The myogenic protein MyoD requires two nuclear histone acetyltransferases, CREB-binding protein (CBP)/p300 and PCAF, to transactivate muscle promoters. MyoD is acetylated by PCAF in vitro, which seems to increase its affinity for DNA. We here show that MyoD is constitutively acetylated in muscle cells. In vitro, MyoD is acetylated both by CBP/p300 and by PCAF on two lysines located at the boundary of the DNA binding domain. MyoD acetylation by CBP/p300 (as well as by PCAF) increases its activity on a muscle-specific promoter, as assessed by microinjection experiments. MyoD mutants that cannot be acetylated in vitro are not activated in the functional assay. Our results provide direct evidence that MyoD acetylation functionally activates the protein and show that both CBP and CBP/p300 are candidate enzymes for MyoD acetylation in vivo.

MyoD, a transcription factor of the myogenic basic helix-loop-helix (bHLH) family, is central to the process of muscle cell differentiation (1). It is involved in skeletal muscle determination and functions at early stages of development (2). Like other members of the myogenic bHLH family, MyoD is able to induce a muscle phenotype in nonmuscle cells, a phenomenon referred to as myogenic conversion (3). MyoD and the other myogenic bHLHs transactivate muscle specific promoters, such as that of the muscle creatine kinase (MCK) gene (4). Myogenic bHLHs are recruited to these promoters through specific sequences referred to as E-boxes, to which they bind as heterodimers with the ubiquitous E12/E47 proteins (5). MyoD is expressed early in the muscle cell lineage; it is expressed but poorly active in proliferating myoblasts, and its activity is regulated by multiple mechanisms. MyoD is a very unstable molecule that is degraded through ubiquitination (6), a process regulated by phosphorylation (7). In addition, MyoD is controlled by a protein inhibitor, Id (8).

Two co-activators, CBP/p300 and PCAF, are required for muscle promoter transactivation by MyoD (9, 10). CBP and p300 are homologous proteins (11). CBP was first characterized as a co-activator for the CREB transcription factor (12), and p300 was characterized as a target for transforming viral proteins such as E1A (11). CBP and p300 share domains of strong homology, including several domains of interaction with a variety of transcription factors (13), and appear to be functionally equivalent in all systems studied so far, with one exception (14). In cells, CBP/p300 are part of large multimolecular complexes, some of which also include general transcription factors (15). CBP/p300 are thought to co-activate transcription by acting as bridges between these sequence-specific transcription factors and the basal transcription machinery (16). In addition, CBP/p300 also display an intrinsic histone acetyltransferase (HAT) activity (17, 18) and are able to recruit other histone acetyltransferases such as PCAF (19). Core histone acetylation appears to be the hallmark of dynamic chromatin and is associated with active transcription, whereas deacetylated histones are most often found in silent, static parts of the genome (20). Some histone acetyltransferases are involved in transcription (21, 22). Acetylation occurs on lysine residues located in the N-terminal histone tails, which protrude from the core nucleosome (23). In addition, histone acetyltransferases also acetylate nonhistone proteins, such as general (24) or sequence-specific (25–27) transcription factors, and for some of them acetylation has been shown to be of functional importance. A proportion of CBP/p300 and PCAF is associated in multimolecular complexes, in which they may exert distinct functions (28), probably through acetylation of distinct lysines on histones (29) or on nonhistone proteins (27).

CBP/p300 (9, 30, 31) and PCAF (10) have been shown to be required for terminal differentiation of myoblasts and transactivation of muscle-specific promoters. CBP/p300 directly interacts with MyoD, apparently through the MyoD transactivation domain located in the N-terminal moiety of the molecule (32). Results from transient transfection assays using mutants of these proteins suggested differential roles for CBP/p300 and PCAF. In particular, transactivation of the p21 promoter by MyoD seemed to require PCAF HAT activity but not CBP/p300 HAT activity (10). HAT targets in the muscle differentiation program have not yet been fully determined. These targets might include both histone and nonhistone proteins. Indeed, Sartorelli et al. (33) have shown that MyoD itself can be acetylated by PCAF in vitro. In their study, MyoD ectopically expressed in muscle cells was found to be acetylated, and the level of MyoD acetylation appeared to increase slightly during terminal differentiation. In vitro, MyoD acetylation by PCAF appeared to increase its affinity for DNA (33). Analysis of mutants of the protein in which specific lysines were replaced by

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**CREB-binding Protein/p300 Activates MyoD by Acetylation**

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arginines suggested that acetylation could play a role in MyoD activity. However, the low activity of the point mutants could result from conformational perturbations of the protein rather than lack of acetylation. Here, we show that endogenous MyoD is acetylated. In contrast to ectopically expressed MyoD (33), the level of acetylation of the endogenous protein did not seem to vary during terminal differentiation. In vitro, MyoD could be acetylated by CBP/p300 as well as by PCAF. The sites of acetylation were determined by mass spectrometry and confirmed using point mutants of the protein. These experiments indicated that two lysines located at the boundary of the DNA binding/dimerization domain (the hHLL domain), lysines 99 and 102, are specifically acetylated. We have used microinjection experiments in which acetylated and nonacetylated MyoD were directly compared to assess the functional effect of acetylation. MyoD was injected into individual cells together with an MCK GFP reporter vector. Acetylation by CBP or p300, as well as acetylation by PCAF, increased the transactivating capability of MyoD. Functional acetylation required the integrity of lysines that are acetylated in vitro. Taken together, these results demonstrate that MyoD acetylation increases its activity, and that acetylation by CBP/p300 is as efficient as acetylation by PCAF for activating the protein.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**

Myoblastic C2C12 and embryonic C3H 10T1/2 cells were maintained in Dulbecco’s minimal essential medium (Life Technologies, Inc.) supplemented with 15% fetal calf serum (Dominique Dutcher) and 10% fetal calf serum, respectively. To induce terminal differentiation, C2C12 were placed in differentiation medium (Dulbecco’s minimal essential medium, 1% fetal calf serum). Phenotypic differentiation was maximal after 72 h.

**Preparation of Recombinant Proteins and Acetylation in Vitro**

The bacterial expression vectors containing wild type MyoD cDNA and all mutated species of MyoD were kind gifts of Dr. K. Breitschopf, A. Ciechanover, and S. Leibovitch and direct the expression of untagged MyoD. BL21 (DE3)/pLysS E. coli cells were used for bacterial expression of MyoD. Following induction with isopropyl-1-thio-β-D-galactopyranoside, cells were lysed, and MyoD was precipitated by 0.6 M NaCl (final volume, 10 μl). Cells received a mixture of dextran/rhodamine (M, 40,000 and 10,000 fixable; Molecular Probes, Inc., Eugene, OR) at 1% final concentration, together with 2 mg/ml MCK-GFP/NLS reporter vector (expressing the green fluorescent protein (GFP) fused to a nuclear localization signal; a kind gift of Dr. S. A. Leibovitch) and 1 μg of MyoD (incubated for 1 h at 30 °C with 100 ng of baculovirus-produced P300 or p300, with or without acetyl-CoA (1 μM final concentration). After 24 h of culture, cells were fixed with 2% paraformaldehyde in PBS. Coverslips were then mounted on slides with VectaShield (Biovalley) and analyzed by fluorescence microscopy.

**Immunoprecipitation and Western Blotting**

Immunoprecipitation and Western blotting were performed using standard procedures. Immunoprecipitated proteins were collected on Protein G-agarose (Sigma). Beads were washed three times in PBS. The samples were resolved on SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with phosphate-buffered saline containing 10% dry milk, incubated with antibodies followed by horseradish peroxidase-conjugated secondary antibodies (Sigma), and developed using the Roche Molecular Biochemicals LumiLight kit as recommended by the manufacturer.

Acetylation of MyoD in live cells was assessed by immunoprecipitation of total cell extracts of proliferating or differentiating C2C12 myoblasts with anti-acetyllysine antibodies (Upstate Biotechnology, Inc.) and the anti-MyoD antibodies (C-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Alternatively, extracts were analyzed by immunoprecipitation with anti-MyoD antibodies (5.8A; Novoceastra, followed by Western blotting with the anti-acetyllysine antibodies (Upstate Biotechnology) and the anti-MyoD antibodies (C-20; Santa Cruz Biotechnology).

**Electrophoretic Mobility Shift Assay**

A synthetic oligonucleotide containing an E box derived from a muscle creatine kinase gene enhancer (5′-CTAGACCCCCAACACTTGCTTC-3′) was 32P-end-labeled using the T4 polynucleotide kinase end-labeling kit (New England Biolabs). Recombinant MyoD (wild type or mutants; 0.5 μg) was incubated with 2 ng of probe in a reaction mixture containing 10 mM Hepes, pH 7.9, 0.1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 10% glycerol, and 300 ng of poly(dI-dC) for 30 min at room temperature. The binding reactions were loaded on nondenaturing acrylamide gels run in 0.25× TBE at 50 V for 1 h at room temperature. Gels were dried and autoradiographed.

**RESULTS**

**MyoD Is Acetylated in Vitro by CBP/p300 and PCAF—**Recombinant MyoD (untagged; see “Experimental Procedures”) was incubated with recombinant HATs (p300 or PCAF) were produced in baculovirus-driven overexpression systems) together with 14C-labeled acetyl-CoA. Both enzymes were able to promote the incorporation of 14C into MyoD and not into gluthathione S-transferase, used as an irrelevant control protein (Fig. 1). Incorporation was estimated at roughly 100 pmol of HAT proteins and [14C]acetyl-CoA. Peptides were directly compared to assess the functional effect of acetylation. MyoD was injected into individual cells together with a MCK GFP reporter vector. Acetylation by CBP or p300, as well as acetylation by PCAF, increased the transactivating capability of MyoD. Functional acetylation required the integrity of lysines that are acetylated in vitro. Taken together, these results demonstrate that MyoD acetylation increases its activity, and that acetylation by CBP/p300 is as efficient as acetylation by PCAF for activating the protein.

**Analysis of Acetylated Peptides**

The acetylated MyoD peptide described above and fragments thereof were microinjected in a buffer containing 10 mM Tris, pH 7.5, and 100 mM KCl (final volume, 10 μl). Cells received a mixture of dextran/rhodamine (M, 40,000 and 10,000 fixable; Molecular Probes, Inc., Eugene, OR) at 1% final concentration, together with 2 mg/ml MCK-GFP/NLS reporter vector (expressing the green fluorescent protein (GFP) fused to a nuclear localization signal; a kind gift of Dr. S. A. Leibovitch) and 1 μg of MyoD (incubated for 1 h at 30 °C with 100 ng of baculovirus-produced P300 or p300, with or without acetyl-CoA (1 μM final concentration). After 24 h of culture, cells were fixed with 2% paraformaldehyde in PBS. Coverslips were then mounted on slides with VectaShield (Biovalley) and analyzed by fluorescence microscopy.

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Acetylation of MyoD in live cells was assessed by immunoprecipitation of total cell extracts of proliferating or differentiating C2C12 myoblasts with anti-acetyllysine antibodies (Upstate Biotechnology, Inc.) and the anti-MyoD antibodies (C-20; Santa Cruz Biotechnology).
immunoprecipitated using this antibody and analyzed by Western blotting using anti-MyoD antibodies. MyoD could be detected in the immunoprecipitates of C2C12 cells obtained with immunoblotting using anti-MyoD antibodies. MyoD could be immunoprecipitated using this antibody and analyzed by Western blotting using the anti-MyoD antibody and an irrelevant antibody did not pull down MyoD in muscle cell extracts. This result strongly suggests that endogenous MyoD is acetylated. In addition, the levels of MyoD acetylation in growing myoblasts appeared to be equivalent to those in differentiated cells (Fig. 2A, compare C2 growth and C2 differentiation). This result was confirmed by reciprocal experiments, in which extracts were immunoprecipitated using the anti-MyoD antibody and analyzed by Western blotting with the anti-acetyllysine antibody (Fig. 2B). Comparison of proliferating and terminally differentiating myoblasts again did not show any increase in the level of MyoD acetylation upon differentiation (Fig. 2B, compare C2 growth and C2 differentiation). The specificity of the anti-acetyllysine antibody was verified using in vitro acetylated MyoD, which demonstrated that the antibody recognized only the acetylated species (Fig. 2C). Taken together, these results demonstrate that acetylated MyoD is detectable in C2C12 muscle cells and suggest that the level of MyoD acetylation is not modulated during terminal differentiation.

Two Lysines Are Acetylated by CBP/p300 and PCAF—Point mutants in which lysines are progressively replaced by arginines, starting at the N terminus (Fig. 3A), were used in in vitro experiments to locate the acetylated lysines. The replacement of three lysines located at the boundary of the bHLH domain, lysines 99, 102, and 104 (Fig. 3B), strongly decreased acetylation by CBP/p300, suggesting that these lysines were acetylated. No further decrease was observed when, in addition, downstream lysines were mutated. This result suggests that CBP/p300 acetylates the same lysines as those acetylated by PCAF (33). This result was confirmed using synthetic peptides corresponding to amino acid sequences of MyoD that include lysines (Fig. 3C). Peptides corresponding to amino acids 109–127 or 130–149 were not acetylated in this assay, whereas the peptide corresponding to amino acids 96–107 of MyoD was acetylated, confirming the results obtained with MyoD mutants (Fig. 3B). Peptide 96–107 was acetylated to similar levels by the two enzymes, as was a peptide corresponding to the histone H3 N-terminal 24 amino acids used as an internal standard. In contrast, only CBP/p300 was able to acetylate a peptide corresponding to H4 N-terminal sequence; no acetylation was observed with this peptide using PCAF, as expected from previously published data concerning PCAF substrate preference (29).

In order to determine the site of acetylation, the MyoD 96–107 synthetic peptide modified by CBP, p300, or PCAF was analyzed by mass spectrometry and chemical sequencing (Fig. 4A and data not shown). Cleavage of the peptide at arginine sites using ArgC protease invariably yielded an unmodified form of the C-terminal peptide (positions 9–18; M + H = 1219.6) (Fig. 4A). The modification was always restricted to the N-terminal portion (positions 1–8), and thus lysine residue 9 (MyoD Lys102) could be excluded as a site of modification. Likewise, the N-terminal α-amino group was not modified, since in all cases, the mass peaks corresponding to the nonacetylated tryptophan immunion ion (m/z = 159.1) and the modified b2 ion (Trp-Ala; m/z = 258.1) were detectable in fragment spectra, whereas the peaks for their acetylated counterparts were missing (not shown).

In the monoacetylated peptide (M + H = 1007.5) the main site of acetylation appears to be Lys7 (MyoD Lys102). This is mainly concluded from the fact that, after mass spectrometry fragmentation (data not shown), we detected the mass signal (m/z = 345.2) for the acetylated y2 ion (Ac-Lys-Arg) and the following y ion series, but the signal for the nonacetylated ion was either absent or extremely weak. Lys4 (MyoD Lys99) is also acetylated, albeit to a lesser extent, as can be deduced from the presence of the acetylated b4 and b5 ions (331.2 and 602.3) besides their nonmodified counterparts (489.2 and 560.3). These findings were confirmed by Edman sequencing of the monoacetylated peptide. No differences could be found when we compared the peptides modified by CBP, p300, and PCAF. To summarize, in all cases the preferential site of acetylation in the in vitro assay corresponds to MyoD Lys102, and Lys99 is also modified by the enzymes.

This results was confirmed by the analysis of mutants of MyoD in which lysines 99 and 102 or 104 were mutated. Whereas replacement of lysine 99 and 102 by arginines decreased MyoD acetylation to background levels, replacement of lysine 104 did not have any effect (Fig. 4B). Identical results...
were obtained with PCAF as the acetylating enzyme (data not shown).

X-ray analysis of a MyoD-DNA complex had previously demonstrated that lysines 99 and 102 are not involved in DNA binding (38). Indeed, their mutation did not have any effect on MyoD binding to DNA in EMSA experiments, and mutants 2–5 displayed a wild type affinity for DNA (Fig. 4C). As expected from x-ray data (38), the mutation of lysine 146, in contrast, destroyed the interaction between MyoD and its target sequence (Fig. 4C, mutants 6 and 7).

Taken together, these results indicate that both p300 and PCAF are able to specifically acetylate MyoD in vitro, most likely on lysines 99 and 102.

Acetylation of MyoD by p300 or PCAF Increases Its Transactivation Capability—Mutation of lysines 99, 102, and/or 104, since triple point mutants of these lysines had no effect on an irrelevant promoter (data not shown). Results (Fig. 5) show that MyoD acetylation increased its ability to transactivate the MCK promoter, as assessed first by the increased number of GFP-positive microinjected cells and second by the fact that GFP-positive cells that received acetylated MyoD displayed a brighter fluorescence than those that received nonacetylated MyoD (Fig. 5A). Injected doses of acetylated or nonacetylated MyoD seemed to be equivalent, as assessed by immunofluorescence on injected cells using an anti-MyoD antibody (data not shown). Acetylation of MyoD resulted in a 3–4-fold increase in its activity. Similar results were obtained with PCAF and with p300, indicating that both enzymes are able to functionally activate MyoD by acetylation. The functional activation was related to acetylation of lysines 99, 102, and/or 104, since triple point mutants of these lysines were not activated by acetylation by p300 (mut2 and mut5, Fig. 5, B and D) nor by PCAF (mut2 and mut5, Fig. 5, C and D). Taken together, these results indicate that acetylation of MyoD by CBP/p300 or PCAF on lysines 99 and 102 positively affects its activity.

**DISCUSSION**

MyoD, a key regulator of muscle cell differentiation, is expressed but inactive in proliferating myoblasts and is activated on terminal differentiation. It is regulated by a number of mechanisms, including ubiquitination, phosphorylation, and inhibitors, such as Id. In addition, Sartorelli *et al.* (33) have recently shown that MyoD can also be acetylated by a nuclear
HAT, PCAF, at least when ectopically expressed in muscle cells. We here show that endogenous MyoD can be detected in an acetylated form (Fig. 2), since it is specifically recognized by anti-acetyllysine antibodies. Interestingly, MyoD appears as a single band when detected using this antibody, whereas it is a doublet of bands when detected using an anti-MyoD antibody. These two bands have previously been shown to correspond to post-translational modifications of the protein by phosphorylation (7). In this regard, it will be interesting to investigate the relationship between acetylation and other post-translational modifications such as phosphorylation.

Our results suggest that the acetylation level of endogenous MyoD did not vary in a significant manner during terminal differentiation, in contrast to what was described by Sartorelli et al. (33) for ectopically expressed MyoD. Endogenous MyoD is thus acetylated in proliferating myoblasts, in which it is inactive; hence, acetylation is not sufficient for MyoD activation when terminal differentiation is triggered. Other mechanisms, such as dissociation from the inhibitor Id, are likely to be involved in this activation. In vitro, MyoD can be acetylated by CBP/p300 as well as by PCAF. Mass spectrometry analysis of synthetic peptides suggested that both enzymes specifically acetylate two lysines, lysine 99 and 102, with the same preference for lysine 102. Analysis of mutants of these lysines supported this conclusion (Figs. 3 and 4). This stands in contrast to what has been previously published concerning PCAF (33), where three lysines were found to be acetylated in the same region (99, 102, and 104) in experiments using synthetic peptides and mutants of these lysines. The determination of the lysines acetylated in live cells is the important issue and is currently under investigation. The acetylated lysines are located at the boundary of MyoD's DNA binding and heterodimerization domain, the bHLH. CBP/p300 and PCAF seem to acetylate the same lysine residues on MyoD, in contrast to what has been observed for other substrates; e.g., CBP/p300 acetylates several lysines on histone H4 as well as on histone H3, whereas PCAF shows a strong preference for lysine 14 of histone H3 (Ref. 29; see also Fig. 3C).

We have used a microinjection assay to demonstrate that acetylation of MyoD by both CBP/p300 and PCAF increases its transactivating capability by 3–4-fold. We believe that this represents a highly significant increase, given the fact that, from our estimation, only 1/3 of the MyoD molecules are actually acetylated in the samples. This activation corroborates previous results obtained in transient transfection experiments (33). The interpretation of these transfection experiments was based on the comparison between wild type MyoD and point mutants in which specific lysines have been replaced by arginines, which cannot be acetylated. However, the effect of these mutations could be the result of a conformational change of the protein, rather than of the lack of acetylation. Our approach using microinjection is more direct. In addition, our approach allowed to directly demonstrate that, like PCAF, CBP/p300 is also able to activate MyoD through acetylation. Although the CBP/p300 HAT domain is dispensable for activation of the p21 promoter by MyoD (10), there are arguments supporting an important role of CBP/p300 HAT in muscle cell differentiation.

MyoD was acetylated in vitro by p300 (A, B, and D) or PCAF (C and D). Acetyl-CoA was omitted from negative controls. Acetylated MyoD was microinjected into C3H embryonic cells, together with an MCK-GFP reporter construct and an injection marker coupled to rhodamine. Cells were analyzed 24 h later. A shows photographs of injected cells. B and C show quantitative results of typical experiments in which 80–100 cells were counted. D shows MyoD activation, as a ratio between the number of GFP-positive cells obtained with acetylated MyoD versus that obtained with nonacetylated MyoD (mean of three independent experiments; S.D. values are also shown).
For example, a mutant of CBP with impaired HAT activity acts as a negative transdominant for terminal differentiation in stable transfectants in which it is inducible. Results described in this paper suggest that the CBP/p300 HAT domain might indeed participate in the process of terminal differentiation through acetylation of MyoD.

The mechanism through which MyoD acetylation increases its activity in our microinjected assay is a matter of conjecture. Experiments in which microinjected MyoD was detected using immunocytofluorescence indicated that the doses of injected protein was similar in the two cases (data not shown) and, thus, that the effect was not due to an artifactual increase of the protein concentration due to the process of acetylation, such as, for example, increased solubility of the acetylated samples.

Taken together, our results directly demonstrate that MyoD acetylation by CBP/p300, as well as by PCAF, increases its activity in our microinjection assay is a matter of conjecture. For example, a mutant of CBP with impaired HAT activity acts as a negative transdominant for terminal differentiation in stable transfectants in which it is inducible. Results described in this paper suggest that the CBP/p300 HAT domain might indeed participate in the process of terminal differentiation through acetylation of MyoD.

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