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Mutagenesis of the myogenin basic region identifies an ancient protein motif critical for activation of myogenesis

(DNA-binding domain/tissue-specific transcription/muscle differentiation)

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ABSTRACT **Myogenin is a muscle-specific nuclear factor that acts as a genetic switch to activate myogenesis. Myogenin, MyoD, and a growing number of proteins implicated in transcriptional control share sequence homology within a basic region and an adjacent helix–loop–helix motif. Here we identify by site-directed mutagenesis a 12-amino acid subdomain of the myogenin basic region essential for binding of DNA and activation of myogenesis. The basic region of the widely expressed helix–loop–helix protein E12 is conserved at 8 of these 12 residues and can mediate DNA binding when placed in myogenin, but it cannot activate myogenesis. Replacement of each of the four nonconserved residues of the myogenin basic region with the corresponding residues of E12 reveals two adjacent amino acids (Ala^{86}-Thr^{87}) that can impart muscle specificity to the basic region. These residues are specific to, and conserved in, the basic regions of all known myogenic helix–loop–helix proteins from *Drosophila* to man, suggesting that they constitute part of an ancient protein motif required for activation of the myogenic program.**

Myogenin is a muscle-specific nuclear protein that is rapidly induced when skeletal myoblasts are triggered to differentiate; its expression is sufficient to activate the muscle differentiation program in a variety of nonmuscle cell types (1, 2). Myogenin belongs to a family of muscle-specific regulatory factors that includes MyoD (3), myf-5 (4), and MRF4/herculin/myf-6 (refs. 5–7; for reviews, see refs. 8 and 9). These factors share extensive homology within a basic region and a putative helix–loop–helix (HLH) motif that mediate DNA binding and factor dimerization (10–12). Basic–HLH domains have also been identified within several gene products that regulate cell fate in *Drosophila* (13–15), the E2A gene products (E12, E47/E2-5) (10, 16), and products of the *myc* family of oncogenes (17).

HLH proteins share the ability to recognize the DNA consensus sequence CANNTG, known as an E-box, which imparts tissue specificity to numerous enhancers and is important for muscle-specific transcription of the muscle creatine kinase (MCK), myosin light chain 1/3, cardiac α-actin, and acetylcholine receptor α-subunit genes (18–25). Analysis of the DNA-binding properties of HLH proteins *in vitro* has revealed that cell type-specific HLH proteins, such as myogenin, MyoD, and gene products from the *Drosophila* achaete scute complex (*AS*-C), preferentially form heterooligomers with ubiquitous HLH proteins, such as the E2A and daughterless gene products (11, 21, 26, 27). HLH proteins that lack functional basic regions and can inhibit DNA binding of other HLH proteins have also been identified (28).

The ability of diverse HLH proteins to recognize a common DNA sequence suggests that conserved amino acids within their basic regions may be required for recognition of the E-box consensus, while those residues that are unique may participate in cell type-specific transcription. Indeed, recent studies have shown that the basic region of MyoD contains a recognition code for myogenesis and that replacement of this basic region with that of other HLH proteins can abolish muscle-specific transcription without affecting DNA binding (11). In the present study, we have dissected the basic region of myogenin to identify the specific amino acids that mediate sequence-specific DNA binding and muscle-specific gene activation. Our results identify a conserved protein motif in the myogenin basic region that is critical for muscle-specific gene activation and suggest that the basic domains of HLH proteins are modular and allow DNA binding and cell type-specific transcription to be independently determined.

MATERIALS AND METHODS

Cell Culture and Transfections. Transfection of C3H/10T½ cells, analysis of chloramphenicol acetyltransferase (CAT) activity, and myosin immunostaining were performed as described (2). Transactivation assays were performed using 5 μg of reporter plasmid and 10 μg of the expression vector pEMSV (3) containing myogenin cDNAs, as described (21). MCK-CAT, referred to previously as pCKCATE4 (19), contains the 246-base-pair (bp) MCK promoter immediately 5′ of the CAT gene and the 300-bp enhancer inserted 3′ of the CAT gene. MCK-tkCAT, also called 4R-tkCAT, contains four copies of the *K*E-2 site from the MCK enhancer inserted immediately 5′ to the thymidine kinase basal promoter (29).

Mutagenesis. Mutant oligonucleotides were used as primers on single-stranded template DNA prepared by the method of Kunkel *et al.* (30). All mutants were confirmed by sequencing. Following mutagenesis, an *Afl* II–*Nco* I fragment that encodes the basic region was subcloned from the mutagenic vector into the same sites of the wild-type myogenin cDNA in the pEMSV expression vector to avoid extraneous mutations.

Gel Mobility-Shift Assays with *in Vitro* Translation Products. *In vitro* transcription/translation reactions and gel mobility-shift assays, using *in vitro* translation products generated in the absence of [^{35}S]methionine, were performed as described (21). The DNA probe corresponded to the right, high-affinity E-box from the MCK enhancer (18–20), which was end-labeled with ^{32}P.

RESULTS

A 12-Amino Acid Subdomain of the Myogenin Basic Region Is Required for Activation of Myogenesis. To pinpoint the amino acids within the myogenin basic region responsible for DNA binding and muscle-specific transcription, we intro-

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Abbreviations: CAT, chloramphenicol acetyltransferase; HLH, helix–loop–helix; MCK, muscle creatine kinase.

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duced mutations in the basic region and examined their consequences on several of myogenin's biological activities. Wild-type and mutant proteins obtained by *in vitro* transcription and translation were compared for their abilities to bind the MCK enhancer in the presence of the widely expressed HLH protein E12. Oligomerization of mutant proteins with E12 was also measured by coimmunoprecipitation of E12 with an anti-myogenin antibody. Myogenic regulatory activity was assayed in 10T½ fibroblasts by transactivation of a reporter plasmid in which the CAT gene was linked to the MCK enhancer and promoter. Because activation of the MCK enhancer may involve cooperative interactions among multiple enhancer-binding factors (29, 31), we also tested each mutant for its ability to transactivate MCK-tkCAT (a gift from A. Lassar), which contains four copies of the MCK high-affinity E-box immediately upstream of the thymidine kinase promoter (29). Activation of the endogenous myogenic program was also assayed by immunostaining for myosin expression in 10T½ cells following transient transfection. We showed previously (32) that endogenous myogenic regulatory factor genes are not expressed at significant levels relative to exogenous myogenin in the time frame of a transient transfection assay. Thus, the activity of each myogenin mutant in the above assays should reflect its intrinsic properties.

The basic region of myogenin, like that of other myogenic HLH proteins (3–7), contains three clusters of basic amino acids, designated B1, B2, and B3 (Fig. 1). Mutagenesis of basic cluster 1 (mutant BS-1) had no effect on the properties of myogenin, whereas mutagenesis of basic clusters 2 and 3 (mutants BS-2 and BS-3) abolished myogenic activity and DNA binding (Fig. 1 *A* and *B*). Mutants BS-2 and BS-3 retained the ability to oligomerize with E12, albeit with lower efficiency than the wild-type protein, supporting the notion that oligomerization does not require DNA binding. The behavior of these mutants is similar to that of MyoD mutants described previously (11).

To further define the region of the basic domain important for binding of DNA and activation of myogenesis, we intro-

A

	B1 B2 B3	oligomerization with E12	DNA binding with E12	MCK-CAT activity (% of wild-type)	MCK-tkCAT activity (% of wild-type)	Myosin staining (% of wild-type)
myogenin	$_{74}$K R K S V S V D R R R A A T L R E K R R$_{93}$	+	+	100	100	100
BS1	T G G - - - - - - - - - - - - - - - -	+	+	226	58	100
BS2	- - - - - - - - G G G - - - - - - - -	+	-	6	0	0
BS3	- - - - - - - - - - - - - - - - M L G	+	-	1	0	0
S-P $_{79}$	- - - - - P - - - - - - - - - - - -	+	+	80	123	61
D-R $_{81}$	- - - - - - - R - - - - - - - - - -	+	+	98	108	98
RR-KK $_{82-83}$	- - - - - - - - K K - - - - - - - -	+	+	2	4	0
R-V $_{84}$ /R-A $_{89}$	- - - - - - - - - - V - - - - A - - - -	+	+	1	0	0
T-G $_{87}$	- - - - - - - - - - - - - G - - - - -	+	+	4	0	0
T-A $_{87}$	- - - - - - - - - - - - - A - - - - -	+	+	0	3	0
T-S $_{87}$	- - - - - - - - - - - - - S - - - - -	+	+	15	3	15
T-D $_{87}$	- - - - - - - - - - - - - D - - - - -	+	-	0	2	0
T-E $_{87}$	- - - - - - - - - - - - - E - - - - -	+	-	0	2	0

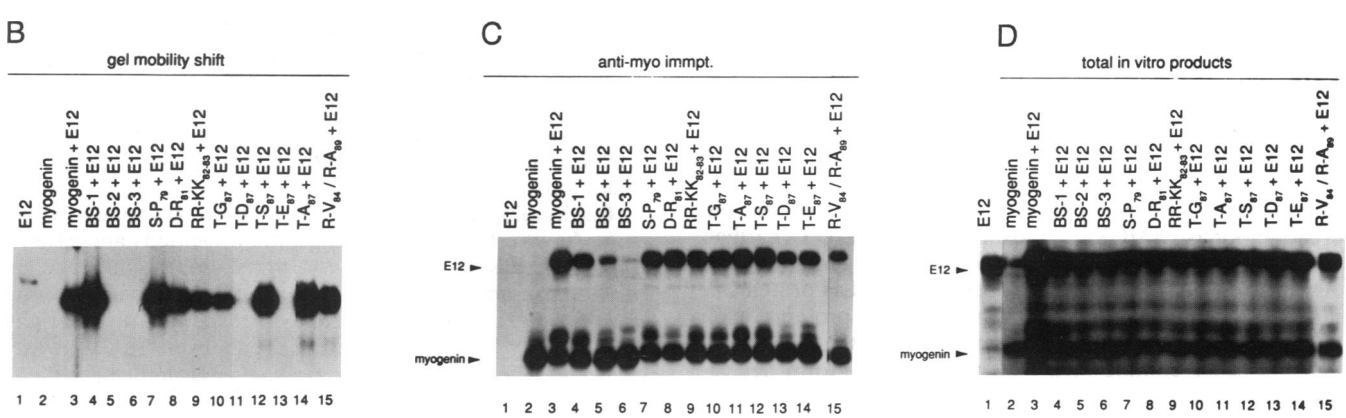

FIG. 1. Mutants within the basic domain of myogenin define a 12-amino acid segment required for muscle-specific gene activation. (*A*) The amino acid sequence of the basic domain of mouse myogenin is shown and the names of mutants are shown to the left. A dash indicates no change at that position. The frequency of myosin-positive cells following transfection with wild-type myogenin was 51 per 6000 cells. No myosin-positive cells were observed with the expression vector lacking a cDNA insert. Values for MCK-CAT, MCK-tkCAT, and myosin heavy-chain expression are expressed relative to wild-type myogenin and represent the average of at least three experiments. (*B*) Gel mobility-shift assays with unlabeled E12 and wild-type and mutant myogenin proteins obtained by *in vitro* translation. The probe corresponds to the right E-box in the MCK enhancer (20, 21), which was end-labeled. Only the region of the gel containing the mobility-shifted complex is shown. Less than 5% of the probe was shifted with myogenin plus E12. (*C*) Immunoprecipitation with anti-myogenin antibody of [^{35}S]methionine-labeled *in vitro* translation products. Immune complexes were denatured and resolved by SDS/PAGE and fluorography. (*D*) Total [^{35}S]methionine-labeled *in vitro* translation products after SDS/PAGE.

duced point mutations between the basic clusters (Fig. 1). Introduction of a proline between B1 and B2 (mutant S-P_{79}) did not significantly alter the properties of myogenin. Similarly, substitution of arginine for aspartate-81 (mutant D-R_{81}), which is conserved at this position in many HLH proteins, had no apparent effect on DNA binding or myogenic activity. In contrast, mutations in the B2–B3 region resulted in dramatic effects on the properties of myogenin. Mutant RR-KK_{82-83}, in which the first two arginines in B2 were replaced with lysines, was devoid of myogenic activity. Similarly, replacement of threonine-87 with glycine (mutant T-G_{87}), alanine (mutant T-A_{87}), serine (mutant T-S_{87}), aspartate (mutant T-D_{87}), or glutamate (mutant T-E_{87}) led to a loss of much or all myogenic activity. By comparison of mutants BS-2 and RR-KK_{82-83}, we conclude that positive charge in the B2 cluster is necessary for DNA binding but is not sufficient for muscle-specific gene activation. These findings differ from previous studies with E47, in which substitution of lysine for arginine at the corresponding position in the basic domain abolished DNA binding (12).

We found by immunoprecipitation and immunostaining that all of the above mutants, as well as those described below, were expressed at comparable levels to wild-type myogenin (data not shown), indicating that their failure to activate myogenesis was not due to differences in expression. To ascertain whether loss of myogenic activity in certain mutants might be due to subtle differences in levels of expression or DNA-binding affinity that result in large differences in transcription, we determined the sensitivity of the transactivation assay to differing levels of expression plasmid and found that 1 μg of wild-type myogenin was more active in the transactivation assay than 20 μg of the myogenin mutants that were biologically inactive (data not shown). Thus, the transactivation assay is sufficiently sensitive to detect differences in myogenic activity exhibited by "weak" alleles of myogenin.

Myogenin/E12 Substitution Mutants Reveal Two Amino Acids That Can Direct Muscle-Specific Transcription. The properties of the above mutants suggested that the 12 amino acids encompassing B2 and B3 are essential for binding of DNA and activation of myogenesis and that these are separable functions. Of these 12 amino acids, 6 are identical and 2 are conserved in E12 (Fig. 2*A*), which is expressed ubiquitously and cannot activate myogenesis. To further define the requirements for muscle-specific gene activation, we replaced the B2–B3 region of myogenin with the corresponding region of E12. This mutant (myo-$E12_{basic}$) formed a heterooligomer with wild-type E12 and bound to the MCK enhancer with high affinity (Fig. 2*B*) but showed no myogenic activity even when transfected at a concentration 20-fold higher than wild-type myogenin. The properties of myo-$E12_{basic}$ are similar to those of the MyoD–E12 chimera described by Davis *et al.* (11).

The failure of mutant myo-$E12_{basic}$ to activate myogenesis suggested that one or more of the four nonconserved amino acids in this region (Arg^{84}, Ala^{86}, Thr^{87}, Arg^{92}) might participate in muscle-specific transcription. We therefore changed each of these residues to the corresponding amino acid in E12 and analyzed the properties of the resulting mutants (Fig. 2). Mutants R-V_{84} and R-L_{92} retained the ability to efficiently activate myogenesis. In contrast, mutant A-N_{86} was completely inactive in transfection assays, whereas mutant T-N_{87} exhibited low activity. With mutant T-N_{87}, we observed a reduced number of myosin-positive cells relative to wild type, but the intensity of staining of those cells that were positive appeared to be equivalent with the wild-type and mutant proteins, consistent with the possibility that activation of the differentiation program is an all-or-none decision at the single-cell level rather than a graded response throughout the population of transfected cells.

To further test the role of Ala^{86}-Thr^{87} in muscle-specific transcription, we replaced Asn^{86}-Asn^{87}, in the basic domain of myo-$E12_{basic}$, with Ala-Thr, leaving the remainder of the E12 basic domain intact [myo-E12(N-A_{86}/N-T_{87}); E12 revertant]. Remarkably, this two-amino acid substitution restored full myogenic activity to this mutant, confirming that Ala^{86}

A

	B2 / B3	oligomerization with E12	DNA binding with E12	MCK-CAT activity (% of wild-type)	MCK-tkCAT activity (% of wild-type)	Myosin staining (% of wild-type)
myogenin	^{82}R R R A A T L R E K R R$_{93}$	+	+	100	100	100
E12; myo-$E12_{basic}$	R R V A N N A R E R L R	+	+	3	1	0
R-V_{84}	- - V - - - - - - - - -	+	+	95	97	54
A-N_{86}	- - - - N - - - - - - -	+	+	3	0	0
T-N_{87}	- - - - - N - - - - - -	+	+	11	8	30
R-L_{92}	- - - - - - - - - - L -	+	+	77	83	100
myo-E12 (N-A_{86}/N-T_{87}) (E12 revertant)	R R V A N N A R E R L R (A T)	+	+	89	128	103

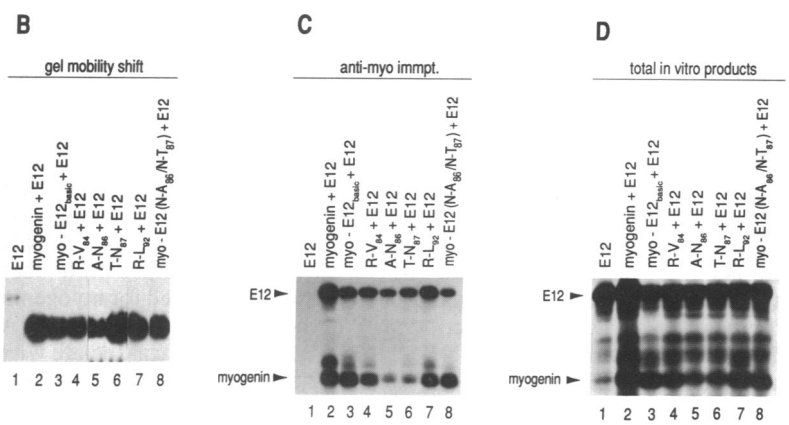

FIG. 2. Mutants that replace the minimal basic region of myogenin with the corresponding amino acids of E12 define two amino acids required for muscle-specific gene activation. (*A*) The amino acid sequence of the B2–B3 region of myogenin and the corresponding region of E12 is shown. Amino acid identity and similarity are indicated with lines and dots, respectively. Dash indicates no change. Myogenic activity was determined as in Fig. 1. (*B*) Mobility-shift assays with the ^{32}P-labeled MCK oligomer and unlabeled wild-type or mutant myogenins. Only the region containing the mobility-shifted probe is shown. (*C*) Immunoprecipitation with anti-myogenin antibody of wild-type and mutant myogenin proteins in the presence of E12. (*D*) Total translation products after SDS/PAGE.

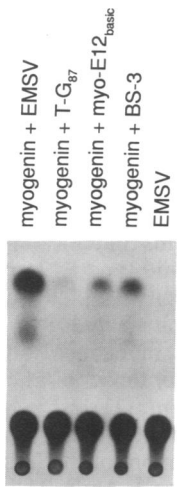

FIG. 3. Mutations in the basic region convert myogenin from an activator to an inhibitor of myogenesis. 10T½ cells were transiently transfected with MCK-CAT (5 μg), EMSV-myogenin (5 μg), and the indicated myogenin mutants in pEMSV (20 μg), and CAT activity was determined as described (2).

and Thr[87], in conjunction with surrounding residues, are sufficient to impart muscle specificity to the basic region of E12 or myogenin (Fig. 2).

Myogenin Mutants That Fail to Activate Myogenesis Inhibit Activity of Wild-Type Myogenin. The myogenin mutants that were unable to activate myogenesis suggested two mechanisms through which HLH proteins could act as negative regulators: by formation of heterooligomers that cannot bind DNA, as has been proposed for the HLH protein Id (28), and by competition with wild-type proteins for binding to muscle control regions. To test these potential mechanisms, we performed cotransfection assays with wild-type myogenin and mutant BS-3, which oligomerizes with E12 but fails to bind DNA, and mutants T-G_{87} and myo-$E12_{basic}$, which bind DNA but do not activate myogenesis. Fig. 3 shows that all three mutants inhibited transactivation of the MCK enhancer by wild-type myogenin. Thus, alteration of residues essential for myogenesis or of the surrounding basic residues that mediate DNA binding can convert myogenin from an activator to an inhibitor of myogenesis.

DISCUSSION

Our results show that a 12-amino acid segment of the myogenin basic domain is necessary for binding of DNA and activation of myogenesis and that two amino acids (Ala[86] and Thr[87]) in the center of this domain can, in conjunction with surrounding residues, mediate muscle-specific transcription. Davis *et al.* (11) have made similar observations with MyoD, in which introduction of Ala-Thr-Met into the corresponding position of a MyoD–E12 chimera will restore the ability to activate myogenesis. Together, these results support a model in which residues in the two basic clusters that are conserved throughout the HLH family of proteins (Fig. 4) mediate recognition of the E-box consensus, whereas the central residues of the basic region that are variable are important for cell-type specificity. Ala[86] and Thr[87] are conserved at this position in every myogenic HLH protein identified to date, in species ranging from *Drosophila* (ref. 36; B. Paterson, personal communication) to man (4, 7, 28), suggesting that they constitute part of an ancient protein motif critical for activation of the myogenic program. In contrast, none of the >30 other HLH proteins that have been identified contain this combination of residues at these positions.

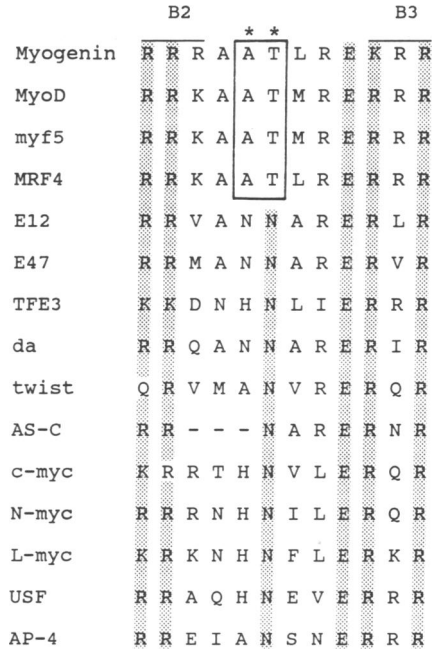

FIG. 4. Minimal basic region required for myogenesis. The region of the myogenin basic domain shown to be essential for DNA binding and muscle-specific transcription is shown. Shaded residues are conserved throughout diverse HLH proteins and are important for sequence-specific DNA binding and transcription activation by myogenin as well as MyoD (11). The conserved myogenic code is indicated with asterisks. Sources for sequences: myogenin (1, 2); MyoD (3); myf5 (4); MRF4 (5); E12 and E47 (10); TFE3 (33); da (14); twist (15); AS-C (13); c-myc, N-myc, and L-myc (28); USF (34); and AP4 (35).

How can the amino acids of the myogenin and MyoD basic domains direct muscle-specific transcription while other residues apparently cannot? One possibility is that the basic domain may be essential for interaction with a coregulator required for muscle-specific gene activation. A requirement for such a coregulator could explain the observation that myogenin and MyoD can activate muscle-specific genes in only certain cell types (37, 38). An alternative possibility (11, 39), which is not mutually exclusive, is that the specific amino acids in the basic regions of myogenin and MyoD allow the proteins to respond to a specific E-box sequence and thereby undergo a conformational change that potentiates transcriptional activation.

While Ala[86]-Thr[87] in the center of the basic region may be necessary, it is apparently not sufficient to direct myogenesis, because introduction of these residues into the complete E12 or E47 protein does not impart muscle specificity (27). Thus, it is likely that the basic region of myogenin acts in combination with other parts of the protein. In this regard, we have recently identified transcription activation domains in the amino and carboxyl termini of myogenin whose activities are modulated by Ala[86]-Thr[87] (J. Schwartz, T.C., and E.N.O., unpublished results).

Among the basic-domain mutants that failed to activate myogenesis, we found two types, those that retained, and those that lost, the ability to bind DNA. Those mutants that bound DNA normally with E12 *in vitro* but failed to activate muscle-specific transcription are similar to a series of MyoD mutants described previously (11) and demonstrate that events in addition to DNA binding are required for myogenin to activate transcription. Whether these mutants bind DNA normally *in vivo*, where they could potentially associate with HLH proteins other than E12, remains to be determined. These mutants also resemble "positive-control" mutants

that have been described for other transcription factors, such as the phage λ repressor (40), the glucocorticoid receptor (41), and the homeodomain protein Oct-1 (42). In the case of Oct-1, positive-control mutations have been mapped to the DNA-binding domain and shown to disrupt interactions with the herpes simplex virus coactivator VP16, which interacts with Oct-1 to induce transcription in virus-infected cells (42).

The second class of nonmyogenic mutants were those that failed to bind DNA. Among these were mutants BS-2 and BS-3, in which the clusters of conserved basic amino acids were altered. These mutants suggest that the spatial arrangement of these positively charged amino acids is necessary for recognition of the E-box consensus. The other mutants that failed to bind DNA were T-D_{87} and T-E_{87}, in which an acidic amino acid was substituted for threonine-87, which is critical for myogenic activity. The ability of a negatively charged residue at this position to abolish DNA binding is intriguing because threonine-87 can be phosphorylated by protein kinase C *in vitro* (L. Li and E.N.O., unpublished results), which also inhibits DNA binding and transactivation and has been implicated in repression of myogenesis (43).

Both types of myogenin mutants that were biologically inactive showed the ability to block activity of wild-type myogenin. Similar transdominant mutants have been described for MyoD (11). These mutants act in a manner similar to Id (28) and presumably inhibit myogenesis by competing with the wild-type protein for an oligomerization partner that is limiting. The observation that alteration of a single codon in the myogenin basic region can convert myogenin from an activator to an inhibitor of myogenesis could provide an explanation for the loss of myogenic potential following mutagenesis of established muscle cell lines (44).

The transdominant negative mutants of myogenin described in this study will be useful for further studies of the roles of HLH proteins in the control of determination and differentiation within the myogenic lineage and should facilitate analysis of the mechanism that underlies the transcriptional specificity of myogenin and other members of the MyoD family.

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