S interacts with MyoD, whereas the additionally truncated A construct does not (Fig. 5), we predicted that the former but not the latter would coactivate MyoD in transient transfection experiments. The truncated peptide S coactivated MyoD with an efficiency similar to that of a vector encoding the entire p300 molecule (Fig. 9A [compare with Fig. 6A]). Surprisingly, the p300 A construct not only failed to coactivate but actually repressed MyoD-mediated transactivation (Fig. 9A) and inhibited myogenesis, as judged by MHC immunostaining of transfected C3H10T1/2 cells (Fig. 9B). These observations suggest that p300 A might be acting to interfere with the coactivation

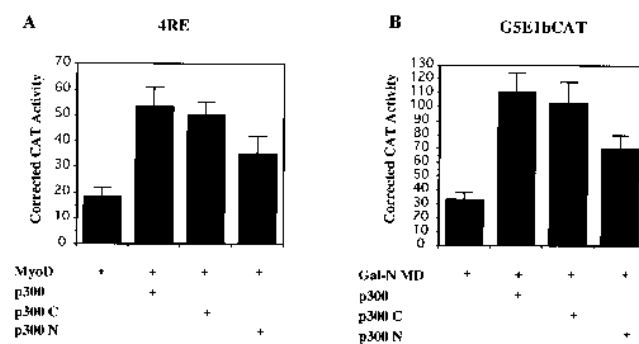


FIG. 7. The two domains of p300 that bind MyoD also coactivate the MyoD activation domain. (A) 3T3 cells were transiently transfected with the 4RE-CAT reporter construct and expression vectors for MyoD and p300, and the CAT assay was performed as described in Materials and Methods. The p300-N vector encodes the first 744 N-terminal amino acids, and the p300-C vector encodes amino acids from 871 to 2377 of p300. (B) The 56 N-terminal amino acids of MyoD fused to the GAL4 DNA-binding domain (Gal-N MD) were assayed for their ability to respond to p300 coactivation in 3T3 cells with expression vectors coding for the entire (p300) or truncated molecules (p300 N and p300 C are those described for panel A).

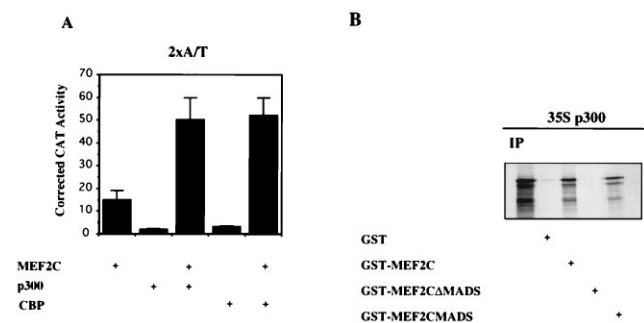


FIG. 8. p300 coactivates MEF2C by interaction with its MADS domain. (A) The MEF2-dependent reporter construct 2xA/T (2xA/T) was introduced in 3T3 cells with expression vectors for MEF2C and for either p300 or CBP, and transcriptional activation was determined by CAT assay 72 h after transfection. The values represent the averages of four independent experiments. (B) In vitro protein-protein interaction assay conducted with agarose-immobilized GST-MEF2C fusion protein (GST-MEF2C), a GST-MEF2C protein with the MADS domain deleted (GST-MEF2CΔMADS), or the MADS domain alone fused to the GST moiety (GST-MEF2CΔMADS) and <sup>35</sup>S-radiolabeled p300. The reactions were resolved by SDS-PAGE. Agarose-immobilized GST protein serves as the control for the specificity of the binding reaction. IP, input protein.

capacity of endogenous p300 in a dominant negative fashion. One mechanism for such inhibition could be the formation of inactive heteromers between p300 and p300 A.

To ascertain whether the p300 A polypeptide can multimerize with a region of p300 competent for MyoD coactivation, the GST-p300 R polypeptide (amino acids 1572 to 2370) immobilized on agarose beads was reacted with radiolabeled p300 S or A polypeptides. The results of these experiments are shown in Fig. 9C and indicate that both p300 S and p300 A are able to interact with the coactivating R domain of p300 (for a summary of these results, see Table 3). One interpretation of these data is that multimerization of the p300 S polypeptide with endogenous p300 would allow the resulting multimer to interact with and coactivate MyoD, whereas the p300 A form, which is itself incapable of binding to MyoD, would generate multimers with endogenous p300 that are no longer able to interact with MyoD. Alternative explanations include the possibility that the truncated p300 A polypeptide, but not the longer p300 polypeptides, could sequester additional factors required for MyoD to function.

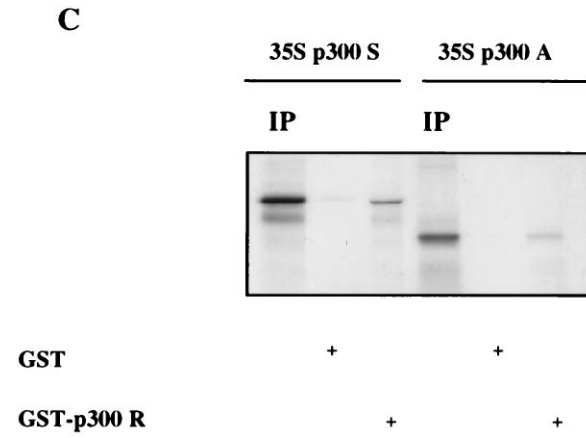
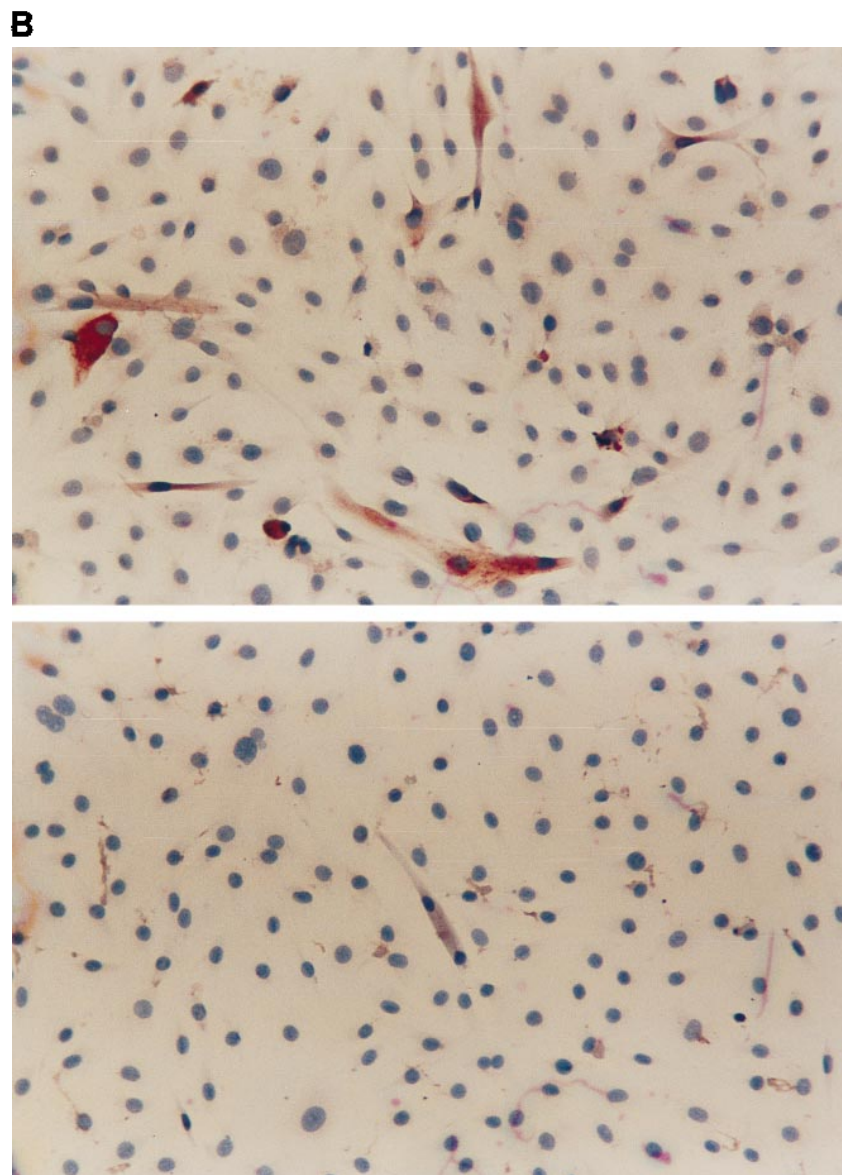
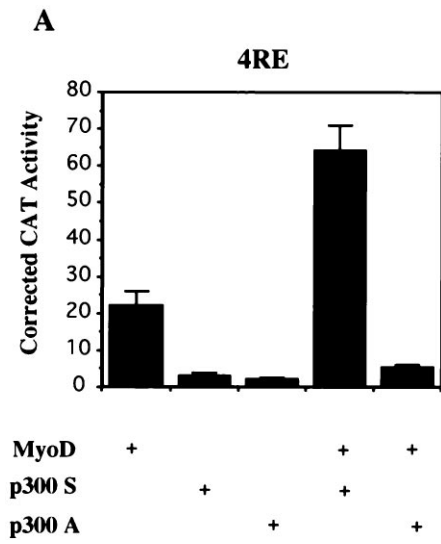
## DISCUSSION

**p300 and myogenesis.** Three coactivators, p300, CBP, and TAFII250, were tested for their abilities to augment MyoD-dependent transactivation. CBP and p300 were both studied on the basis of the observation that they show extensive amino acid homology, including a bromodomain, which is also conserved in TAFII250. We have found that p300 and the related CBP protein (but not TAFII250) increase the ability of MyoD and myogenin to transactivate several muscle-specific regulatory regions and to promote myogenesis (Fig. 1 and 2). While this study was in progress, Yuan et al. reported that p300 is a coactivator of MyoD (85). The results of both physical binding and functional transcription assays indicated that two domains of p300 can act independently to contact and transactivate MyoD and that, reciprocally, the N-terminal region of MyoD, containing its major activation domain, is required for the binding and coactivation by p300. One of the two domains of p300 engaged in contacting MyoD, the L segment, is located at the N terminus of the molecule (amino acids 1 to 596). This segment overlaps a region known to interact with retinoic acid

receptors (RAR) (41). The second domain of p300 that binds MyoD is its C terminus, a region known to bind the oncoprotein E1A (26). However, a p300 mutant (p300Δ30) that fails to interact with E1A retained its ability to coactivate MyoD, thus eliminating this region from contention. This result implies that p300 counteracts the inhibition of muscle differentiation by E1A (85) (our unpublished result) by a mechanism other than binding and sequestering the oncoprotein. Our findings are consistent with the observation that an E1A mutant protein unable to interact with p300 retains the ability to repress muscle-specific transcription (75). p300 binding to the bHLH-E1A complex might relieve a negative transcriptional constraint imposed by the oncoprotein on the myogenic activators or might indirectly displace E1A.

p300 collaborates with MyoD to increase the number of converted C3H10T1/2 fibroblast cells, suggesting that endogenous p300 may be rate limiting in the recipient cells. A similar hypothesis has been put forward regarding the rate-limiting effects of CBP (19, 44) on coactivation of the thyroid hormone receptor and for AP1-dependent transcription in HeLa cells (41). A further indication for a rate-limiting role of p300 in muscle transcription derives from our circumstantial observation of a very low level of p300 expression in myogenic cells (Fig. 1B). Since p300 functions as a common coactivator for several factors (22, 41, 47), we speculate that the artificial introduction of one such activator (i.e., MyoD) in cells that do not normally contain it (i.e., fibroblasts) requires a concomitant increase of the p300 levels to allow the activator to function optimally. We have observed that p300 increases MyoD-mediated transcription of a p21 reporter construct (Fig. 1A). The cyclin-dependent kinase inhibitor p21 controls cell-cycle progression and differentiation. Indeed, analysis of p21 transcripts in several cell lines, including those deriving from erythroid (40, 74), epidermal (55) and muscle cell lineages (35, 73) and from developing embryos (64), has shown a correlation between p21 expression, cell cycle withdrawal, and differentiation. The observation that MyoD induces p21 expression during muscle differentiation and that p300 is capable of coactivating this process provides a conceptual framework to explain how differentiation could be initiated by a cell-type-specific transcriptional activator in association with a coregulator of transcription.

**p300 regulates muscle-specific transcription by coactivating both the myogenic bHLH and MEF2 transcription factors.** MyoD activates transcription by using a discrete domain of 50 amino acids located at its N-terminal region (79). Evidence has accumulated that the activation domain normally assumes a conformation unavailable to promote transcription and that the activation domain is released by proper interactions of a coactivator with the myogenic code of both MyoD (23, 79) and myogenin (14). The two myogenic amino acids alanine and threonine are conserved in the basic domains of each of the four myogenic determinant factors and are absent in other nonmyogenic bHLH proteins. Although the details of how such interactions with coactivators would result in exposing the distally located activation domain of MyoD have not yet been clarified, members of the MEF2 family have been shown to physically interact with the two myogenic amino acids of MyoD (42, 56). Such interaction coincides with the ability of MEF2C to synergize with MyoD and provides evidence that alanine 114 and threonine 115 of MyoD are essential for coregulation by MEF2C. The interaction of MEF2C with MyoD might induce the direct conformational change of the MyoD protein resulting in the unmasking of the activation domain. Alternatively, as a consequence of MEF2-MyoD interaction, additional proteins could be recruited by the activation domains of MyoD or



MEF2 and mediate transcriptional competency. Is p300 such a protein? Our experiments indicate that p300 coactivates both MyoD and MEF2C. The N-terminal 50 amino acids of MyoD are sufficient to sense and respond to p300 coactivation, whereas the integrity of other domains of MyoD that mediate DNA recognition, heterodimerization with E proteins, and the myogenic amino acids seems to be dispensable. The ability of p300 to confer increased transcription to the activation domain of MyoD is paralleled by our observation that the N-terminal region of MyoD is, by itself, capable of interacting both in vitro and in vivo with p300. These findings are most consistent with a direct action of p300 on the activation domain of MyoD.

Among the transcriptional targets of the myogenic bHLH proteins are the MEF2 genes themselves (21, 46, 52). The MEF2 gene products are in turn likely to control myogenic bHLH expression, since the myogenin promoter (18, 27, 83) and regulatory regions of the *Xenopus myoDa* gene (49) contain functionally relevant DNA binding sites for MEF2. Thus, it is likely that the myogenic bHLH and the MEF2 gene products would establish a positive loop by regulating the transcriptional activity of each other (56). By coactivating both MyoD and MEF2C, we propose that p300 maintains and enhances the transcriptional circuitry that regulates the activities of these two pivotal muscle transcriptional activators.

**p300 coactivates MyoD by targeting specific amino acid residues common to other myogenic bHLH proteins.** Amino acids 412 to 490 of the VP16 protein contain an acidic transcriptional activation domain that fully complements that of MyoD (79) and myogenin (72). A detailed mutagenesis study has determined that a Phe442→Pro substitution abolished the potency of the VP16 activation domain (20, 66). Several hydrophobic amino acids located in cluster III of the VP16 activation domain are conserved in the MyoD activation domain (38a). Our data imply that at least some of these conserved amino acids may delineate a common conformation and activation mechanism for VP16 and the myogenic bHLH proteins. Substitution of one of these conserved amino acids, the phenylalanine at position 29 of MyoD, reduced the transcriptional activity of MyoD and three additional mutations in Gal-N MD(PPA+P) not only abolished transcription but also abolished p300 coactivation (Fig. 4). The mutations introduced in Gal-N MD(PPA+P) destroy the conserved FYD motif present in the activation domains of each myogenic bHLH (FYL in MRF4) and a phenylalanine residue at position 42 of MyoD. Because of this conservation in a critical location of the molecule and because of the deleterious effect of the mutations on MyoD transactivation, we propose that the FYD motif is likely to be important in conferring transactivation properties to all of the myogenic bHLH proteins. The cyclic side chains of the three prolines introduced in the construct Gal-N MD(PPA+P) are incompatible with the predicted  $\alpha$ -helical structure of the N-terminal region of MyoD (38a). Alternative amino acid substitutions will be necessary to determine whether the loss of both transcriptional activation and p300 coactivation by the Gal-N MD(PPA+P) mutations is related to a general struc-

tural modification or to a more specific effect related to the side chains of the substituted amino acids.

Our and others' findings that p300 coactivates numerous transcription factors indicate that p300 is not a dedicated coactivator. Most likely, different amino acid residues present in several activators are able to sense its action. The transcriptional activators that we have tested and that are coactivated by p300 (i.e., MyoD, VP16, Myc, and p53) have a common feature; that is, they contain several acidic residues in their respective activation domains. On the other hand, p300 does not seem to behave without specificity, because at least two transcriptional activators, Sp1 and E2-5, do not sense its activity, at least on Gal4-dependent and 4RE reporters (Fig. 1C and 3C). Promoter context seems to influence the ability of the E2A gene products to activate transcription (80). Therefore, we cannot exclude the possibility that E2A gene products might be coactivated by p300 on more complex reporter constructs, as suggested by the observation of a physical association, at least in vitro, of p300 and E12 (Fig. 5H).

**Domains of p300 involved in contacting MyoD and other transcriptional activators.** The two L and R domains of p300 (encoding, respectively, the N-terminal 596 amino acids and a C-terminal region encompassing amino acids 1572 to 1869) independently interact with the MyoD N-terminal domain with roughly comparable affinities. The C and A segments of p300 consisting of amino acids 744 to 1571 (containing the conserved bromodomain) and amino acids 1869 to 2414, respectively, fail to bind MyoD. The N terminus of p300 is also the site of interaction with the RAR, and it has been recently reported that the Jun protein can independently bind the C-terminal and the bromodomain-containing regions of p300 (47). Taken together, these observations suggest that p300 consists of flexible modules that can accommodate interactions with multiple activators of transcription and that can integrate signals deriving from multiple pathways. Amino acids 1 to 596 and 1737 to 2413 of p300 activate transcription when fused to the GAL4 DNA-binding domain (85). These regions overlap with those contacted by MyoD, the TATA-binding protein (TBP), and the basal transcription factor IIB (TFIIB) (85). Analogously, the RAR recognizes a region of p300 that interacts with TBP while c-Fos interacts with a region of CBP that is also contacted by TFIIB (7). The observation that p300 can interact with MyoD, c-Fos, RAR, and Jun as well as TBP (1) and TFIIB, suggests that p300 might constitute a physical nexus between enhancer-binding proteins and components of the basal transcriptional machinery. This observation further suggests that transcriptional activation exerted by the activation domains of enhancer-binding proteins contacting p300 could be transmitted to the basal transcriptional apparatus by the activation domains of p300 itself.

**p300 as a coregulator of cellular differentiation.** p300 coregulates transcription mediated by a number of activators engaged in controlling different aspects of cellular differentiation. These include the bHLH myogenic proteins (reference 85 and this study); the oncogene product Myb (22); the retinoic

FIG. 9. A truncated form of p300 acts as a dominant negative regulator of MyoD and multimerizes with a functionally competent portion of p300. (A) Carboxyl region A of p300 MyoD suppresses MyoD transactivation functions. A MyoD expression vector plus the 4RE-CAT reporter construct were transfected in 3T3 cells in the absence or presence of expression vectors encoding the polypeptide domains S and A of p300. The values represent the averages of three independent experiments. (B) p300 A inhibits MyoD-dependent conversion of C3H10T1/2 cells. C3H10T1/2 cells were transiently transfected with either MyoD alone (top panel) or MyoD plus the vector encoding the p300 A domain (bottom panel). The cells were cultured for 4 days in differentiation medium. While the cells transfected with MyoD alone show clear signs of myogenic differentiation, including elongated shape, multinucleation, and positivity for staining with an MHC antibody, the cells transfected with p300A in combination with MyoD maintain the monotonous morphology of undifferentiated fibroblasts and are negative for MHC staining. (C) p300 A forms homomultimers with GST-p300. Agarose-immobilized GST-p300 R polypeptide (amino acids 1572 to 2370) was allowed to interact with radiolabeled p300 polypeptides expressed by the p300 S and p300 A vectors. After elution, the bound polypeptides were analyzed by SDS-PAGE. Both p300 S and A proteins are retained on GST-p300, but not on the GST moiety. IP, input radiolabeled proteins.

TABLE 3. Properties of p300 constructs

p300 domain <sup>a</sup>	MyoD binding	Transcription	Phenotypic conversion
p300	Yes	Coactivates	Myogenic
S	Yes	Coactivates	Myogenic
A	No	Inhibits	Inhibitory
GST-p300 R	Yes	NA <sup>b</sup>	NA

<sup>a</sup> Domain designations are as in Fig. 5.

<sup>b</sup> NA, not applicable.

acid, estrogen, glucocorticoid, and thyroid hormone receptors; and AP1 activity (41, 47). The effects of the oncoprotein E1A are also influenced by p300 by a direct interaction of the two proteins (36, 57) that possibly results in mutual titration. The ability of E1A to relieve transcriptional repression induced by YY1, a member of the human Krüppel family of proteins, is in fact mediated by p300 through a mechanism entailing physical association of the two proteins (48). Interaction of E1A and myogenic as well as ubiquitous bHLH proteins has been demonstrated (75), and rescue of E1A-mediated repression of muscle enhancers by p300 has been documented. The level of complexity at which p300 operates is increased by the observation that the targets of p300 coactivation are themselves regulators of p300. For instance, the retinoic acids and E1A induce p300 phosphorylation, a phenomenon associated with cellular differentiation of F9 cells (43). Furthermore p300 is an *in vitro* substrate for the Cdc2 cyclin-dependent kinase (6) and has been found associated in a phosphorylated form with cyclin A (8). That such posttranscriptional modifications can affect p300 function is demonstrated by the fact that p300 phosphorylation and ubiquitination influence the ability of p300 to interact with either E1A or simian virus 40 T antigen, respectively (5, 25). In this context, we have found that two reporter constructs, 4RE and MCK, are coactivated by p300 when introduced in C2C12 muscle cells (Fig. 1D). Their behavior indicates that exogenous p300 better coactivates when the cells are kept in culture conditions that favor cell cycling and that, at least for the 4RE construct, cell differentiation induced by serum removal adversely affects the activity of exogenous p300. This observation might be interpreted as the consequence of saturating levels of p300 in differentiated and not in cycling C2C12 cells. As an alternative explanation, endogenous p300 might be prevented from coactivating muscle transcription in myoblasts because of posttranscriptional modification or complex formation with inhibiting proteins. Such negative constraints, not sensed by stoichiometrically relevant amounts of exogenous p300, would be released during differentiation, making p300 available to more efficiently coactivate transcription and promote differentiation.

The considerable size of p300 and the plethora of proteins interacting with it prompt the view of p300 as a scaffold on which disparate molecules including transcription factors, kinases, and coactivators converge to regulate transcription pertinent to cellular differentiation.

#### ACKNOWLEDGMENTS

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#### ADDENDUM IN PROOF

During the revision of the manuscript, we learned that two studies have reported observations on MyoD and p300 similar to those presented here (R. Eckner, T.-P. Yao, E. Oldread, and D. M. Livingston, *Genes Dev.* **10**:2478–2490, 1996; P. L. Puri, M. L. Avantaggiati, C. Balsano, N. Sang, A. Graessmann, A. Giordano, and M. Levrero, *EMBO J.*, in press).

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