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Research Articles

MyoD1: A Nuclear Phosphoprotein Requiring a Myc Homology Region to Convert Fibroblasts to Myoblasts

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Expression of a complementary DNA (cDNA) encoding the mouse MyoD1 protein in a variety of fibroblast and adipoblast cell lines converts them to myogenic cells. Polyclonal antisera to fusion proteins containing the MyoD1 sequence show that MyoD1 is a phosphoprotein present in the nuclei of proliferating myoblasts and differentiated myotubes but not expressed in 10T1/2 fibroblasts or other nonmuscle cell types. Functional domains of the MyoD1 protein were analyzed by site-directed deletional mutagenesis of the MyoD1 cDNA. Deletion of a highly basic region (residues 102 to 135) interferes with both nuclear localization and induction of myogenesis. Deletion of a short region (residues 143 to 162) that is similar to a conserved region in the c-Myc family of proteins eliminates the ability of the MyoD1 protein to initiate myogenesis but does not alter nuclear localization. Deletions of regions spanning the remainder of MyoD1 did not affect nuclear localization and did not inhibit myogenesis. Furthermore, expression of only 68 amino acids of MyoD1, containing the basic and the Myc similarity domains, is sufficient to activate myogenesis in stably transfected 10T1/2 cells. Genetic analysis maps the MyoD1 gene to mouse chromosome 7 and human chromosome 11.

THE IDENTIFICATION OF THE MYOD1 COMPLEMENTARY DNA (cDNA) (*1*) depended on the initial observation that a brief treatment of C3H10T1/2 fibroblasts (10T1/2 cells) with 5-azacytidine yields, at high frequency, colonies capable of forming muscle (up to 50 percent) (*2*), presumably because 5-azacytidine incorporation into DNA results in the demethylation and subsequent expression of specific loci involved in myogenesis. It was later shown (*3*) that the frequency of myogenic conversion after treatment with 5-azacytidine is consistent with the activation of a small number of genes and possibly a single gene. Furthermore, genomic DNA transfections (*4, 5*) have shown that myoblast DNA, but not DNA from 10T1/2 cells, has the capacity to convert 10T1/2 cells to muscle at a frequency consistent with the transfer of a single genetic locus, an indication that 5-azacytidine does, in fact, lead to an altered structure (that is presumably related to demethylation) of 10T1/2 DNA.

We used subtracted cDNA hybridization to isolate the MyoD1 cDNA (*1*). MyoD1 is not expressed in 10T1/2 cells but is expressed in myoblast lines derived from 10T1/2 cells after treatment with 5-azacytidine, as well as in other myoblast lines and primary muscle both in vivo and in vitro (*1*). The MyoD1 cDNA, when expressed under the control of a viral long terminal repeat (LTR) and transfected into several different fibroblast or adipoblast cell lines, converts these cells to myoblasts (*1*). It is important to distinguish between the azacytidine gene (that is, the gene activated by 5-azacytidine treatment of 10T1/2 cells), the genomically transferred myogenic gene, and MyoD1 (the myogenic regulatory gene ultimately identified by subtracted cDNA screening). Whether these are all the same or different genes remains to be determined. In this regard, it was recently shown (*6*) that transfection of a genomic cosmid DNA sequence distinct from MyoD1 can result in the activation of myogenesis in 10T1/2 cells.

The sequence of the MyoD1 cDNA contains a major open reading frame of 318 amino acid residues. The sequence has a region of 22 amino acid residues with a marked similarity to a region that is

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highly conserved in the family of Myc proteins. Also, this 22–amino acid sequence is within a region of Myc that is important for two-step oncogenic transformation (*7*). The Myc-MyoD1 similarity is shared with the predicted protein products of four transcription units of the *achaete-scute* complex of *Drosophila* (*1, 8*), which are involved in neuronal determination and the development of the nervous system.

To characterize the MyoD1 protein, we have generated polyclonal antisera to trpE-MyoD1 fusion proteins and have used site-directed deletional mutagenesis to study functional domains of the MyoD1 protein. Our results show that MyoD1 is a nuclear phosphoprotein, and we identify two domains that are both necessary and sufficient for myogenic activity: a highly basic region and the region of Myc similarity.

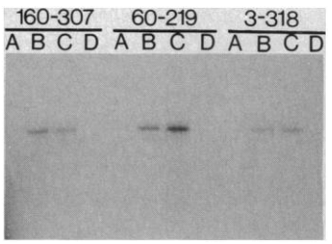

Fig. 1. Immunoprecipitation of in vitro translated MyoD1 protein. Antisera from rabbits immunized with each of the three fusion protein constructs containing MyoD1 amino acid residues 160 to 307, 60 to 219, and 3 to 318, as indicated, were used to precipitate MyoD1 protein translated in vitro in a rabbit reticulocyte translation system (NEN Research Products). (Lanes A) Preimmune sera; (lanes B) immune sera; (lanes C) immune sera competed with trpE protein; (lanes D) immune sera competed with corresponding fusion protein. For the immunoprecipitations, the in vitro translation mixture was diluted into RIPA buffer (10 m*M* tris, *p*H 7.4, 150 m*M* NaCl, 1 percent NP-40, 1 percent sodium deoxycholate, 0.1 percent SDS), incubated for 30 minutes on ice with Pansorbin at 50 μl/ml (10 percent weight to volume; Calbiochem), centrifuged at 12,000*g* for 20 minutes, and divided into 1-ml portions; 10 μl of the appropriate antiserum was then added to each. Competitions were performed by direct addition of 20 μl of unfractionated bacterial cell extract containing the induced trpE or trpE-MyoD1 fusion polypeptide (1 to 2 mg/ml). Immunoprecipitates were incubated overnight at 4°C and collected the next day on 20 μl (packed volume) of protein A agarose (Repligen). After 1 hour at 4°C with shaking, precipitates were centrifuged, washed once with RIPA buffer, and then once with 10 m*M* tris (*p*H 7.4). The pellets were resuspended in SDS gel sample buffer, heated to 90°C, centrifuged, and processed on 10 percent polyacrylamide gels. Each antiserum specifically precipitates the in vitro translated MyoD1 protein, which has a gel mobility of about 45 kD.

Antisera to MyoD1 protein and immunoprecipitation. To study the localization of the MyoD1 protein in myoblasts and myotubes, we prepared rabbit antisera to bacterial fusion proteins. Three restriction fragments of the MyoD1 cDNA were inserted into a bacterial trpE expression vector, fusing the bacterial trpE polypeptide with the MyoD1 major open reading frame covering amino acid residues 160 to 307, 60 to 219, and 3 to 318, respectively (*9*). Whereas the last two fusion proteins contain the region of MyoD1 that has a high degree of similarity to a conserved region of the Myc proteins, the first fusion protein construct lacks all but three amino acids of the region of Myc similarity. After these vectors were expressed in *Escherichia coli*, the trpE-MyoD1 fusion proteins were gel-purified from bacterial lysates by SDS–polyacrylamide gel electrophoresis (PAGE) and used to immunize rabbits (*10*).

We synthesized MyoD1 protein in an in vitro rabbit reticulocyte translation system primed with full-length MyoD1 RNA generated by T3 RNA polymerase, and immunoprecipitated the translation product with each antiserum. The in vitro translation product has a gel mobility of approximately 45 kD. This protein is precipitated by all three antisera (Fig. 1). The addition of a molar excess of the trpE-MyoD1 fusion protein competitively blocks its precipitation, whereas the trpE protein alone does not compete successfully.

Having established that the antisera precipitate in vitro translated MyoD1 protein, we used the antisera to precipitate proteins from in vivo labeled cells. Either 10T1/2 cells or a myogenic cell line expressing MyoD1 messenger (mRNA) that was derived from 10T1/2 cells by treatment with 5-azacytidine (aza-myoblasts) were cultured for 2 hours in methionine-free medium supplemented with [^{35}S]methionine and a total cell lysate was used for immunoprecipitation. All three antisera to MyoD1 precipitate a set of proteins ranging between 45 and 48 kD from preparations of aza-myoblasts (Fig. 2A, lanes B) but not from 10T1/2 cells (Fig. 2B, lanes B). The precipitation of this set of proteins was blocked by the addition of the trpE-MyoD1 fusion protein (lanes D) but not by the trpE protein alone (lanes C). Two of the antisera also precipitate larger proteins from both 10T1/2 cells and aza-myoblasts, although in this case the precipitated proteins are different for each antiserum. These

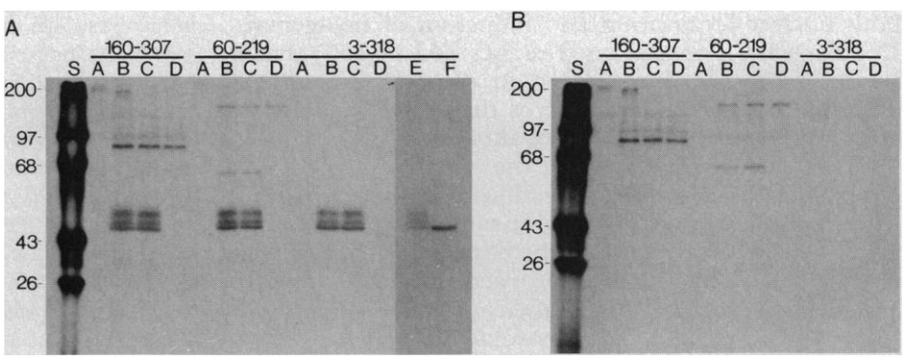

Fig. 2. Immunoprecipitation of in vivo labeled MyoD1 protein. Immunoprecipitations of proliferating aza-myoblasts (**A**) or 10T1/2 cells (**B**) are shown for antisera to each of the three fusion proteins, as indicated. (Lanes S) Molecular size standards (kilodaltons); (lanes A), pre-immune sera; (lanes B) immune sera; (lanes C) immune sera competed with trpE protein; (lanes D) immune sera competed with immunizing fusion protein. (Lane F) Alkaline phosphatase treatment was performed on second-round immunoprecipitates. (Lane E) Mock phosphatase treatment. Proliferating aza-myoblasts or 10T1/2 cells on 10-cm tissue culture dishes, were washed twice with tris-saline and labeled for 2 hours in methionine-free medium supplemented with 15 percent dialyzed fetal calf serum and 125 μCi/ml L-[^{35}S]methionine (>1000 Ci/mmol, NEN). Cells were then washed with tris-buffered saline, scraped from the dish in phosphate-buffered saline (PBS) with 5 m*M* EDTA, centrifuged, and lysed by addition of RIPA buffer with 10 m*M* iodoacetamide and 0.1 percent aprotinin, and then sonicated. Lysates were first cleared with Pansorbin (as described in Fig. 1), and 10 μl of antiserum was added to each. Immunoprecipitates were collected on protein A agarose; washed two times with RIPA buffer, once with RIPA lacking SDS and with 1*M* NaCl; once again with RIPA; and once with 10 m*M* tris *p*H 7.4. The pellets were then resuspended in 50 μl of 10 m*M* tris (*p*H 7.4) with 1 percent SDS, heated to 90°C for 10 minutes, spun, and resuspended in RIPA buffer containing the appropriate antiserum for a second immunoprecipitation and processed similarly. For alkaline phosphatase treatment the protein A agarose matrix was washed twice in 100 m*M* tris (*p*H 8.0), 50 m*M* $MgCl_2$, 0.1 percent aprotinin, and then resuspended in 45 μl of the same buffer without (lane E) or with (lane F) the addition of 5 μl (0.7 units) of bacterial alkaline phosphatase (BAP) (Sigma). (BAP has no reported diesterase activity.) Reactions were incubated for 45 minutes at 30°C, washed twice in RIPA buffer and once in 10 m*M* tris (*p*H 7.4), and resuspended in SDS gel sample buffer. As a control, similar reactions were performed in the presence of 20 m*M* Na_2HPO_4 which inhibited the phosphatase reactions by competition.

proteins can be distinguished from MyoD1 in subsequent analysis because of their cell type distribution and by comparison of immunoreactivity with each different antiserum.

Since phosphorylation could account for the apparent size variation of the MyoD1 protein, we incubated a precipitated sample with bacterial alkaline phosphatase (BAP) (lane F) or without BAP (lane E) prior to gel electrophoresis. The phosphatase treatment resulted in a single intensified band at 45 kD, supporting the conclusion that the larger species represent phosphorylated forms of MyoD1 protein.

Localization of MyoD1 protein to the nuclei of myoblasts and myotubes. The 10T1/2 cells have a low spontaneous myogenic potential [of the order of 1 in 10^8 cells (*4*)] and do not contain detectable amounts of MyoD1 mRNA or immunoprecipitable MyoD1 protein. In contrast, aza-myoblasts are highly myogenic and contain MyoD1 mRNA and protein. In proliferating aza-myoblasts, the antiserum to MyoD1 demonstrates nuclear localization in a diffuse, punctate pattern that is excluded from the nucleoli (Fig. 3A). Adsorption of the antiserum with the trpE protein does not diminish the nuclear staining but does block the cytoplasmic staining (Fig. 3B). Adsorption of the antiserum with the trpE-MyoD1 fusion protein used for immunization extinguishes the nuclear staining (Fig. 3C). In contrast to the aza-myoblasts, the nuclei of 10T1/2 cells do not stain with the antiserum (Fig. 3D), nor do the nuclei from a wide variety of nonmyogenic cells. The antiserum does stain nuclei in other myogenic cells, including mouse C2C12 myoblasts (*11*) (Fig. 3E) and chicken primary myoblasts. During mitosis the bulk of MyoD1 is not associated with chromosomes, suggesting that it is either not associated with DNA or, like many other nuclear proteins, including Myc (*12*), it becomes

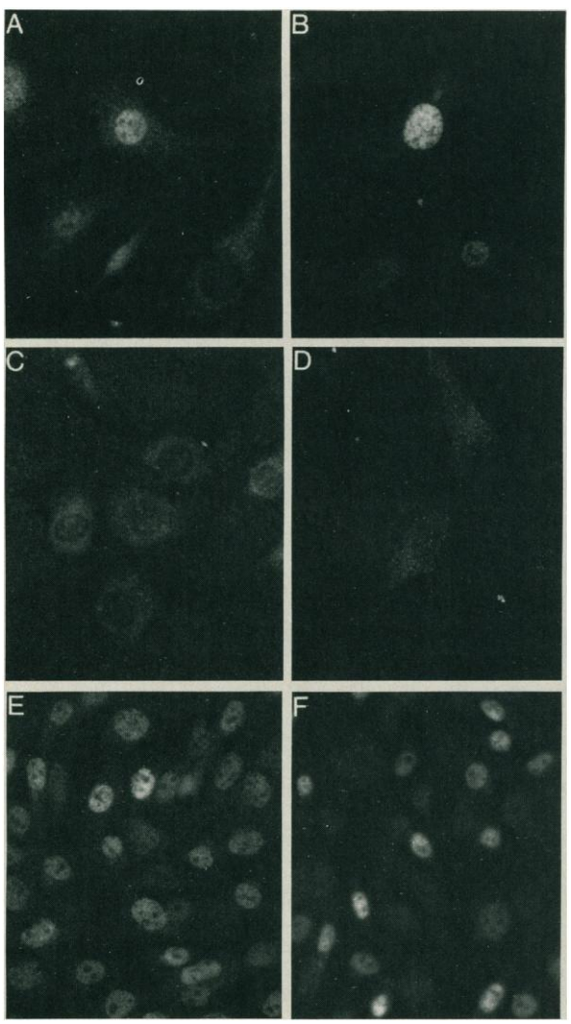

Fig. 3. Immunofluorescent localization of MyoD1 protein in myoblasts and 10T1/2 cells. (**A**) Aza-myoblasts in growth medium labeled with antiserum to MyoD1 (anti-MyoD1) without competition. (**B**) Aza-myoblasts in growth medium labeled with anti-MyoD1 competed with trp-E protein. (**C**) Aza-myoblasts in growth medium labeled with anti-MyoD1 competed with fusion protein. (**D**) 10T1/2 cells in growth medium labeled with anti-MyoD1 competed with trp-E protein. (**E**) C2C12 myoblasts in growth medium labeled with anti-MyoD1 without competition. (**F**) C2C12 myoblasts after 24 hours in differentiation medium labeled with anti-MyoD1 without competition. Cells were fixed for 10 minutes in PBS with 2 percent paraformaldehyde, permeabilized in PBS with 0.1 percent Triton X-100 and stained with diluted (1:250) rabbit anti-MyoD1 followed by diluted (1:500) rhodamine conjugated donkey antiserum to rabbit IgG (Jackson Laboratory). The antiserum was blocked by prior incubation with a molar excess of either trpE protein or fusion protein as indicated.

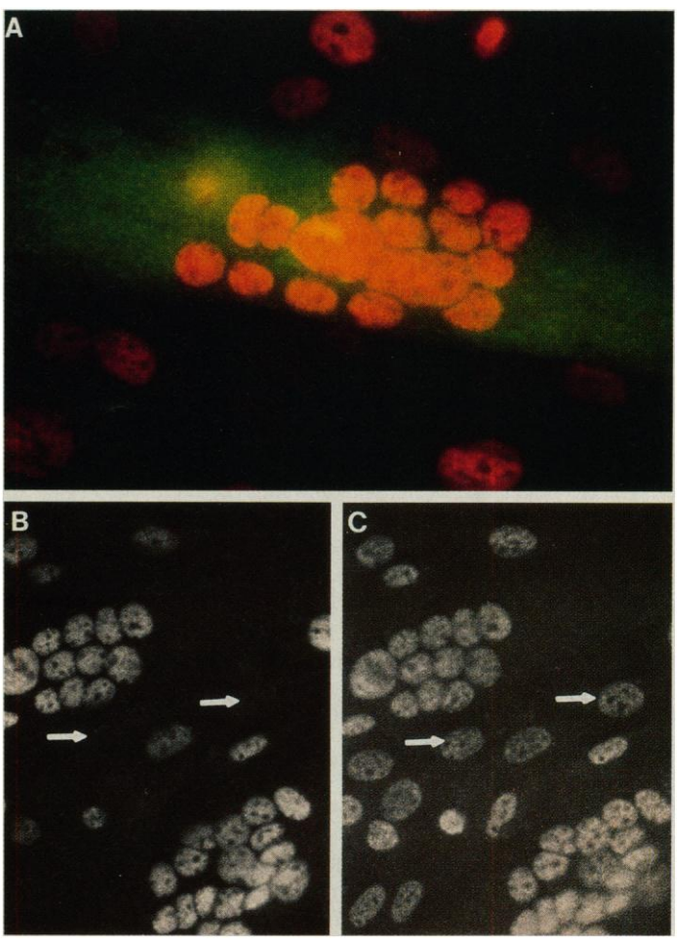

Fig. 4. Myoblasts and myotubes double-labeled with anti-MyoD1 (A and B) and either a monoclonal antibody to myosin heavy chain (MF20) (*23*) (A) or a monoclonal antibody to snRNP (anti-Sm) (C), demonstrating the variability of MyoD1 expression in the mononucleated cells in differentiating cultures, while the myotube nuclei uniformly express high levels of MyoD1. (**A**) Aza-myoblasts after 20 hours in differentiation medium. A newly formed myotube, whose nuclei all express MyoD1, is surrounded by undifferentiated mononucleated cells expressing variable levels of MyoD1 (rhodamine, MyoD1; fluorescein, myosin heavy chain). (**B** and **C**) Aza-myoblasts 20 hours in differentiation medium showing localization of anti-MyoD1 (B) and anti-Sm (C). Clustered MyoD1 positive nuclei are in early myotubes. Arrows indicate nuclei with low levels of MyoD1. Cells were fixed for 20 minutes in 1 percent paraformaldehyde and permeabilized in PBS with 0.25 percent Triton X-100. Secondary antibodies specifically generated for double-labeling were used (Jackson Laboratory).

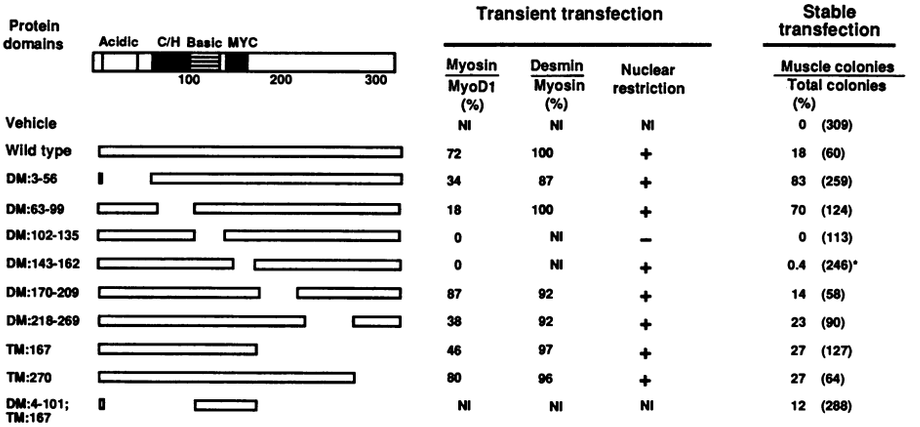

Fig. 5. The MyoD1 expression vector and each of the mutated MyoD1 expression vectors were transfected into 10T1/2 cells and assayed for MyoD1 expression and myogenic activity. For the transient transfections cultures were double-stained with a monoclonal antibody to myosin heavy chain (MF-20) and either anti-MyoD1 or antibody to desmin (anti-desmin) (*24*), as described in the legend to Fig. 6. The domains of the MyoD1 protein are the acidic region (amino acids 1 to 60); the cysteine and histidine rich region (amino acids 62 to 101); the basic region (amino acids 102 to 124); the Myc similarity region (amino acids 141 to 162); and the COOH-terminal region (amino acids 163 to 318). Deletion numbers refer to amino acid residues deleted, inclusive. DM, deletion mutant; TM, truncation mutant; myosin/MyoD1, percentage of MyoD1-positive cells that contain myosin heavy chain; desmin/myosin, percentage of myosin heavy chain–positive cells that also contain desmin; nuclear restriction, + indicates restriction of MyoD1 immunoreactivity to the nucleus and – indicates both cytoplasmic and nuclear immunoreactivity; muscle colonies/total colonies, percentage of total colonies showing myogenesis, parenthesis shows total number of colonies; NI, no immunoreactivity seen with anti-MyoD1, anti-myosin, or anti-desmin, as indicated; *, one colony of 500 to 1000 cells contained 28 myosin-positive cells in a sectored distribution that is suggestive of spontaneous myogenic conversion of 10T1/2 cells (*4*).

cytoplasmic during mitosis. It is possible, however, that a small amount of MyoD1 protein is specifically bound to a subset of nuclear sites even during mitosis.

In the replicating myoblast population, there is a significant variability in the intensity of the nuclear MyoD1 immunoreactivity. An apparently continuous gradient of MyoD1 immunoreactivity exists with some cells staining relatively intensely, most cells in a mid-range, and some with no detectable MyoD1 immunoreactivity (see Fig. 3, A, B, and E). The same degree of variability is seen in every subclone analyzed. When myoblasts are switched from growth medium—Dulbecco's modified essential medium (DMEM) plus 15 percent fetal calf serum—to differentiation medium (DMEM plus 2 percent horse serum), the variability of MyoD1 expression becomes more extreme and the overall effect is a loss of the middle range of expression (Fig. 3F).

When aza-myoblasts or C2C12 myoblasts are cultured in differentiation medium at sufficiently high cell density, many of the myoblasts fuse to form myotubes. We examined MyoD1 immunoreactivity in the myotube nuclei and in the mononucleated cells that fail to differentiate. Myoblasts were grown to confluence in growth medium and cultures were processed for immunohistochemistry at varying times after the medium was changed to differentiation medium. At both the earliest time point (20 hours, when myotubes have only a few nuclei) (Fig. 4, A and B) and at the latest time point examined (6 days after the switch to differentiation medium) all of the nuclei in the myotubes show relatively uniform MyoD1 immunoreactivity. In contrast, the myosin negative mononucleated cells still present in these cultures continue to demonstrate significant variability in MyoD1 expression at all times. (Nuclei with low levels of MyoD1 are indicated by arrows in Fig. 4B.)

One possible explanation for the variation in staining would be variable penetrance of antibodies into the nuclei. Therefore we double-labeled aza-myoblasts and C2C12 myoblasts with antisera to MyoD1 and antibodies to either small nuclear ribonucleoproteins (snRNP), DNA, histone H2b, or histone H3 (*13*). While the MyoD1 immunoreactivity demonstrates significant cell to cell variation (Fig. 4B), the antibodies to the non-MyoD1 nuclear elements show a fairly uniform staining of the myoblast nuclei (as shown for antibodies to snRNP in Fig. 4C).

Site-directed deletional mutagenesis of MyoD1. We have demonstrated that MyoD1 is a nuclear protein that is modified by phosphorylation. We have shown previously that MyoD1 confers a myogenic potential in several different fibroblast cell lines (*1*). Presumably, specific domains of the MyoD1 protein interact with other cellular elements to confer the properties of nuclear localization and the potential to initiate myogenesis. In order to identify functionally significant domains of the MyoD1 protein, we undertook a site-directed deletional analysis of the MyoD1 protein. The sequence of the MyoD1 cDNA contains several regions of interest regarding potential functional domains (see Fig. 5): the amino

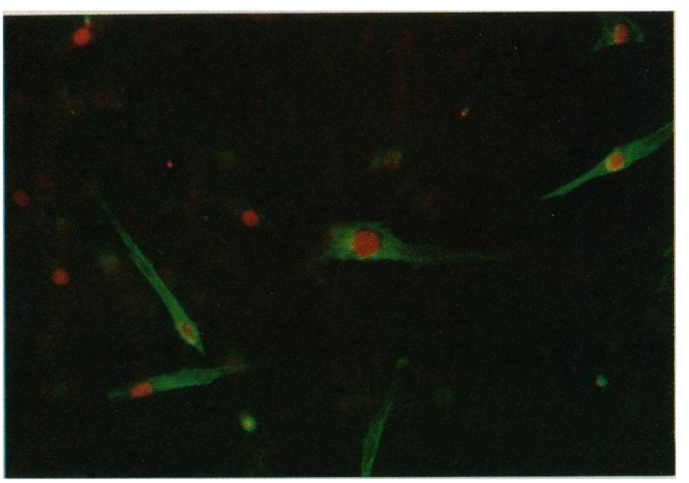

Fig. 6. Transfection of the MyoD1 expression vector into 10T1/2 cells activates myosin heavy chain expression in a transient assay. In this field, several cells contain MyoD1 protein in their nucleus (rhodamine) and a subset of these cells also are expressing myosin heavy chain (fluorescein). Approximately 2 to 5 percent of cells in cultures transfected with the MyoD1 expression vehicle show MyoD1 immunoreactivity. Parallel cultures transfected with the expression vehicle lacking the MyoD1 insert, as a control, do not contain any cells with either MyoD1 or myosin heavy chain immunoreactivity. Subconfluent cultures of 10T1/2 cells were transfected with 5 μg of the MyoD1 expression vector as calcium phosphate precipitate in DMEM containing 10 percent fetal calf serum and 30 μ*M* chloroquine. After 18 hours the media was replaced with serum-free DMEM containing insulin (10 μg/ml) and transferrin (5 μg/ml). Two days later, cells were fixed with 2 percent paraformaldehyde in PBS for 10 minutes, permeabilized in PBS with 0.25 percent Triton X-100 for 10 minutes, and double-labeled with anti-MyoD1 and a monoclonal antibody to myosin heavy chain (MF-20). A rhodamine conjugated donkey antiserum to rabbit IgG was used to localize anti-MyoD1 and a fluorescein-conjugated goat antiserum to mouse IgG was used to localize the MF-20 monoclonal antibody.

Table 1. Chromosome mapping of the MyoD1 gene in the murine and human genomes. Hybrid cell line DNA's were probed with the MyoD1 cDNA. The cell lines ABM-31, ABM-30, ABM-28, and 17T-1 were mouse-hamster hybrids, and the cell lines FF4-3a, F(8.14)Q, A4-3a, F(2.8)D, F(7.8)A, and F(11)Y were mouse-rat hybrids. The HDM series clones were human-mouse hybrids. The presence or absence of mouse chromosome 7 was concordant with the presence or absence of the MyoD1 diagnostic band. The MyoD1 sequences also map to the homologous human chromosome, 11. The murine and human chromosome complement of the hybrid cell lines was determined by karyotyping and by DNA markers.

Source of DNA	Chromosome complement	MyoD1
	Mouse	
FF4-3a	8, 9, 10, 12, 13, 14, 15, 16, 18, 19	−
ABM-31	2, 6, 7, 9, 12, 14, 15, 17	+
ABM-30	2, 6, 7, 9, 10, 12, 15, 16	+
F(8.14)Q	1, 8, 12, 13, 14	−
ABM-28	2, 4, 17	−
A4-3a	9, 14, 15	−
F(2.8)D	2, 8	−
F(7.8)A	7, 8	+
17T-1	8, 17	−
F(11)Y	9, 11	−
	Human	
HDM-4	4, 20	−
HDM-5	14	−
HDM-9	14, 20, 21	−
HDM-15	4, 11, 21	+
HDM-18	4, 11	+
HDM-20	4, 7	−

terminus is highly enriched in acidic residues; several clustered histidines and cysteines are present between amino acids 62 and 101; a highly basic region is present between residues 102 and 124; and a short, 22-residue segment between amino acids 141 and 162 shows a strong similarity to a conserved segment in mouse, chicken, and human c-Myc, chicken v-Myc, and mouse N-Myc and L-Myc proteins, as well as to the predicted protein products of four transcription units in the *Drosophila achaete-scute* complex (*1, 8*).

Specific in-frame, oligonucleotide-directed deletions were made in a MyoD1 cDNA expression vehicle (*14*) and both a transient transfection assay and stable transfected polyclones were analyzed to determine the myogenic competence of each mutant. In the transient assay the wild-type MyoD1 expression vehicle induced expression of myosin heavy chain and desmin in approximately 72 percent of the MyoD1 positive 10T1/2 cells (Fig. 5). An example of such a transient assay is shown in Fig. 6. For each of the mutants, the percentage of MyoD1-positive cells that have activated myosin heavy chain and desmin expression is shown in Fig. 5.

As demonstrated by deletion mutant DM:3–56 and truncation mutant TM:167, remarkably large regions of the MyoD1 protein can be deleted from either the NH_2-terminal end or the carboxyl-terminal end without interfering with either myogenic activity or nuclear localization. Only two regions appear necessary for myogenic activity. Deletion of the region of similarity to Myc and the proteins of the *achaete-scute* complex (DM:143–162) completely eliminates myogenic activity but does not alter nuclear localization. A second, highly basic, region (DM:102–135) appears to be necessary both for nuclear partitioning of the MyoD1 protein and for myogenic activity. In this case, MyoD1 immunoreactivity is present in both the cytoplasmic and nuclear compartments, making it doubtful that the failure to activate the myogenic program is merely a consequence of the loss of nuclear partitioning. While other regions were not demonstrated to be necessary for myogenic activity, deletion of the cysteine- and histidine-rich domain (DM:63–99), while leading to a clear myogenic signal, did result in a substantial reduction of the percentage of transfected cells expressing myosin heavy chain. One unexplained finding in this set of experiments is that the frequency of MyoD1-positive cells after transfection with the two nonmyogenic deletion mutants (DM:102–135 and DM:143–162) was approximately 10 to 50 times lower when compared to wild type. Since these two deletion mutant expression vectors were highly efficient in producing stable transformants of 10T1/2 cells expressing the mutant MyoD1 protein with the same nonmyogenic phenotype (see below), we have not pursued an explanation for their reduced penetrance in the transient assay system.

A second analysis of the activity of the mutants was performed in stable polyclones of transfected 10T1/2 cells. We cotransfected 10T1/2 cells with pCMVneo (*15*) and either the wild-type or a mutant MyoD1 expression vehicle and then selected for resistance to G418. All of the mutants that showed myogenic activity in the transient assay gave rise to myogenic clones, as determined by fusion and expression of myosin heavy chain (Fig. 5). Colonies derived by transfection with deletion mutants DM:102–135 and DM:143–162 did not show significant amounts of myogenesis although they did demonstrate significant MyoD1 immunoreactivity, supporting the results from the transient assays that these two domains are necessary for MyoD1 myogenic activity. It is not clear whether the very low level of myogenesis seen after transfection with DM:143–162 represents a cDNA-mediated event or a spontaneous myogenic conversion (see legend to Fig. 5).

Since the above results indicated that the basic and Myc similarity regions were necessary for myogenesis, we undertook to determine if, within the context or our assay system, expression of these two regions would be *sufficient* to convert 10T1/2 cells to myoblasts. A deletion mutant expression vehicle coding for a 68–amino acid polypeptide containing the basic and Myc homology region was constructed (DM:4–101;TM:167) and analyzed for myogenic activity in both transient and stable transfection assays (Fig. 5). In the transient assay, a few cells were very faintly fluorescent for MyoD1 and myosin, but the amount of fluorescence was not sufficiently above background to be definitely scored as either MyoD1 immunoreactivity or myogenic conversion. In contrast, expression of this 68–amino acid region in stable transfections is sufficient for myogenic conversion at a frequency similar to that of the wild-type protein (Fig. 5).

Chromosomal assignment of the MyoD1 gene. To establish the genetic localization of the MyoD1 gene within the murine and human genome and to determine whether this assignment correlates with any other previously defined myogenic locus, we probed DNA from a series of mouse × hamster or mouse × rat somatic cell hybrids with a 1.3-kb fragment of the MyoD1 cDNA. Hind III restriction fragments of 10.7 kb and 2.6 kb were detected in the mouse control DNA. Cross-hybridizing bands of 12.0 kb and 2.1 kb were present in the rat genome and a single band of 1.5 kb was present in hamster control DNA. Scoring the hybrid cell lines for the mouse-specific MyoD1 fragments assigns this genomic locus to mouse chromosome 7 (Table 1). There are no discordancies between the presence or absence of chromosome 7 in the hybrids and the presence or absence of the murine restriction fragments.

Comparative mapping of mouse and human genomic sequences has revealed conservation of syntenic linkage association between mouse chromosome 7 and human chromosome 11. Therefore, we analyzed six human × mouse somatic cell hybrid clones, two of which contained human chromosome 11, for retention of human-specific MyoD1 restriction fragments. The MyoD1 probe hybridized with a 6.4-kb Hind III fragment in human control DNA. Scoring these hybrid cell lines for the human-specific fragment assigns this locus to human chromosome 11 (Table 1). Children

with Beckwith-Wiedeman syndrome have a greatly increased risk for the development of three embryonal tumors—hepatoblastoma, rhabdomyosarcoma, and Wilm's tumor. This syndrome has been associated with the somatic development of homozygosity for loci residing on chromosome 11 (*16*). It is interesting that MyoD1 and a locus involved in the development of rhabdomyosarcomas both map to human chromosome 11.

MyoD1: A master regulatory protein with multiple levels of control. MyoD1 is a nuclear phosphoprotein whose expression is restricted to proliferating myoblasts and differentiated myotubes. While MyoD1 activates lineage markers (MyoA and MyoH) (*1*) in replicating myoblasts, these cells do not activate muscle structural genes until growth factors are withdrawn or depleted. In this regard, the "activity" of MyoD1 is at least partially regulated by exogenous factors, possibly through phosphorylation and possibly by interactions with serum induced proteins, including c-fos or c-Myc. Not only does the activity of the MyoD1 protein seem to be controlled, but once the gene is developmentally or experimentally activated, the amount of MyoD1 protein also seems to be controlled since individual myoblast nuclei, but not myotube nuclei, vary in their MyoD1 immunoreactivity. Our initial experiments suggest several factors that affect the quantitative variation in amounts of MyoD1: exogenous mitogens, cell density, and, possibly, position in the cell cycle. Tight control over the amount of MyoD1 and its activity may be required to ensure that myoblasts fuse only in the G1 period of the cell cycle. An extension of this rationale would also explain why serum withdrawal, which prevents entry into S phase, is required for activation of the terminal myogenic program.

Deletional analysis of MyoD1 demonstrates that of the recognizable motifs in the protein—the acidic region, the cysteine:histidine region, the basic region, the Myc similarity region, and the COOH-terminal region—only the Myc similarity region and the basic region seem to be necessary for conversion of 10T1/2 cells to myoblasts. In fact, expression of only 68 amino acids of MyoD1, containing the basic and Myc similarity domains, is sufficient to activate myogenesis in stably transfected 10T1/2 cells. We assume, although cannot yet prove, that many of the domains that are not necessary for myogenesis serve important regulatory functions that are not easily assessed in our assay system.

Recent data (*17*) indicates that the MyoD1 protein expressed from a viral LTR can activate the endogenous gene in stably transfected 10T1/2 cells. Whether a similar activation of the endogenous gene occurs during the time course of a transient transfection assay is not clear. While this consideration must temper our discussion of the mechanism by which the functional deletion mutants activate myogenesis, it does not alter our conclusion that the region of Myc similarity and the basic region are necessary and sufficient for myogenesis within the context of our assay system.

MyoD1, Myc, and the proteins of the *achaete-scute* complex regulate different aspects of cell determination or differentiation (or both), yet all three contain a similar 22–amino acid sequence. This region is necessary for the myogenic activity of MyoD1 and is within a region of Myc known to be important for two-step transformation (*8*). While the molecular function of this conserved region remains unknown, it does not seem unreasonable to suggest that other proteins with similar areas of homology may regulate determination and differentiation in other lineages or modulate differentiation within the muscle lineage. For example, both C2C12 cells and the rat myoblast line L6 express a protein during differentiation, mdf1 (previously BU65) (*18*), which contains 73 amino acids with 75 percent identity to a corresponding region of MyoD1 that includes both the basic and the Myc similarity domains (*19*). It is possible that mdf1 may either participate in a parallel myogenic pathway or modulate a MyoD1-dependent pathway. The conservation of the basic and Myc similarity domains between these two proteins emphasizes the potential importance of these regions in effecting DNA or protein interactions (or both). We have some data showing that MyoD1 is a sequence-specific DNA binding protein (*17*).

MyoD1 protein activates the expression of lineage markers (MyoA and MyoH) in proliferating myoblasts, as well as muscle structural genes in differentiating myocytes (*1*), behaving as if it were a master regulatory gene for muscle determination and differentiation. RNA analysis of various fetal and adult mouse tissues has revealed that MyoD1 RNA is expressed only in skeletal muscle (*1*). This has recently been confirmed by in situ hybridization studies in developing mouse embryos (*20*). Because the expression of MyoD1 is apparently restricted to skeletal muscle, it is unlikely that MyoD1 participates in a combinatorial network, such as has been postulated for the *Drosophila* segmentation and homeotic genes (*21*), to effect the differentiation of cell types other than skeletal muscle. It is possible, however, that such a combinatorial system, established during the very early stages of embryogenesis, converges to activate the MyoD1 gene, which, in turn, participates in a divergent pathway to activate the entire myogenic program. Furthermore, since MyoD1 is able to initiate myogenesis not only in fibroblast and adipoblast cell lines but also in some nonmesodermal cell types (*22*), it is not unreasonable to consider whether, in coordination with constitutive factors present in many cell types, MyoD1 alone may be capable of activating the myogenic program. These questions are now experimentally approachable.

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9. Fusion proteins containing MyoD1 coding sequences linked to trpE gene product sequences were expressed in *E. coli*, with the use of pATH vectors [for example, C. L. Dieckmann and A. Tzagoloff, *J. Biol. Chem.* **266**, 1513 (1985)]. A Pst I fragment (nucleotides 640 to 1082), a Msp I fragment (nucleotides 337 to 820), and an Alu I fragment (nucleotides 168 to 1203) of the MyoD1 cDNA were isolated and ligated to Pst I cut pATH 11, Cla I cut pATH 10, and Sma I cut pATH 10, respectively. These fragments fuse MyoD1 major open reading frame residues 160 to 307, 60 to 219 and 3 to 318 (termination) in-frame to the trpE polypeptide fragment. Prospective clones in *E. coli* HB101 were analyzed by restriction digest analysis, and correct ones were transferred to *E. coli* C600. The restriction site for the Msp I and Alu I fragments were not regenerated so that the cloning junctions were directly confirmed by sequencing. Small-scale fusion protein inductions revealed induced proteins of the expected sizes on SDS-PAGE gels.
10. Fusion proteins were induced as described [K. R. Spindler *et al.*, *J. Virol.* **49**, 132 (1984)], except that the final concentration of 3β-indoleacrylic acid was 20 μg/ml and induced cultures were harvested after 6 hours. Cells were centrifuged at 6000 rpm for 10 minutes and stored as frozen cell pellets at −20°C. For fusion protein purification, frozen cell pellets were thawed, solubilized in SDS gel sample buffer (about 5 ml per 100 ml of culture), sonicated, heated to 90°C for 20 minutes and centrifuged at 10,000*g* for 5 minutes, and the supernatant was transferred to a preparative 10 percent SDS-PAGE gel. The region of the gel containing the fusion protein was determined by fixing and staining side lanes containing molecular markers. For immunizations, polyacrylamide gel slices containing trpE-MyoD1 fusion proteins were finely chopped with a razor blade, placed in a sterile 5-ml syringe, mixed 1:1 with incomplete Freund's adjuvant, and serially passaged through 16-, 18-, and 21-gauge needles. New Zealand White female rabbits were injected subcutaneously at several sites along the back. The first immunization contained about 400 μg of fusion protein per rabbit, followed by three booster injections 3 to 4 weeks apart, each containing about 200 μg of fusion protein per rabbit. Further boosts were 2 to 3 months apart, containing about 100 μg of fusion protein per rabbit. Rabbits were bled 7 to 10 days after each boost.
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13. Antibodies to histone were provided by B. David Stollar. Antibodies to DNA and to snRNP were provided by J. Steitz.
14. Site directed mutagenesis of the MyoD1 cDNA was done directly in the expression vector, pEMSVscribe, previously used for transfection and expression of MyoD1 (*1*). Since this vector is a Bluescribe (Stratagene) derivative, it can be grown as a single-stranded vector in *E. coli* TG1, with the use of the M13 K07 helper phage. Selected in-frame deletions (described above) were generated as follows. Oligonucleotides (30 to 36 bp) were synthesized to contain sequences evenly flanking the desired deletion. In addition, two COOH-terminal sequence truncation mutants were generated from smaller oligonucleotides synthesized to create termination codons. These oligonucleotides were used as suggested with the Amersham oligonucleotide-directed mutagenesis kit, which is based on a mutant strand selection step [J. W. Taylor *et al.*, *Nucleic Acids Res.* **13**, 8765 (1985)]. In vitro synthesized mutant plasmids were transformed into *E. coli*. For each in-frame deletion, the newly generated junction resulted in a diagnostic restriction enzyme site which was used to analyze randomly picked clones by restriction digests. Clones that appeared correct were then used to prepare single-stranded DNA for sequencing with the use of the T7 Sequenase kit (U.S. Biochemical Corp.) and [α^{35}S]thio-dATP (1000 Ci/mmol; NEN). Two to three random clones of prospective COOH-terminal truncations were picked and sequenced directly.
15. 10T1/2 cells were transfected with 0.2 μg of pCMVneo [M. Lineal, *Cell* **49**, 93 (1987)], 4.0 μg of pEMSVscribe, pEMSVscribe-MyoD1, or pEMSVscribe-MyoD1 (mutant), and 10.0 μg of 10T1/2 large molecular size carrier DNA as a calcium phosphate precipitate (M. Wigler *et al. Proc. Natl. Acad. Sci. U.S.A.* **76**, 1373 (1979).
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24. The antibody to desmin was provided by H. Holtzer.
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