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Biochemistry

A point mutation in the MyoD basic domain imparts c-Myc-like properties

(transcription factor/helix–loop–helix proteins/transformation/skeletal muscle)

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ABSTRACT **MyoD and c-Myc, members of the large "basic–helix–loop–helix" family of proteins, regulate diverse aspects of both normal and neoplastic growth and specific gene regulation. These two proteins differ at 9 of the 14 amino acids that comprise the basic domains necessary for DNA binding and transcriptional control. Individual amino acids in the MyoD basic domain were mutated to those found at the analogous positions in c-Myc. Four classes of mutants were obtained: (*i*) those with no effects on MyoD-site binding or activation of MyoD-responsive genes, (*ii*) those with no effect on MyoD-site binding but with a loss of activation potential, (*iii*) those with a loss of both DNA binding and activation potential, and (*iv*) one mutant (mut 9, Leu^{122} → Arg) that left MyoD-site binding unaffected but imparted a new c-Myc-site binding capability. mut 9 competed with wild-type protein for the activation of MyoD-responsive reporter genes but could, like c-Myc, also suppress the adenovirus major-late promoter, which contains a c-Myc binding site. Our studies thus identify specific amino acid residues in the MyoD basic domain that are important for its activity as a DNA-binding transcriptional activator. Most significantly, our results with mut 9 indicate that Leu^{122} of MyoD is a critical determinant of specific DNA binding and that mutation at this residue can alter this specificity.**

The basic–helix–loop–helix (bHLH) family of proteins consists of >20 members involved in myogenesis, proliferation, oncogenesis, and other aspects of tissue-specific gene expression (1–3). bHLH domains consist of a 12- to 15-amino acid segment containing a high content of basic residues and a region consisting of two amphipathic α-helices of $\approx$15 amino acids each (1). The basic motif comprises a sequence-specific DNA-binding domain whereas the HLH region is required for dimerization (4, 5). DNA binding is greatly enhanced with dimerized proteins (4, 6, 7), although dimerization does not require the DNA-binding domain (4).

bHLH proteins bind a consensus DNA element termed an E-box motif, whose precise sequence is determined by the amino acids comprising the basic domain (7, 8). Basic-domain mutations that allow for E-box binding but not for subsequent expression of an adjacent reporter gene indicate that DNA binding and transcriptional activation are distinct and separable functions of bHLH proteins (4, 9, 10).

MyoD, in heterodimeric association with its companion protein E12 (or E47), binds specifically to E-box sites (CACCTG) located in the promoters of muscle-specific genes (8, 11–14). Myc proteins have been shown to dimerize with the bHLH-containing protein Max (15, 16) and to bind an E-box element of a sequence (CACGTG) different from that bound by MyoD (17–20). Myc and Max comprise a distinct subset of the bHLH family based upon two major criteria: (*i*) all members contain an additional leucine zipper domain that is also important for dimerization and *in vivo* activity (15, 16, 21, 22) and (*ii*) neither protein dimerizes with any other members of the bHLH family (1, 15, 23).

MyoD and c-Myc differ at 9 of the 14 amino acids of their basic domains. We have altered individual or multiple amino acids in the MyoD basic domain to those found at the analogous positions in c-Myc and have thus identified four classes of mutations. Of greatest interest is a mutation that allows MyoD protein to bind to a c-Myc DNA site with high affinity and to regulate a gene containing a c-Myc site. Surprisingly, this mutant protein retains the ability to bind a MyoD site but can no longer trigger myogenesis or induce the expression of a MyoD-responsive gene. Naturally occurring MyoD mutations, such as those described here, might contribute to the genesis of skeletal muscle tumors.

MATERIALS AND METHODS

Mutagenesis and *in Vitro* Expression of MyoD cDNAs. A full-length murine MyoD cDNA in the vector pEMc11S (24) was excised with *Eco*RI and recloned in M13mp18. Single-stranded phage DNA was used for mutagenesis (25) with a Bio-Rad Muta-Gene kit and specific mutagenic oligonucleotides (Fig. 1). Mutant cDNAs were excised from double-stranded phage DNAs and cloned into either pBluescript SK(+) (Stratagene) or the pRcCMV eukaryotic expression vector (Invitrogen, St. Louis). *In vitro* transcribed RNAs were translated in rabbit reticulocyte lysate (Promega) with either [^{35}S]methionine or nonradioactive methionine. In some cases, translation mixes included either E12 RNA [from the plasmid pE12R (1) (gift of D. Baltimore, Rockefeller University)] or chimeric Max/E12 RNA (see below). Radioactively labeled proteins were analyzed by SDS/PAGE. In some cases, proteins were immunoprecipitated with either a polyclonal anti-MyoD antibody (gift of S. Tapscott, University of Washington) or an anti-Max antibody generated with a bacterially expressed full-length Max protein (unpublished work).

Cell Culture and DNA Transfection. C3H/10T½ cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% supplemented calf serum (HyClone). Transfections (4) included equivalent amounts of the β-galactosidase-encoding plasmid pCH110 to correct for transfection efficiencies prior to chloramphenicol acetyltransferase (CAT) assays. To study myogenesis, transfected cells were incubated in DMEM plus 2% heat-inactivated horse serum for 4 days. Colonies were fixed (24) and incubated with the anti-myosin heavy chain monoclonal antibody MF-20 (26).

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Abbreviations: bHLH, basic–helix–loop–helix; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility-shift assay; MCK, muscle creatine kinase; MLP, major late promoter.

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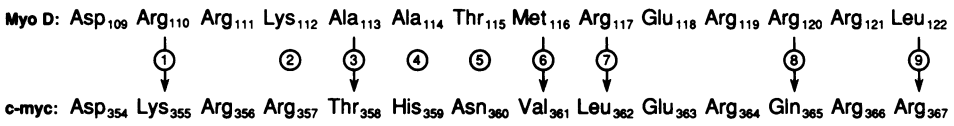

FIG. 1. MyoD mutations. Upper line shows the 14 amino acids comprising the murine MyoD basic domain [amino acids 109–122 of the published sequence (24)], which, for convenience in the text, are arbitrarily denoted as 1–14. Lower line shows the amino acid sequence of the murine c-Myc basic domain (amino acids 354–367). The two sequences are aligned for maximal homology with differences indicated by numbers. By using mutagenic oligonucleotides, the indicated amino acids in the MyoD basic region were changed to those found in the analogous position in c-Myc (numbers with arrows).

Staining was performed with the alkaline phosphatase-based Vectastain ABC kit (Vector Laboratories).

CAT vectors included p3300MCKCAT, which contains the MyoD-responsive murine muscle creatine kinase (MCK) promoter (11), and pMLP-CAT, which contains the adenovirus major late promoter (MLP) abutted to a CAT gene (gift from L. Resar and C. V. Dang, Johns Hopkins University).

Chimeric Max/E12 cDNA. Specific oligonucleotide primers were used in a standard PCR to generate a chimeric cDNA encoding the Max basic domain (codons 1–36; ref. 15) and the E12 HLH domain (codons 349–440; ref. 1). For expression in cultured cells, the 400-base-pair chimeric cDNA was excised from its parental Bluescript vector and blunt-end ligated into pRc/CMV.

Electrophoretic Mobility-Shift Assays (EMSAs). A 25-nucleotide ^{32}P-end-labeled oligonucleotide containing a high-affinity MyoD binding site (4) was annealed with a 10-fold excess of unlabeled complementary-strand DNA for DNA-binding assays. For a c-Myc site, we used the 26-nucleotide palindromic E0(GAC) oligonucleotide (19). The end-labeled oligonucleotide was allowed to self-anneal before use in EMSAs. Binding reaction mixtures, containing 10 μl of unlabeled reticulocyte lysate and 10 μl of 2× binding buffer [40 mM Hepes, pH 7.6/100 mM KCl/2 mM dithiothreitol/2 mM EDTA with 2 μg of poly(dI-dC)/1.0 ng of double-stranded ^{32}P-labeled oligonucleotide ($\approx 10^6$ dpm), and 10% (vol/vol) glycerol], were incubated at room temperature for 15 min, immediately loaded on 5% polyacrylamide gels (30:1 acrylamide/*N,N'*-methylenebisacrylamide weight ratio), and electrophoresed in 45 mM Tris/45 mM boric acid/2 mM EDTA for ≈2.5 hr at 8 V/cm. Gels were then dried and autoradiographed at −70°C for 4–16 hr using a single intensifying screen.

RESULTS

Mutagenesis of the MyoD Basic Region. We used oligonucleotide-mediated mutagenesis of full-length MyoD cDNA to change one or more amino acids in the MyoD basic domain to those found at analogous positions in c-Myc (Fig. 1). Six mutations encoded single amino acid changes (designated mut 1, 3, 6, 7, 8, and 9; Fig. 1), one encoded a double mutation [mut (7+9)], and one encoded a quadruple mutation [mut (1–4)]. Each mutant was transcribed *in vitro*, translated in the presence of E12 mRNA, and then immunoprecipitated with anti-MyoD antibody. The precipitates were subjected to SDS/PAGE and autoradiography. Dimerization between E12 and each MyoD protein was readily detected (Fig. 2*B*). These experiments demonstrate that all eight mutants were translated equivalently and dimerized equally well with E12.

Binding of MyoD Mutants to a MyoD Recognition Site. Each MyoD mutant was next tested for its DNA-binding ability. Unlabeled *in vitro* translated proteins were incubated with a ^{32}P-end-labeled oligonucleotide containing a MyoD binding site (4) and subjected to EMSA. Very weak DNA binding was obtained with individual E12 and MyoD proteins, whereas the combination of the two proteins allowed for readily detectable DNA binding (Fig. 2*C*) (4, 12). Most of the MyoD mutants retained the ability to bind a MyoD site. However, mut 7 and, to a lesser extent, mut (7+9), showed a greatly reduced capacity for binding. The specificity of binding was tested in two ways. First, the addition of anti-MyoD antibody to the lysate prior to the addition of the oligonucleotide abolished binding whereas a control antibody was without effect (Fig. 2*D*). Second, formation of the radioactive complex was blocked by the addition of excess unlabeled oligonucleotide but not by the addition of an equivalent amount of an oligonucleotide containing a mutant MyoD binding site (4) (Fig. 2*E*).

That most of the mutations still allowed binding of the dimer to a MyoD site suggested that the MyoD basic domain could tolerate substantial change and/or that a significant contribution to binding was made by the E12 component of the dimer. We thus asked how binding to a MyoD site would be affected if the dimerization partner contained a basic domain for Max, the natural partner for c-Myc. We therefore expressed a chimeric protein (Max/E12) containing the basic domain of Max and the HLH domain of E12. Immunoprecipitations using a polyclonal anti-Max antibody demonstrated that the Max/E12 chimera dimerized equally well with each of the MyoD proteins (Fig. 2*F*).

The ability of MyoD–Max/E12 dimers to bind to the ^{32}P-labeled MyoD site was examined (Fig. 2*G*). Upon prolonged autoradiographic exposure, the expected low level of DNA binding with wild-type MyoD homodimers was seen. All of the MyoD mutants, in dimeric complex with Max/E12, showed, at best, only trace levels of binding that was actually less than that observed with wild-type MyoD homodimers. These data, in conjunction with those shown in Fig. 2*C*, indicate that the E12 basic domain is a critical determinant of specific DNA binding by MyoD and that its role cannot be duplicated by Max.

Binding of MyoD Mutants to a c-Myc Recognition Site. Given our ability to express a MyoD dimerization partner containing either an E12 or a Max DNA-binding domain, we asked whether, in either of these two contexts, mutant MyoD proteins could bind to a consensus c-Myc DNA site. Each MyoD mutant was therefore cotranslated with either E12 or Max/E12 RNA in the presence of nonradioactive methionine and used in an EMSA employing a double-stranded, ^{32}P-labeled E0 (GAC) c-Myc binding site (19). Wild-type MyoD–E12 dimers were incapable of binding the c-Myc site, as were most of the mutants (Fig. 2*H*). However, strong binding was noted with mut 9 and mut (7+9). Thus, mut 7 strongly inhibits MyoD site binding (Fig. 2*C*) but has no effect on the ability of mut 9 to bind a c-Myc site. Therefore, in association with E12, mut 9 not only allows continued recognition of a MyoD site but unmasks a potent capacity to bind a c-Myc site.

When MyoD–Max/E12 dimers were tested by EMSA for binding to a c-Myc site, a similar pattern was seen in that only mut 9 and mut (7+9) bound (Fig. 2*I*). Overall, the double mutant was significantly better at binding this site, unlike the case for MyoD site binding, where both mutant proteins bound equally well.

Expression of MyoD Mutants in Cultured Cells. The MyoD mutants were tested for their abilities to activate muscle-specific gene expression. cDNAs in the pRc/CMV expression vector were transfected into C3H/10T½ cells along with the p3300MCKCAT reporter plasmid (11). After induction of myogenic differentiation, CAT activity was measured after

A

Myo D protein	In vitro translation	Dimerization with: E12	Dimerization with: max-E12	Binding with E12 to: Myo D site	Binding with E12 to: c-myc site	Binding with max-E12 to: Myo D site	Binding with max-E12 to: c-myc site	MCK-CAT activity (with E12) (% of wild type)	Myosin staining (% of wild type)	MLP-CAT activity (% of pRC CMV myc 1)
wild type (WT)	+	+	+	+	−	−	−	100	100	97
mut 1	+	+	+	+	−	−	−	97	90	115
mut 3	+	+	+	+	−	−	−	94	102	ND
mut 6	+	+	+	+	−	−	−	20	10	ND
mut 7	+	+	+	−	−	−	−	9	3	ND
mut 8	+	+	+	+	−	−	−	116	89	ND
mut 9	+	+	+	+	++	−	+/−	2	0	20
mut (7+9)	+	+	+	−	++	−	+	3	1	ND
mut (1-4)	+	+	+	+	+/−	−	−	17	26	ND

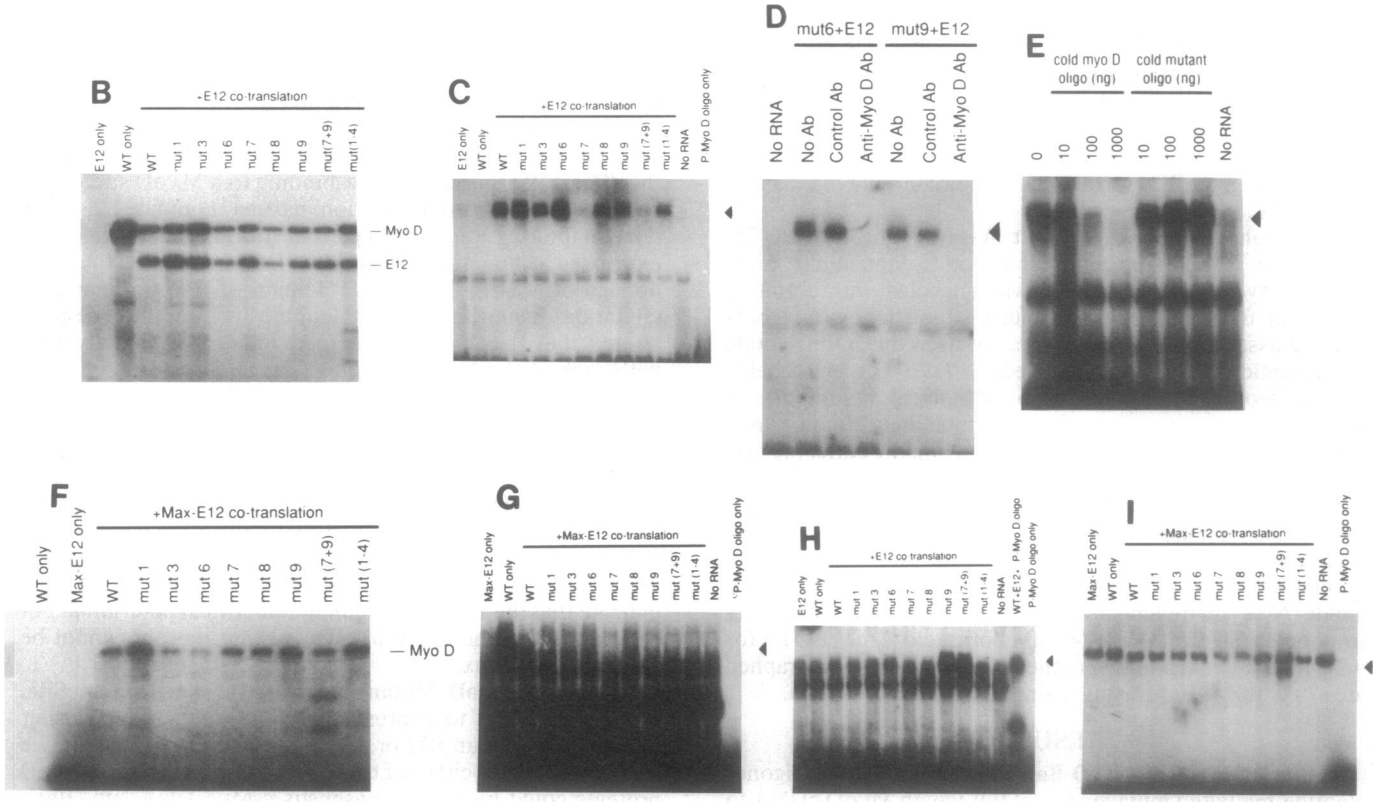

FIG. 2. (*A*) Summary of behaviors of MyoD mutants *in vitro* and in transfected cells. (*B*) Coimmunoprecipitations of *in vitro* translated MyoD and E12 proteins. Wild-type (WT) MyoD and MyoD mutants, with or without E12, were transcribed *in vitro* and translated in rabbit reticulocyte lysates in the presence of [^{35}S]methionine. One-tenth of each reaction mixture was then subjected to immunoprecipitation using a polyclonal anti-MyoD antibody followed by protein A-agarose beads. Precipitates were resolved by 15% SDS/PAGE and processed for autoradiography. (*C*) Binding of a consensus MyoD site by MyoD–E12 dimers. MyoD proteins were cotranslated with E12 in the presence of unlabeled methionine. Aliquots were mixed with double-stranded, ^{32}P-end-labeled MyoD oligonucleotide (Oligo) and subjected to EMSA. (*D*) Inhibition of MyoD oligonucleotide binding by anti-MyoD antibody (Ab). mut 6 and mut 9 were each cotranslated with E12. Standard EMSAs were performed except that 1 μl of either a control antibody or an anti-MyoD antibody was added to the lysate prior to the addition of ^{32}P-labeled MyoD binding site. (*E*) Competition for MyoD-site binding. Wild-type MyoD and E12 were cotranslated in the presence of unlabeled methionine and then incubated in a standard EMSA mixture with a constant amount of ^{32}P-labeled MyoD oligonucleotide (4) and increasing amounts of either unlabeled ("cold") homologous double-stranded oligonucleotide or an unlabeled double-stranded oligonucleotide containing a mutant MyoD binding site (CGCCTG). Only the homologous oligonucleotide competed with the labeled probe. (*F*) Coimmunoprecipitations of MyoD and Max/E12 chimeric proteins. Individual MyoD RNAs were translated *in vitro* with or without the addition of Max/E12 RNA. Immunoprecipitations were performed as described in *B* except that a polyclonal anti-Max antibody was used. (*G*) Lack of binding of MyoD–Max/E12 dimers to a MyoD site. MyoD mutants and Max/E12 chimeric protein were cotranslated as described in *C* and then tested by EMSA for binding to the end-labeled MyoD oligonucleotide (Oligo). Binding in all cases was significantly less than that observed for wild-type MyoD homodimers (second lane). (*H*) Binding of MyoD–E12 dimers to a c-Myc site. *In vitro* translation mixtures from *C* were tested by EMSA for binding to the E0 (GAC) c-Myc binding site. As a comparison for relative degree of probe binding, the second lane from the right shows binding of ^{32}P-labeled MyoD binding-site oligonucleotide by cotranslated wild-type plus E12 (see *C*). Binding of the c-Myc site by mut 9–E12 and mut (7+9)–E12 heterodimers was greater than any other binding observed. (*I*) Binding of MyoD–Max/E12 dimers to a c-Myc site. Binding reactions were as in *G* except that the E0(GAC) oligonucleotide was used as a probe.

appropriate adjustments for transfection efficiencies. Based on CAT enzyme assays and *in vitro* DNA-binding profiles, three types of mutations were observed: (*i*) those with only minimal effects on both MyoD-site binding and MCK promoter activation (mut 1, mut 3, and mut 8); (*ii*) those with markedly reduced DNA binding and transcriptional activation potential [mut 7 and mut (7+9)]; and (*iii*) those with normal or increased DNA binding but impaired MCK pro-

moter activation [mut 6 and mut (1–4)]. As expected from the *in vitro* DNA binding profiles, transcriptional activation of the MCK promoter was also greatly impaired with all MyoD constructs when they were cotransfected with a Max/E12 expression vector (data not shown). The low level of activation that was observed in some cases was most likely the result of functional dimerization between transfected MyoD proteins and endogenous E12 or E47 proteins. The myogenic capacity of each of the mutants was further tested by measuring the ability of transfected C3H/10T½ cells to express myosin heavy chains. The results (Fig. 2*A*) were in general agreement with those obtained by transient assays with the MCK-CAT reporter plasmid.

The ability to bind DNA but not to activate target-gene expression suggested that mut 9 would compete with wild-type MyoD for MCK promoter activation, thus serving as a type of dominant negative mutant. We tested this directly in cotransfection experiments using constant amounts of the pRcCMV/wtMyoD expression plasmid and p3300MCKCAT reporter plasmid and varying amounts of pRcCMV/mut 9 (Fig. 3). As the amount of this latter plasmid DNA increased, CAT activity in cellular extracts progressively declined, eventually reaching background levels. That competition with wild-type MyoD was virtually complete with only a 2-fold molar excess of mut 9 suggested either that binding of the mutant protein to the MyoD site was extremely efficient or that it effectively competed for a limiting partner (e.g., E12) in the C3H/10T½ cells.

The adenovirus MLP contains a consensus c-Myc site at positions −63 to −52 relative to the start of transcription. The bHLH protein USF also binds to this site and results in a 10- to 20-fold enhancement of transcription in an *in vitro* assay with other critical transcription factors (28). Using an MLP-CAT vector (pMLP-CAT), we asked how c-Myc and several

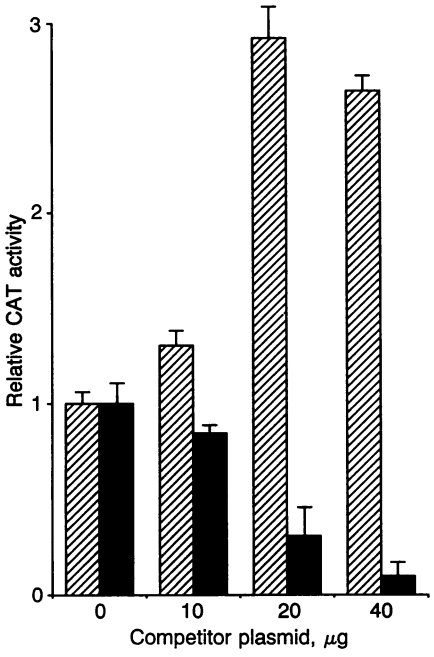

FIG. 3. mut 9 protein competes with wild-type MyoD for binding to and activation of the MCK promoter. C3H/10T½ cells were transfected with 5 μg of p3300MCK-CAT and 20 μg of pRcCMV/wtMyoD plus the indicated amount of either pRcCMV/mut 9 (filled bars) or additional pRcCMV/wtMyoD as a control (hatched bars). Differences in DNA concentrations were adjusted with pRcCMV vector DNA. Myogenic differentiation was induced and CAT enzyme determinations were performed 2 days later on extracts from pooled, duplicate plates after adjusting for differences in transfection efficiencies. Bars indicate SE determined from four experiments.

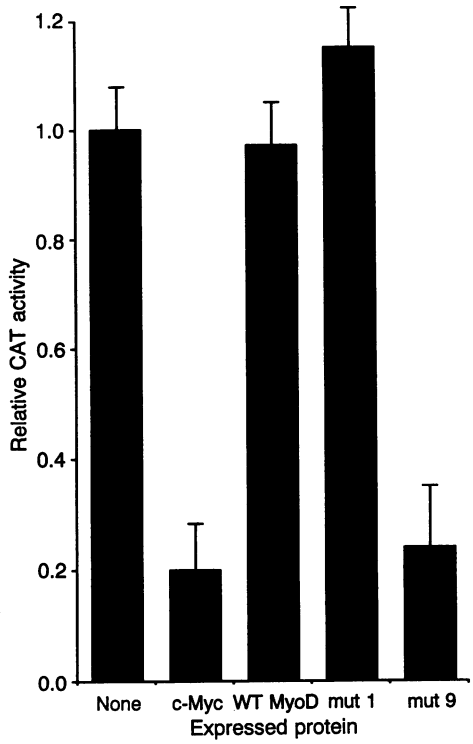

FIG. 4. Suppression of the adenovirus MLP by c-Myc and mut 9. C3H/10T½ cells were transfected with 5 μg of an MLP-CAT construct plus 20 μg of pRcCMV containing the indicated cDNA inserts. CAT activities in cytoplasmic extracts were determined 2 days after induction of myogenic differentiation, with adjustment for transfection efficiencies. Bars indicate SE determined from three experiments. WT, wild type.

MyoD mutants would affect its expression. C3H/10T½ cells were transfected with pMLP-CAT DNA and the pRcCMV expression vector alone or containing c-Myc, wild-type MyoD, mut 7, or mut 9 cDNAs. Neither wild-type MyoD nor mut 7 had any significant effect on CAT levels (Fig. 4). However, the expression of both c-Myc and mut 9 caused a consistent 4- to 6-fold suppression of CAT activity. These results, together with those in Fig. 2, demonstrate that the mut 9 mutation expands the DNA-binding specificity of MyoD to include the c-Myc site, causes it to lose the ability to activate genes containing MyoD sites, and imbues it with the ability to regulate a gene containing a c-Myc binding site in a manner indistinguishable from that seen with authentic c-Myc.

DISCUSSION

Previous analyses of bHLH proteins have begun to elucidate the contribution of the basic domains to specific DNA binding and transcriptional activation. Thus, replacement of the MyoD basic domain with that from E12 abolishes transcriptional activation without affecting DNA binding, indicating that these are distinct and separable functions (4). In the related bHLH protein myogenin, Ala^{86} and Thr^{87}, two amino acids highly conserved among all myogenic proteins described to date, and corresponding to positions 4 and 5 of c-Myc and MyoD (Fig. 1), are essential for muscle-specific transcription (6). In the E47 bHLH protein, two arginine residues, flanking position 8 in the aligned sequences shown in Fig. 1, are necessary for DNA binding (5). Finally, a chimeric gene containing the c-Myc basic domain embedded in the yeast *CBF1* gene can rescue a *cbf1* null mutant strain of *Saccharomyces cerevisiae* from methionine auxotrophy whereas a similar gene containing an AP4 basic domain cannot (29). The difference in biological function between these two basic domains maps to a single amino acid corre-

sponding to position 9 (Fig. 1). In c-Myc this position is the main determinant for recognition of the variant inner dinucleotide in the CANNTG motif (30).

We have identified four distinct classes of MyoD mutants: (*i*) those with no or minimal effects on MyoD-site binding and transcriptional activation (mut 1, mut 3, and mut 8; Fig. 1), (*ii*) those with markedly reduced binding and transcriptional activation activity [mut 7 and mut (7+9)], (*iii*) those with normal or even increased DNA binding but reduced transcriptional activation [mut 6 and mut (1–4)], and (*iv*) one with reduced MyoD-site binding and transcriptional activation but with markedly enhanced binding to a c-Myc site (mut 9). Leu^{122} in the MyoD basic domain is thus a major determinant of DNA binding specificity and is likely to play a key role in recognizing the inner dinucleotide sequence within the CANNTG motif. These results provide a rationale for the highly conserved nature of the position 9 residue among all bHLH myogenic proteins (including E12 and E47), where it is always a leucine or valine, and among all proteins of the Myc/Max family, where it is always an arginine. It is significant that mut 9, even when dimerized with Max/E12, altered DNA binding specificity to such an extent that it permitted the recognition of a c-Myc site. That such dimers also allow binding to a MyoD site suggests that, rather than changing DNA-binding specificity, the effect of mutation is to expand the subset of dinucleotides within the CANNTG motif that can be recognized by MyoD.

Although c-Myc is related to other transcriptional activators of the bHLH family and contains a transactivation domain (31), we found that c-Myc, rather than inducing a MLP-CAT construct, actually caused a suppression, a property that has been described in the case of three collagen genes (32). More important, whereas neither wild-type nor mut 7 MyoD affected MLP-CAT expression, mut 9 produced a suppressive effect indistinguishable from that of c-Myc. These results, together with those in Fig. 2, indicate that, at least in some contexts, mut 9 protein can functionally substitute for c-Myc.

That mutation of Leu^{122} to Arg in MyoD can occur as a result of a single nucleotide substitution (CTG → CGG) suggests a mechanism for skeletal muscle oncogenesis. During terminal differentiation, which requires withdrawal from the cell cycle (33), myoblasts cease to express c-Myc (34, 35) and will fail to differentiate if the expression of this protein persists (36–39). A MyoD protein with the position 9 (Leu^{122} → Arg) mutation might, however, be able to functionally substitute for c-Myc as the expression of the c-*myc* gene is extinguished, thus providing a sustained proliferative signal. This might also occur through other myogenic proteins (40–43) that contain this conserved position 9 leucine. The MyoD gene is also under positive autoregulatory control (44), and its continued expression is required to maintain the differentiated state (45) and perhaps also to aid in the induction of quiescence during terminal myogenic differentiation (27, 46). Because mut 9 is transcriptionally inactive toward muscle-specific genes, it or similar mutations might also compete with wild-type MyoD to suppress the expression of myogenic proteins and to stimulate proliferation through c-Myc-independent pathways.

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