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# Degradation of Myogenic Transcription Factor MyoD by the Ubiquitin Pathway In Vivo and In Vitro: Regulation by Specific DNA Binding

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**MyoD is a tissue-specific transcriptional activator that acts as a master switch for skeletal muscle differentiation. Its activity is induced during the transition from proliferating, nondifferentiated myoblasts to resting, well-differentiated myotubes. Like many other transcriptional regulators, it is a short-lived protein; however, the targeting proteolytic pathway and the underlying regulatory mechanisms involved in the process have remained obscure. It has recently been shown that many short-lived regulatory proteins are degraded by the ubiquitin system. Degradation of a protein by the ubiquitin system proceeds via two distinct and successive steps, conjugation of multiple molecules of ubiquitin to the target protein and degradation of the tagged substrate by the 26S proteasome. Here we show that MyoD is degraded by the ubiquitin system both in vivo and in vitro. In intact cells, the degradation is inhibited by lactacystin, a specific inhibitor of the 26S proteasome. Inhibition is accompanied by accumulation of high-molecular-mass MyoD-ubiquitin conjugates. In a cell-free system, the proteolytic process requires both ATP and ubiquitin and, like the in vivo process, is preceded by formation of ubiquitin conjugates of the transcription factor. Interestingly, the process is inhibited by the specific DNA sequence to which MyoD binds: conjugation and degradation of a MyoD mutant protein which lacks the DNA-binding domain are not inhibited. The inhibitory effect of the DNA requires the formation of a complex between the DNA and the MyoD protein. Id1, which inhibits the binding of MyoD complexes to DNA, abrogates the effect of DNA on stabilization of the protein.**

MyoD is a tissue-specific transcriptional activator that acts as a master switch for skeletal muscle development. Following binding to specific upstream DNA regulatory elements, it leads to activation of a wide array of muscle-specific genes and consequently to conversion of proliferating myoblasts to terminally differentiated mature myotubes (10, 30, 43). MyoD belongs to the family of muscle-specific basic helix-loop-helix (bHLH) proteins, which also includes Myf5, myogenin, and MRF4 (42, 43). These myogenic regulators have 80% homology within a segment of about 70 amino acid residues that encompasses the basic and helix-loop-helix motifs. These motifs mediate DNA binding and dimerization, respectively (41). MyoD binds to DNA as a homodimer; however, a more stable complex is generated when MyoD heterodimerizes with other ubiquitously expressed bHLH proteins, such as E2A, E12, and E47 (28). The activity of MyoD is negatively regulated by members of the Id (inhibitors of differentiation) family of proteins. These proteins can heterodimerize with E12/E47 or MyoD, but since they lack the basic region, the complexes cannot bind to DNA and are therefore inactive (2, 24, 37).

Like many other transcriptional factors, MyoD is an extremely short-lived protein, with a half-life of ~30 min (38). However, the proteolytic system(s) that targets the protein, as well as the underlying regulatory mechanisms involved, has not been identified. Recent evidence implicates the ubiquitin proteolytic system in the degradation of many short-lived key regulatory proteins (8, 9, 11, 20). Among these are mitotic and $G_1$ cyclins, tumor suppressors and oncoproteins, transcriptional activators and their inhibitors, and cell surface receptors for growth-promoting factors. Degradation of a protein via the ubiquitin system involves two discrete and successive steps, conjugation of multiple molecules of ubiquitin to the target protein and degradation of the tagged substrate by the 26S proteasome complex with the release of free and reutilizable ubiquitin. Conjugation proceeds in a three-step mechanism. Initially, ubiquitin is activated in its C-terminal Gly residue by the ubiquitin-activating enzyme, E1. Following activation, one of several E2 enzymes (ubiquitin carrier proteins or ubiquitin-conjugating enzymes [Ubcs]) transfers ubiquitin from E1 to a member of the ubiquitin-protein ligase family, E3, to which the substrate protein is specifically bound. E3 catalyzes the last step in the conjugation process, covalent attachment of ubiquitin to the substrate. Following transfer of the first ubiquitin moiety to the target protein, a polyubiquitin chain is synthesized by processive transfer of additional activated ubiquitin moieties to the previously conjugated molecule. The chain serves, most probably, as a recognition marker for the protease. The binding of the substrate to E3 is specific and implies that E3 enzymes, which belong to a growing family of proteins, play a major role in recognition and selection of substrates for conjugation and subsequent degradation. An important yet unresolved problem involves the mechanisms that underlie specific recognition of the many cellular substrates of the system. A few proteins may be recognized via their free and destabilizing N-terminal residue (N-end rule [40]); however, most cellular proteins are recognized by signals that are distinct from the N-terminal residue. For some proteins that are degraded constitutively, the recognition motifs reside downstream from the N-terminal residue. Degradation of many other proteins is regulated by a specific posttranslational mod-

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ification, such as phosphorylation, or following association with ancillary proteins and nucleic acids.

Here we show that the conjugation and subsequent degradation of the transcriptional activator MyoD are mediated by the ubiquitin system. The protein can be stabilized following complex formation with its specific DNA binding sequence. Formation of a proteolysis-resistant complex is dependent on dimerization of proteins that have intact bHLH domains. Formation of complexes with proteins that lack the basic motif (for example, Id) does not allow association with DNA and consequently renders MyoD susceptible to degradation. The physiological implications of this novel type of regulation are discussed.

## MATERIALS AND METHODS

**Materials.** Materials for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were from Bio-Rad. Hexokinase was from Boehringer Mannheim. Polynucleotide kinase and poly(dI-dC) were from Promega. L-[$^{35}$S]methionine and [$\gamma$-$^{32}$P]ATP were obtained from New England Nuclear. Ubiquitin, dithiothreitol (DTT), ATP, phosphocreatine, phosphocreatine kinase, 2-deoxyglucose, glutathione, glutathione-agarose, IPTG (isopropyl-$\beta$-D-thiogalactopyranoside), Tris buffer, and polyethyleneimine were purchased from Sigma. DEAE-cellulose (DE52) was from Whatman. Tissue culture reagents were purchased from Biological Industries, Kibbutz Bet Haemek, Israel, and from Life Technologies. Lactacystin was purchased from Calbiochem. Oligonucleotides were synthesized by Biotechnology General, Rehovot, Israel. Reagents for enhanced chemiluminescence were from Amersham. Immobilized protein A was from Pharmacia. Anti-MyoD antibodies were purchased from Santa Cruz (polyclonal) and Novocastra (monoclonal). All other reagents were of high analytical grade.

**Plasmids and expression of MyoD and E47N.** cDNA encoding MyoD in a pEMCIIs vector for cellular expression was generated in the laboratory of the late Harold Weintraub and was obtained from Stephen Tapscott. The pRK171a bacterial expression vector containing the wild-type (wt) MyoD cDNA was obtained from the same source and was described elsewhere (39). A mutant species of MyoD that lacks the DNA-binding domain (basic region, amino acid residues 102 to 114) ($\Delta$basic) was generated by site-directed mutagenesis. Following IPTG induction in *Escherichia coli* BL21(DE3)/pLysS, cells were lysed by sonication and nucleic acids were removed by precipitation in 0.3% polyethyleneimine. MyoD (~90 to 95% pure) was precipitated with 0.6 M ammonium sulfate as described previously (39). Construction of the expression vector and purification of E47N, an N-terminally truncated form of E47, was described elsewhere (36).

**Cell lines and transfection.** Cos cells were grown at 37°C in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and 2 mM L-glutamine. Cells were transfected with MyoD by using the DEAE-dextran method (14) or the Superfect kit (Qiagen) according to the manufacturer's instructions.

**Stability of MyoD in vivo.** The cellular stability (half-life) of MyoD was monitored in a pulse-chase labeling experiment followed by immunoprecipitation. At 48 h following transfection, Cos cells growing in a monolayer were washed with a medium that lacks methionine and labeled for 1 h in the presence of 200 $\mu$Ci of [$^{35}$S]methionine per ml in a complete medium that lacks unlabeled methionine. The proteasome inhibitor lactacystin (10 $\mu$M) or dimethyl sulfoxide (in which the inhibitor was dissolved) was added, when indicated, 30 min after the addition of the label and was present throughout the experiment. The lysosomal proteolysis inhibitor chloroquine (100 $\mu$M) was added, when indicated, for the duration of the chase period. Following labeling, the medium was replaced with a complete medium that contains also 2 mM unlabeled methionine. Cells were harvested (time zero; pulse) or were further incubated for the indicated time periods (chase). Cell lysates were initially treated with preimmune immunoglobulin G (IgG). Following removal of the preimmune IgG, labeled MyoD was immunoprecipitated with anti-MyoD antibody. Both the preimmune and immune complexes were precipitated with immobilized protein A. Following SDS-PAGE (10% polyacrylamide), proteins were visualized with a phosphorimager (Fuji, Tokyo, Japan). For visualization of MyoD-ubiquitin conjugates, cells were treated with lactacystin as described above, and extracts were precipitated with anti-MyoD antibody. MyoD was detected by Western blot analysis with anti-MyoD antibody. To confirm that the high-molecular-mass compounds generated are MyoD-ubiquitin adducts, the nitrocellulose membrane was stripped and reblotted with antiubiquitin antibody (17).

**Preparation and fractionation of crude reticulocyte lysate.** Reticulocytes were induced in rabbits, and lysates were prepared as described previously (18). The lysate was fractionated over DEAE-cellulose into unabsorbed material (fraction I) and high-salt eluate (fraction II) as described previously (18). Fraction II was further fractionated with $(NH_4)_2SO_4$ into fraction IIA (0 to 38%) and fraction IIB (42 to 80%) as described previously (18). Purified E1 and E2-14kDa were prepared by covalent affinity chromatography of fraction II over immobilized ubiquitin as described previously (18). When indicated, E2-14kDa was further purified via anion-exchange chromatography over a MonoQ column (Pharmacia) as described previously (15, 32). The right margin of the fraction eluted at 220 mM KCl contains only E2-14kDa. Bacterially expressed E2-14kDa cloned into the pET11d expression vector (obtained from Simon S. Wing) was expressed in *E. coli* BL21(DE3)/pLysS cells (44). Because of the low expression level, the protein was not purified, and instead we used fraction IIB, which is known to contain E2-14kDa. The ubiquitin-conjugating enzyme E2-F1 was prepared from fraction I as described previously (5).

**Conjugation and degradation assays.** Conjugation and degradation were performed essentially as described previously (4, 5, 18). Briefly, the reaction mixture contained, in a final volume of 25 $\mu$l, crude reticulocyte lysate (10 $\mu$l; ~1 mg of protein) or fraction II (100 $\mu$g of protein), Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 2 mM DTT, 5 $\mu$g of ubiquitin, and MyoD. Fraction I (250 $\mu$g), fraction IIA (50 $\mu$g), fraction IIB (50 $\mu$g), E1 (2 $\mu$g), E2-14kDa or E2-F1 (0.3 $\mu$g), and E47N (100 ng) were added when indicated. Anion-exchange chromatography- and affinity-purified E2-14kDa was added at 0.15 $\mu$g, whereas extract from bacteria expressing E2-14kDa was added at 50 $\mu$g. Double-stranded oligonucleotides were added when indicated. The nucleotides were preincubated for 15 min at 30°C in the presence of MyoD prior to their addition to the reaction mixture. Reactions were carried out in the presence of either 0.5 mM ATP and an ATP-regenerating system (10 mM phosphocreatine and 0.5 $\mu$g of phosphocreatine kinase) or 0.5 $\mu$g of hexokinase and 10 mM 2-deoxyglucose to deplete endogenous ATP. Conjugation assay mixtures contained 800 ng of MyoD protein and 0.5 $\mu$g of the isopeptidase inhibitor ubiquitin aldehyde (19), whereas degradation reaction mixtures contained 200 ng of the substrate and no ubiquitin aldehyde. Conjugation reaction mixtures were incubated for 20 min at 37°C, and degradation reaction mixtures were incubated for 2 h at the same temperature. Reactions were terminated by the addition of 12.5 $\mu$l of threefold-concentrated sample buffer and, following boiling, were resolved via SDS-PAGE (10% polyacrylamide). Electrophoresed proteins were blotted onto nitrocellulose paper. MyoD was detected by enhanced chemiluminescence after initial incubation with anti-MyoD by incubation with a secondary horseradish peroxidase-conjugated antibody (Amersham).

**Electrophoretic mobility gel shift assay.** Probes were end labeled by using polynucleotide kinase and [$\gamma$-$^{32}$P]ATP. Unincorporated labeled ATP was removed by using a Sephadex G-50 spin column (Pharmacia). The reaction mixture contained, in a final volume of 20 $\mu$l, 25 mM Tris-HCl (pH 7.9), 50 mM KCl, 5 mM $MgCl_2$, 7.5% glycerol, 0.1 mM EDTA, 1 mM DTT, 0.5 $\mu$g of poly(dI-dC), bacterially expressed wt and mutant MyoD proteins in the indicated amounts, and 10 to 20 fmol of labeled probe. Following preincubation of MyoD proteins for 10 min at 30°C, the labeled probe was added and the reaction mixture was incubated for an additional 20 min. The mixture was then applied to a 4% polyacrylamide gel in 0.25× TBE (1× TBE is 90 mM Tris, 64.6 mM boric acid, and 2.5 mM EDTA, pH 8.3) and electrophoresed at 20 mA at 4°C. Gels were vacuum dried, and DNA-protein complexes were visualized by fluorography.

**Purification of Id protein.** A *Bam*HI-*Eco*RI fragment (922 bp) of pMH18$\Delta$R (2) containing the entire coding region of the Id1 protein was subcloned in frame with glutathione *S*-transferase into *Bam*HI-*Eco*RI-digested pGEX-2T. Following transformation into *E. coli* BL21(DE3)/pLysS cells and induction of the protein with IPTG, glutathione *S*-transferase–Id1 was affinity purified over immobilized glutathione. Purified Id was added to the degradation reaction mixture as indicated.

**Determination of the N-terminal residues of MyoD.** Determinations of the N-terminal residues of bacterially expressed and eukaryotic cell-expressed MyoD were carried out by Arie Admon and Tamar Ziv. Bacterially expressed MyoD was resolved via SDS-PAGE, blotted onto polyvinylidene difluoride paper, and subjected to three cycles of automated Edman degradation (Applied Biosystems). The identities of the amino acids were determined chromatographically. $^{35}$S-labeled MyoD was immunoprecipitated from transfected Cos cells (see above), resolved via SDS-PAGE, blotted onto polyvinylidene difluoride paper, and subjected to a single cycle of automated Edman degradation. The material containing the first-cycle reaction products was collected and lyophilized, and radioactivity was determined with a beta scintillation counter. As a control we used mock-transfected labeled cells that were treated in a manner identical to that for the MyoD cDNA-transfected cells.

**Determination of protein.** Protein concentrations were determined by the Bradford method (7). Bovine serum albumin was used as a standard.

## RESULTS

**MyoD is a short-lived protein that is degraded in the cell by the ubiquitin-proteasome pathway.** The majority of protein substrates targeted by the ubiquitin system are short-lived. A pulse-chase experiment was carried out in order to assess the stability of the MyoD protein in vivo. As shown in Fig. 1A, the half-life of the protein in Cos cells is ~20 min. A similar half-life was observed also in muscle cells (38). To identify the proteolytic system involved, cells were incubated in the presence of chloroquine, an inhibitor of lysosomal proteolysis, or

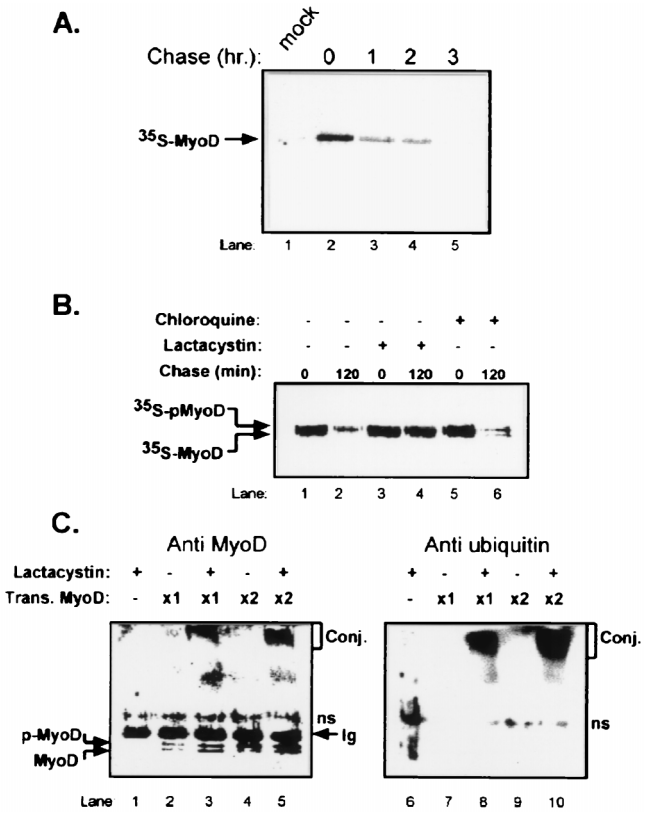


FIG. 1. MyoD is a short-lived protein, and its degradation in cells is mediated by the ubiquitin-proteasome pathway. (A) Stability of MyoD in vivo. The half-life of MyoD in Cos cells was measured in a pulse-chase experiment followed by immunoprecipitation of the labeled protein as described in Materials and Methods. (B) Sensitivity of MyoD degradation to cellular proteolysis inhibitors. Degradation of MyoD in Cos cells was monitored in a pulse-chase experiment followed by immunoprecipitation of the labeled protein in the presence of the proteasome inhibitor lactacystin or the lysosomal inhibitor chloroquine as described in Materials and Methods. (C) Ubiquitin-MyoD conjugates in cells. Cos cells were transfected with either 5 or 10 μg of MyoD expression vector. Following 1 h of incubation in the presence or absence of lactacystin, cells were disrupted and extracts were immunoprecipitated with anti-MyoD antibody and resolved by SDS-PAGE. Proteins were detected by Western blot analysis. Lanes 1 to 5, detection with anti-MyoD antibody. Lanes 6 to 10, following stripping of membrane, proteins were redetected with antiubiquitin antibody. pMyoD, phosphorylated form of MyoD; Conj., conjugates; Trans. MyoD, transfection with MyoD expression vector; ns, nonspecific cross-reacting protein; Ig, heavy chain of the Ig molecule.

lactacystin, a specific inhibitor of the 26S proteasome (12). As shown in Fig. 1B, lactacystin completely inhibited degradation. In contrast, chloroquine had no effect. As expected, incubation in the presence of lactacystin leads to accumulation of high-molecular-mass ubiquitin conjugates of MyoD (Fig. 1C). Two forms of MyoD can be precipitated from cells. The slower-migrating form is the phosphorylated protein; following treatment of the immunoprecipitate with calf intestine alkaline phosphatase, it "collapses" into the faster-migrating form (not shown).

**MyoD is degraded by the ubiquitin proteolytic system in vitro.** To study in detail the mechanisms involved in ubiquitin-mediated degradation of MyoD, it was necessary to monitor the degradation of MyoD in a reconstituted cell-free system. As shown in Fig. 2A, when incubated in a crude reticulocyte lysate, MyoD generates high-molecular-mass adducts in a process that requires ATP. To demonstrate that the adducts are generated in a ubiquitin-dependent manner and to further identify the conjugating enzymes involved, it was necessary to fractionate the lysate. As shown in Fig. 2B, conjugation of MyoD requires ubiquitin and ATP. Interestingly, it appears that all of the conjugating enzymes are contained within fraction II (lane 5): addition of E2-F1, which is contained in fraction I (lane 6), does not support the generation of conjugates by itself and does not increase their generation beyond the level generated by fraction II (lane 7). To identify the E2 enzyme involved in the conjugation of MyoD, we further fractionated the system. As can be seen in Figure 2C, panel 1, addition of fraction IIB, which contains several E2 enzymes, reconstituted conjugation. An important E2 enzyme contained in this fraction and involved in proteolysis of several substrates of the system is E2-14kDa (15, 31, 32). As shown in Fig. 2C, panel 1, addition of ubiquitin affinity-purified E2-14kDa reconstitutes conjugation. We noted the appearance of a band of ~90 kDa that reacts with the MyoD antibody. This band can be either a cross-reacting protein or, more probably (since it appears in a system that does not contain any additional components), a dimer of MyoD that is resistant to boiling in the sample buffer. To further corroborate the role of E2-14kDa in conjugating MyoD, we used both recombinant E2-14kDa and affinity-purified enzyme that was further purified to remove other E2 species. As shown in Fig. 2C, panel 2, both preparations gave a similar pattern of conjugates, strongly suggesting that E2-14kDa is involved in the conjugation of MyoD.

To examine whether tagging of MyoD with ubiquitin leads also to degradation of the protein, we reconstituted a cell-free proteolytic system. Incubation of wt MyoD in the presence of fraction II, ubiquitin, and ATP leads to complete degradation of the protein (Fig. 3A, lane 3). In contrast, omission of ubiquitin inhibits degradation completely (lane 2). Not surprisingly, the process requires also ATP (Fig. 3A, compare lane 1 to lane 3), which is required for activation of ubiquitin and the activity of the 26S proteasome complex. To examine the physiological relevance of the proteolytic process in vitro, we generated heterodimers of MyoD with E47. Formation of heterodimers was verified by a gel shift assay, and under the conditions employed, all MyoD was incorporated into heterodimers (data not shown). As can be seen in Fig. 3B, MyoD is efficiently degraded in its heterodimeric form as well. One important potential regulatory mechanism that governs MyoD stability can be the association of the protein with its cognate DNA. Therefore, we studied the degradation of a MyoD mutant that lacks the DNA-binding domain (Δbasic). As shown in Fig. 3A (lanes 4 to 6) and 3B (lanes 3 and 4), the pattern of degradation of the mutant protein is identical to that of the wt protein.

**Regulation of MyoD degradation by specific DNA binding.** To study the effect of DNA binding on the stability of MyoD, we designed two oligonucleotides, one that contains the two specific MyoD binding sites (E box) and a second one in which the two sites were mutated (Fig. 4A). Addition of increasing amounts of the double-stranded E-box-containing DNA significantly inhibits the degradation of wt MyoD (Fig. 4B, lanes 1 to 5). In striking contrast, the added DNA had no effect whatsoever on the degradation of the Δbasic MyoD protein (lanes 6 to 10). Not surprisingly, addition of mutant DNA did not affect the degradation of either the wt or the Δbasic protein (Fig. 4C). Again, to demonstrate the physiological relevance of the DNA inhibition, we examined the effect on MyoD-E47 heterodimers. As can be seen in Fig. 4D, DNA exerts a similar inhibitory effect on MyoD-E47 heterodimers. Moreover, the effect appears to be linear and stoichiometric. With a DNA/MyoD molar ratio of 2, the inhibition is complete. In molar ratios of less than 1, the effect is only partial.

To analyze the step in the ubiquitin proteolytic cascade in

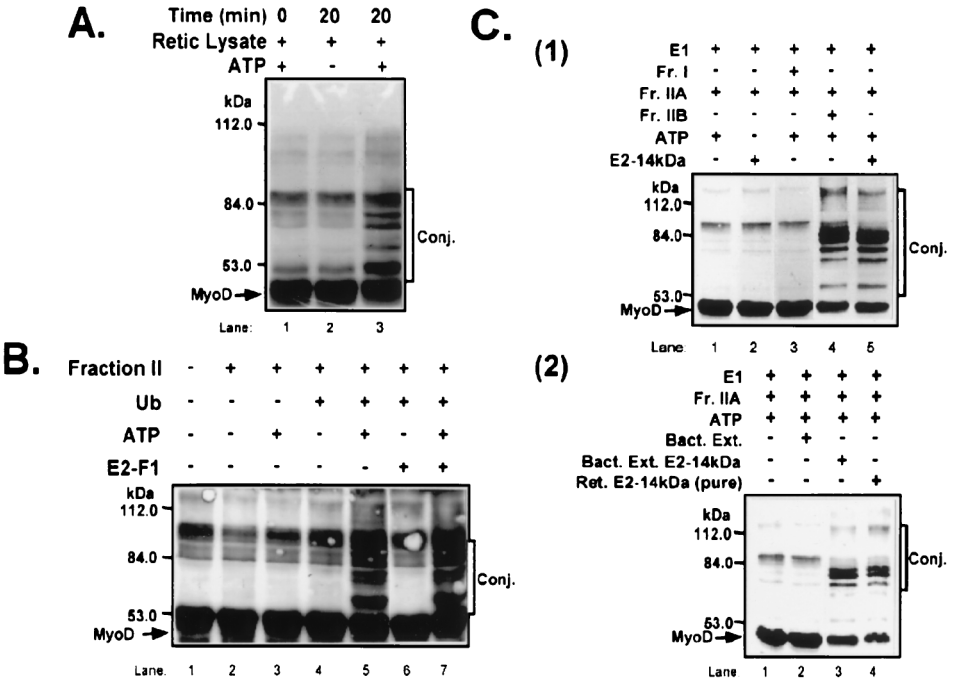


FIG. 2. ATP- and ubiquitin-dependent conjugation of MyoD in crude (A) and fractionated (B) reticulocyte lysate and in a system reconstituted from purified and partially purified enzymes (C). (A) ATP-dependent conjugation of MyoD. Conjugation of MyoD to ubiquitin in crude reticulocyte (Retic) lysate was monitored as described in Materials and Methods. Reaction mixtures containing ATP also contain an ATP-regenerating system. Reaction mixtures without ATP contain hexokinase and 2-deoxyglucose. Lane 1, reaction mixture with ATP incubated on ice; lanes 2 and 3, reaction mixtures incubated at 37°C. Conj., conjugates. (B) Conjugation of MyoD in fractionated reticulocyte lysate. Conjugation of MyoD in fractionated reticulocyte lysate was monitored as described in Materials and Methods. (C) Conjugation of MyoD requires E2-14kDa. Panel 1, conjugation of MyoD was monitored in reaction mixtures containing purified E1 and E2-14kDa and crude reticulocyte fraction (Fr.) I, IIA, and IIB as described in Materials and Methods. Panel 2, conjugation of MyoD was determined in the presence of control BL21 extract (Bact. Ext.), BL21 extract prepared from cells that express E2-14kDa, and E2-14kDa initially purified via ubiquitin affinity chromatography and further purified via anion-exchange chromatography to resolve the enzyme from other species of E2. E1, fraction IIA, and ATP were added as described for panel 1.

which DNA affects MyoD stability, we investigated the effect of the oligonucleotides on MyoD conjugation. Conjugation of ubiquitin to MyoD is markedly reduced in the presence of specific DNA (Fig. 5A, compare lanes 4 and 5 to lanes 2 and 3). In contrast, mutant DNA did not have any effect on the conjugation pattern (lanes 6 and 7). Similar to its inability to affect the degradation of Δbasic mutant MyoD, the specific DNA did not affect conjugation of this molecule (Fig. 5B).

Different mechanisms that regulate the binding of MyoD to DNA have been proposed. One such mechanism involves the cooperative binding of MyoD to more then one DNA recognition motif. Indeed, multiple MyoD binding sites on several MyoD target genes, such as the muscle creatine kinase gene (25), have been described. It has been shown that while MyoD binds poorly to DNA that contains a single binding site, it binds tightly to DNA that contains two adjacent sites. This effect is not due to binding of the same MyoD molecule to the two sites. Rather, it is the result of a cooperative binding of two MyoD dimers to the two sites (3, 41). To test the effect of cooperative binding on the protective effect of DNA on MyoD degradation, we designed an oligonucleotide that contains a single binding site. As can be seen in Fig. 6, this DNA has only a minor protective effect (compare complete degradation of MyoD in the absence of DNA [lane 2] to degradation of MyoD in the presence of a single-site DNA [lane 3]). The two-site-containing DNA inhibits degradation completely (Fig. 6, compare lanes 3 and 4).

MyoD is negatively regulated by members of the Id family of proteins. These proteins can heterodimerize with E12/E47 or MyoD, but since they lack the basic region, the complexes cannot bind to DNA and are therefore inactive (2, 24, 37). To test the effect of heterodimerization on MyoD stability, we incubated either MyoD homodimers (Fig. 7A) or MyoD-E47 heterodimers (Fig. 7B) in the presence of increasing concentrations of Id1. Increasing concentrations of Id abrogate the inhibitory effect of DNA on the degradation of MyoD and render the protein susceptible to degradation (Fig. 7).

To test the notion that the removal of MyoD from its DNA binding site by the formation of defective heterodimers destabilized the protein, we tested the effect of DNA on such heterodimers. Increasing concentrations of the Δbasic MyoD abrogate the binding of MyoD to its cognate DNA binding site (Fig. 8A). As expected, wt MyoD generates two types of complexes with DNA, one in which a single homodimer binds to a single site and another in which two homodimers are anchored to the two binding sites (Fig. 8A, lane 1). The inhibitory effect is probably due to the formation of wt MyoD-Δbasic MyoD heterodimers that cannot bind DNA. Consequently, the wt MyoD moiety in these heterodimers is also unstable and is degraded in the presence of otherwise inhibitory concentrations of DNA (Fig. 8B).

## DISCUSSION

We have shown that MyoD is a short-lived protein with a half-life of ~20 min (Fig. 1A). Its degradation is mediated by the ubiquitin-proteasome pathway: lactacystin, a specific inhibitor of the 26S proteasome, inhibits the degradation of the transcriptional factor and leads to accumulation of high-molecular-mass MyoD-ubiquitin adducts (Fig. 1B and C).

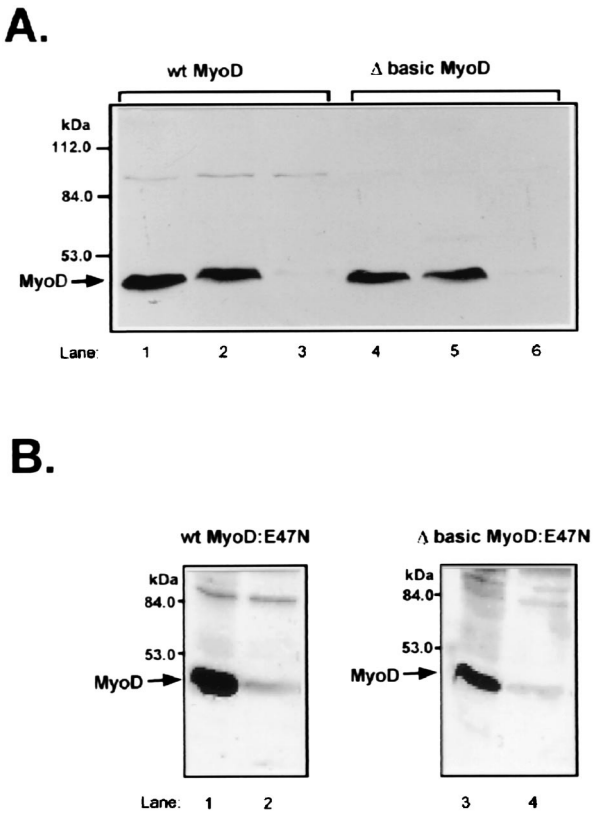


FIG. 3. ATP- and ubiquitin-dependent degradation of wt and Δbasic homo- and heterodimers of MyoD with E47N in crude reticulocyte fraction II. (A) Degradation of wt and Δbasic MyoD homodimers. Proteolysis was monitored in the presence of crude reticulocyte fraction II by Western blot analysis as described in Materials and Methods. Lanes 1 and 4, reaction mixtures were incubated in the presence of ubiquitin but in the absence of ATP. Lanes 2 and 5, mixtures were incubated in the presence of ATP and in the absence of ubiquitin. Lanes 3 and 6, mixtures were incubated in the presence of ubiquitin and ATP. (B) Degradation of wt and Δbasic MyoD heterodimers with E47N. Proteolysis of MyoD-E47N heterodimers was monitored in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of ATP as described in Materials and Methods and for panel A.

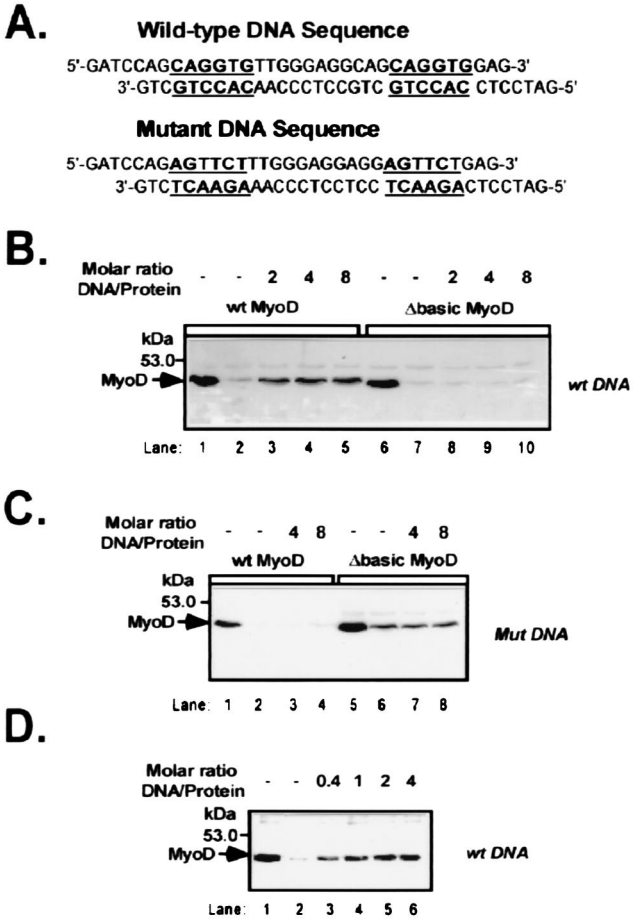


FIG. 4. Binding of MyoD to its specific DNA recognition motif protects the protein from degradation. Degradation of the MyoD proteins was carried out in the presence of crude reticulocyte fraction II as described in Materials and Methods. (A) wt and mutant DNA binding sites of MyoD. The double-stranded oligonucleotide sequence containing two MyoD binding sites and its mutant form to which MyoD does not bind are shown. (B) The specific DNA sequence to which MyoD binds inhibits the degradation of wt but not Δbasic homodimers of MyoD. Lanes 1 and 6, MyoD proteins were incubated in a complete reaction mixture but in the absence of ATP. Lanes 2 and 7, same as lanes 1 and 6, but mixtures were incubated in the presence of ATP. Lanes 3 to 5 and 8 to 10, same as lanes 2 and 7, but MyoD proteins were incubated in the presence of the indicated molar excess of DNA over the protein substrates. (C) A mutant DNA recognition motif does not inhibit the degradation of homodimers of MyoD. Lanes 1 and 5, MyoD proteins were incubated in the presence of crude reticulocyte fraction II but in the absence of ATP. Lanes 2 and 6, same as lanes 1 and 5, but reaction mixtures were incubated in the presence of ATP. Lanes 3, 4, 7, and 8, same as lanes 2 and 6, but MyoD proteins were incubated in the presence of the indicated molar excess of DNA over the protein substrates. (D) Quantitative analysis of the inhibitory effect of DNA on the degradation of wt MyoD-E47N heterodimers. Degradation of the heterodimers was monitored in the presence of the indicated molar excess of DNA over the protein substrates as described above and in Materials and Methods.

Reconstitution of a cell-free system demonstrates that ATP-dependent conjugation of ubiquitin precedes the proteolytic process (Fig. 2 and 3). Interestingly, fraction II contains all of the enzymes necessary to catalyze the conjugation and degradation reaction (Fig. 2B). This finding is particularly intriguing with respect to the E2 enzyme involved in the process, as it is fraction I that contains the universal E2 enzymes (UbcH5s, UbcH6, and UbcH7 [E2-F1]) that appear to be involved in the degradation of the bulk of cellular proteins (5, 29, 34, 35). Further corroborating these observations is the finding that E2-14kDa, an E2 enzyme contained in fraction II, reconstitutes conjugation (Fig. 2C). In contrast, E2-F1, an E2 enzyme contained in fraction I, does not stimulate conjugation (Fig. 2B). It should be noted that E2-14kDa is involved in the degradation of N-end-rule (40) protein substrates but also of certain non-N-end-rule protein substrates (13). Furthermore, E3α, the N-end-rule ubiquitin ligase, is also involved in targeting of non-N-end-rule substrates (13). According to the rules that govern processing of N-terminal amino acid residues, it is predicted that the initiator Met of MyoD is not removed (6); this is because the second amino acid in MyoD is Glu, a destabilizing residue according to the N-end rule. Thus, it appears that MyoD can be targeted via an E2-14kDa-mediated pathway that traverses a non-N-end-rule pathway. To further corroborate this notion, we incubated both the conjugation and degradation reaction mixtures in the presence of Lys-Ala and Phe-Ala, two peptides known to inhibit targeting of substrates with basic or bulky-hydrophobic N-terminal residues, respectively (33). The peptides did not have any effect on either process (not shown). In addition, we used Edman degradation to identify the N-terminal residues of both the bacterially expressed and mammalian cell-expressed MyoD proteins. Both proteins retain the initiator Met, a stabilizing residue, at the N-terminal position. Thus, it is unlikely that

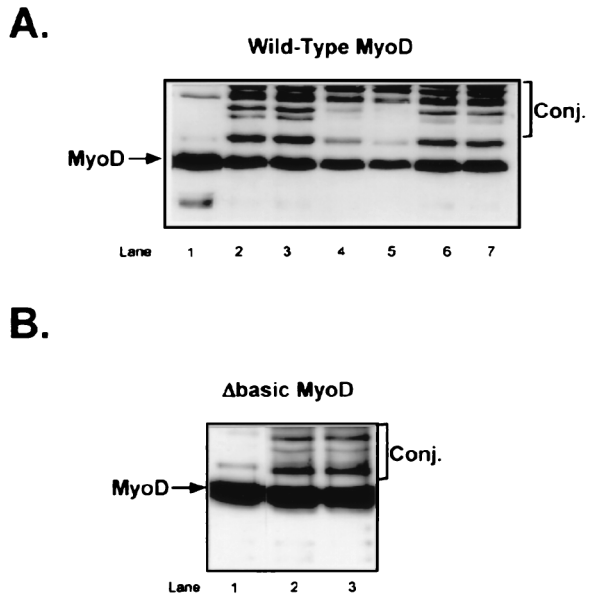


FIG. 5. Binding of MyoD to its specific DNA recognition motif inhibits its conjugation to ubiquitin. Conjugation of ubiquitin to MyoD was monitored as described in Materials and Methods. (A) The specific DNA to which MyoD binds, but not mutant DNA, inhibits conjugation of ubiquitin to wt MyoD. Lane 1, wt MyoD protein was incubated in the presence of crude reticulocyte fraction II, ubiquitin, and ubiquitin aldehyde but in the absence of ATP. Lanes 2 and 3, same as lane 1, but MyoD was incubated in the presence of ATP. Lanes 4 and 5, same as lanes 2 and 3 but with two- and fourfold molar excesses of specific DNA over the protein substrate, respectively. Lanes 6 and 7, same as lanes 2 and 3 but with four- and eightfold molar excesses of mutant DNA over the protein substrate, respectively. Conj., conjugates. (B) Specific DNA does not inhibit conjugation of ubiquitin to Δbasic MyoD. Lane 1, mutant MyoD incubated in the presence of crude reticulocyte fraction II, ubiquitin, and ubiquitin aldehyde but in the absence of ATP. Lane 2, same as lane 1 but with ATP. Lane 3, same as lane 2 but with an eightfold molar excess of specific DNA over the protein substrate.

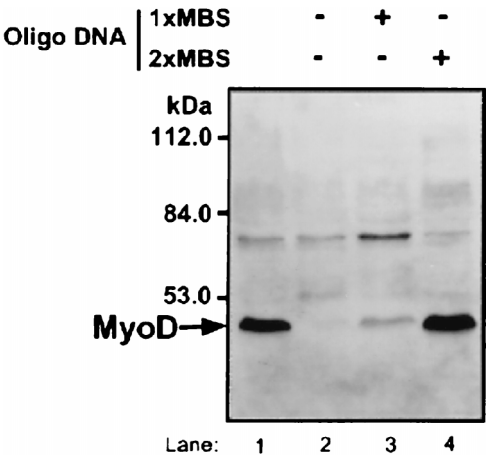


FIG. 6. Cooperative binding of MyoD to two recognition sequences in its cognate DNA is necessary for inhibition of degradation of the protein. Degradation of MyoD was monitored as described in Materials and Methods. Lane 1, MyoD was incubated in the presence of crude reticulocyte fraction II and ubiquitin but in the absence of ATP. Lane 2, same as lane 1, but the reaction mixture was incubated in the presence of ATP. Lane 3, same as lane 2 but with a DNA oligonucleotide that contains one wt and one mutant binding motif. Lane 4, same as lane 2 but with a DNA oligonucleotide that contains two DNA binding motifs. MBS, MyoD binding site.

MyoD is degraded by the N-end-rule pathway. We are currently studying the identity of the E3 enzyme involved, but as noted, even E3α, the N-end-rule ligase, targets non-N-end-rule substrates as well. As shown in Fig. 3A, ubiquitination of MyoD leads also to its degradation. Heterodimers of MyoD with E47 or E12 appear to be the more physiological complexes of MyoD, although it has not been ruled out that homodimers are not functional (22, 26). Therefore, we examined the effect of the ubiquitin system on MyoD-E47 heterodimers. As can be seen in Fig. 3B, these heterodimers are also susceptible to degradation by the ubiquitin system. To study the regulatory mechanisms involved in the degradation of MyoD, we tested the effect of the specific upstream cognate DNA fragment to which MyoD binds in most of its target genes. This DNA fragment stabilizes the protein (Fig. 4). A mutant MyoD species that lacks the DNA-binding domain or a DNA species that harbors mutated binding sites has no effect on the stability of the protein (Fig. 4B and C). Here too, the inhibitory effect of DNA on MyoD-E47 heterodimers could be demonstrated (Fig. 4D). Dissection of the underlying inhibitory mechanism reveals that the nucleic acid inhibits conjugation of ubiquitin to MyoD (Fig. 5). The inhibitory effect can be due to a change in the conformation of the protein that sterically hinders either the ubiquitin ligase (E3) binding site or the Lys residue(s) that serves as a ubiquitination site(s). Alternatively, the DNA binding site in the protein is adjacent or identical to the E3 anchoring domain or the critical ubiquitination site(s), and thus DNA binding does not allow binding of the ligase or transfer of activated ubiquitin moieties. Interestingly, the protective effect of DNA is exerted only when the DNA contains two binding sites to which two homodimers bind. A single-site-containing DNA does not protect the protein (Fig. 6). This is due to the instability of the binding of a single MyoD homodimer to the DNA. Binding of two homodimers has a cooperative effect that is reflected in a dramatic decrease in the rate of dissociation ($K_{off}$) of the protein from the DNA (3, 41). Further dissection of the inhibitory effect of DNA on MyoD degradation revealed that it requires homodimerization or formation of heterodimers with E47. Id1 rendered both MyoD homodimers and heterodimers with E47 susceptible to degradation (Fig. 7). While one known mechanism is that it disrupts MyoD-E47 (or -E12) heterodimers by removing the E proteins from the complex, our data suggest that it can also act via direct interaction with MyoD. Such heterodimerization has been demonstrated both biochemically and in the yeast two-hybrid system (2, 24), and its possible involvement in regulation of MyoD in vivo has not been ruled out. To further dissect the mechanism of sensitivity of such defective MyoD heterodimers to degradation, we studied the effect of the Δbasic species of MyoD, which lacks the DNA-binding domain, on the wt protein. As can be seen in Fig. 8A, formation of such heterodimers abrogates the ability of wt MyoD to bind to its specific DNA binding site. Consequently, these heterodimers are also sensitive to degradation in the presence of otherwise inhibitory concentration of DNA (Fig. 8B).

What can be the physiological significance of the protective effect of DNA on MyoD stability? Like other short-lived transcriptional activators, and unlike stable proteins, MyoD is subjected to a variety of regulatory mechanisms, including autoregulation of its own transcription (38). Rapid removal of the protein is an important regulatory element that can tightly control the expression and activity of a protein in the cell. However, when the protein is involved in transcription, its removal will be a waste. Thus, it makes biological sense to stabilize the fraction of the transcriptional factor that is DNA bound. Indeed, in undifferentiated proliferating myoblasts, MyoD is complexed with Id, which renders it inactive (2, 21) and probably susceptible to degradation. It is interesting that

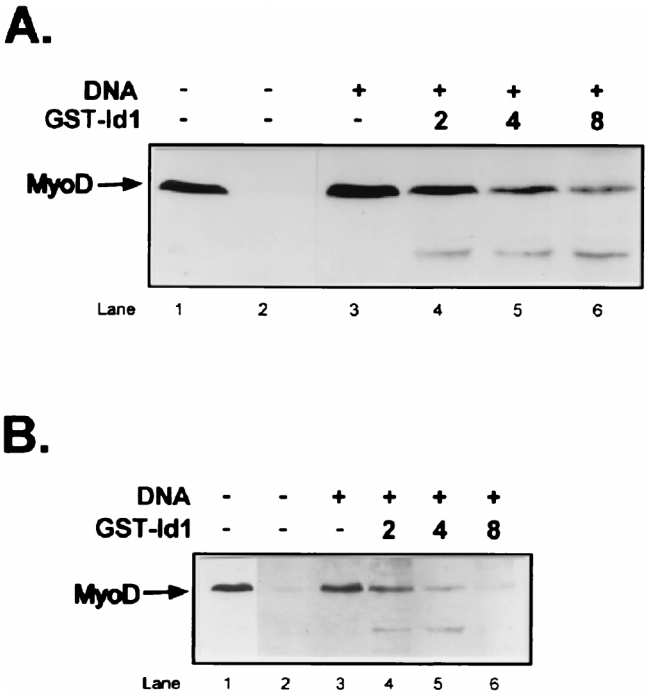


FIG. 7. Association of MyoD homodimers (A) or MyoD-E47N heterodimers (B) with Id1 abolishes the inhibitory effect of DNA on the degradation of MyoD. Degradation of MyoD was carried out as described in Materials and Methods. (A) Association of MyoD homodimers with Id1 abolishes the inhibitory effect of DNA on MyoD degradation. Lane 1, MyoD was incubated in the presence of crude reticulocyte fraction II and ubiquitin but in the absence of ATP. Lane 2, same as lane 1, but the reaction mixture was incubated in the presence of ATP. Lane 3, same as lane 2, but specific DNA was added at a fourfold molar excess over the protein substrate. Lanes 4 to 6, same as lane 3, but Id was added at the indicated fold molar excesses over MyoD. GST, glutathione *S*-transferase. (B) Association of MyoD-E47N heterodimers with Id1 abolishes the inhibitory effect of DNA on MyoD degradation. Degradation of the heterodimers was monitored as described for panel A and in Materials and Methods.

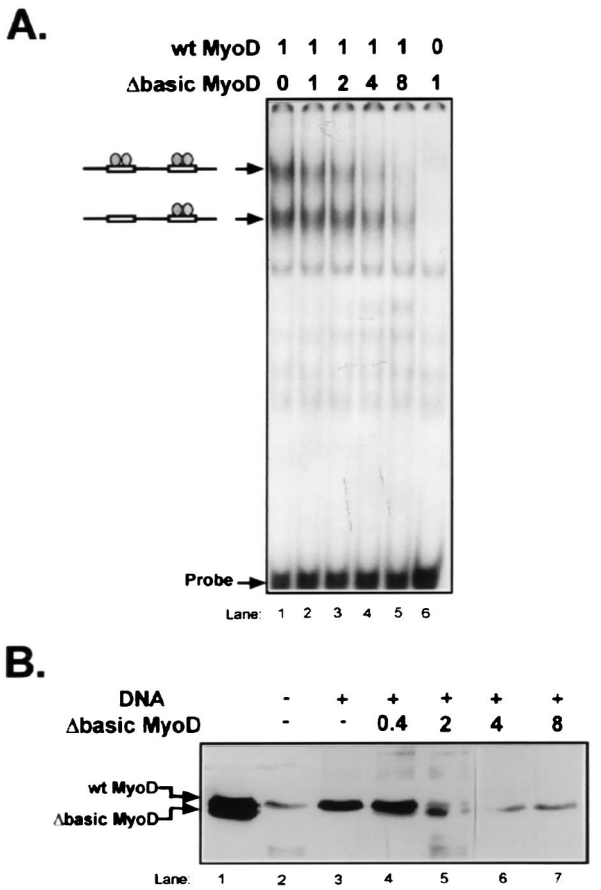


FIG. 8. Heterodimerization of MyoD with Δbasic mutant MyoD prevents binding of MyoD to DNA and consequently abolishes the inhibitory effect of DNA on MyoD degradation. (A) Formation of heterodimeric wt-Δbasic MyoD complex abolishes the specific DNA binding capacity of homodimeric wt MyoD. wt MyoD was incubated in the presence of a labeled DNA probe that contains two MyoD recognition sites and the indicated increasing molar ratios of Δbasic MyoD over the wt protein. Following incubation, the mixture was subjected to electrophoretic mobility shift assay as described in Materials and Methods. DNA-MyoD complexes that contain one and two wt homodimers are indicated. (B) Effect of heterodimerization of wt MyoD with Δbasic MyoD on the inhibitory effect of DNA on MyoD degradation. Lane 1, wt and Δbasic MyoD proteins were incubated in the presence of crude reticulocyte fraction II and ubiquitin but in the absence of ATP. Lane 2, wt MyoD was incubated in the presence of crude reticulocyte fraction II, ubiquitin, and ATP. Lane 3, same as lane 2, but specific DNA was added at a fourfold molar excess over the protein substrate. Lanes 4 to 7, same as lane 3, but Δbasic MyoD was added at 0.4-, 2-, 4-, and 8-fold molar excesses over wt MyoD, respectively.

p53 is stabilized in vivo following binding to DNA in response to DNA damage (1). Repair of the damage leads most probably to dissociation of the p53-DNA complex and to destabilization of p53 by the ubiquitin system, a process that appears to be mediated by Mdm2 (16, 23). Indeed, studies with cell-free system have shown that binding to DNA in the presence of the destabilizing targeting human papillomavirus oncoprotein E6 protects p53 from degradation by the ubiquitin system (27). One prediction is that the level of MyoD in differentiating cells should be higher then that observed in undifferentiated or fully differentiated muscle cells. While this prediction is currently being tested, it may not be true. It is possible, for example, that the DNA-bound MyoD represents only a small fraction of the total cellular MyoD. In this case, the majority of MyoD which is free will be unstable in all cells, differentiating as well as nondifferentiating, and only the small bound fraction that may not be detectable is stable. In this case it will be not the proteolytic machinery that regulates MyoD activity but rather other, upstream elements that regulate binding of MyoD to the specific enhancers at particular time points during differentiation.

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The first two authors contributed equally to this work.

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