Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD

(muscle regulatory gene/MyoD retrovirus)

HAROLD WEINTRAUB, STEPHEN J. TAPSCOTT, ROBERT L. DAVIS, MATHEW J. THAYER, MOHAMMED A. ADAM, ANDREW B. LASSAR, AND A. DUSTY MILLER

Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, WA 98104

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ABSTRACT MyoD is a master regulatory gene for myogenesis. Under the control of a retroviral long terminal repeat, MyoD was expressed in a variety of differentiated cell types by using either a DNA transfection vector or a retrovirus. Expression of muscle-specific proteins was observed in chicken, human, and rat primary fibroblasts and in differentiated melanoma, neuroblastoma, liver, and adipocyte lines. The ability of MyoD to activate muscle genes in a variety of differentiated cell lines suggests that no additional tissue-specific factors other than MyoD are needed to activate the downstream program for terminal muscle differentiation or that, if such factors exist, they are themselves activated by MyoD expression.

MyoD is a master regulatory gene for skeletal myogenesis. It is expressed only in skeletal muscle, and, when transfected into a variety of fibroblast or adipoblast cell lines, it converts these cells to muscle (1-4). The MyoD protein is a nuclear protein that contains a region of ~60 amino acid residues homologous to the c-myc family of proteins (see ref. 5). This region is both necessary and sufficient for conversion of C3H/10T½ (10T½) fibroblasts to muscle (3). MyoD is a DNA-binding protein that binds to the enhancer sequence of the muscle-specific creatine phosphokinase (M-CPK) gene. Preliminary data suggest that it also binds to the enhancers for a number of other muscle-specific genes (6). The 60-residue myc homology region is both necessary and sufficient for this specific DNA binding (6). It has also been shown that MyoD activates its own transcription (7). Autoactivation of MyoD could provide either a positive feedback loop to keep cells committed to myogenesis once MyoD is activated or a mechanism to increase MyoD levels once the gene is activated by upstream factors. Two different genes, myogenin and Myf-5, both with a high degree of homology to MyoD, particularly throughout the myc region, can convert 10T½ cells to muscle (8, 9). The biological relationships between these genes as well as a fourth gene, myd (10), are presently not clear.

Here we explore the potential of MyoD to activate muscle markers in primary cells and in differentiated tissue culture cell lines. The results demonstrate that a variety of cell types (melanoma, neuroblastoma, liver, and adipocyte) can be induced to express muscle markers by MyoD, which supports the notion that MyoD is a master regulatory locus and also suggests that in these circumstances the activation of the muscle program does not require additional tissue-specific factors other than MyoD.

Abbreviations: M-CPK, muscle-specific creatine phosphokinase; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; MHC, myosin heavy chain.

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RESULTS

Conversion of Primary Fibroblast Cells to Muscle by MyoD. MyoD can convert a variety of stable fibroblast lines to muscle (e.g., 10T½, Swiss 3T3, NIH 3T3, L cells, BALB 3T3, etc.) (2). We first tested whether MyoD could convert primary “nontransformed” fibroblasts to muscle. A population of dermal fibroblasts from chicken embryos was transfected with an LTR-driven MyoD cDNA expression vector (2). These cells were converted to elongated, sometimes multinucleated, MHC-positive (Fig. 2A), desmin-positive cells (Fig. 2B) at a frequency consistent with that generally obtained with a transient assay (3). In parallel cultures transfected with a control vector lacking MyoD, small numbers of faintly desmin-positive cells were seen, but none of these stained for MHC and none displayed the elongated, myosin- and desmin-positive phenotype seen in the LTR-MyoD transfected cultures.

Because the frequency of conversion is low in transient assays and because chicken cells do not yield stable clones, we decided to extend these conclusions by using a MyoD-encoding amphotropic retrovirus (LMDSN) in which the neo gene is transcribed from an internal simian virus 40 early promoter–enhancer (15) and MyoD is expressed from the viral LTR (see Experimental Procedures) (Fig. 1). Primary human (Fig. 2C) or rat (Fig. 2D) fibroblasts were infected with the virus and then transferred to serum-free medium to induce muscle. After 3 days, both types of cells become elongated, and the rat cells begin to fuse and form large multinucleated myotubes (Fig. 2D Upper). Often up to 50% or more of the rat cell nuclei in these cultures are in myotubes, depending on the multiplicity of infection. In contrast to rat cells, the human cells fused poorly following infection. However, both infected rat and human cells (Fig. 2C) stained intensely for MHC and desmin, with as many as 50% of the cells being positive for both markers. Although neither the rat nor human cells showed a background of MHC-positive cells, about 0.4% of the primary human cells were desmin positive (but MHC negative) in the absence of infection. For both rat and human cells, expression of muscle markers was dependent on induction by serum withdrawal, indicating that the induction process is not cell type specific (see below as well). Infection with a virus that only expresses neo or with an antisense MyoD virus (LDMSN) did not induce myogenesis in primary rat (Fig. 2D Lower) and human cells. These experiments clearly demonstrate that MyoD induces muscle gene expression in primary chicken, rat, and human cells.

Muscle Markers Can Be Activated in Differentiated Cell Lines by MyoD. We previously demonstrated that, besides fibroblasts, three adipocyte lines will convert to muscle when transfected with the MyoD expression vector (2). Because fibroblasts and muscle can be derived from a common cell (18) and because adipocytes, muscle, and chondrocytes can all be derived from 10T½ cells (19) and all of these cells are mesodermal in origin, we decided to ask whether differentiated cell types derived from ectodermal and endodermal germ layers could convert to muscle.

By using either DNA transfection or viral infection, both B16 (Fig. 3 A and B) and B78 (data not shown) mouse melanoma cell lines as well as a rat neuroblastoma cell line...
(B50 clone 5) (Fig. 3D) (20) convert to muscle as assayed by simultaneous staining of individual cells with MHC and desmin antibodies. Both of these cell types are derived from ectoderm. BNL liver cells (derived from endoderm) also activate MHC and desmin (Fig. 3C). In experiments of others, baby hamster kidney cells can be converted to muscle by transfection with LTR-MyoD (R. Palmieri and S. Hauschka, personal communication), and rat smooth muscle cells can be converted to skeletal muscle by MyoD virus infection (J. Medina and S. Schwartz, personal communication); P19 embryonal carcinoma cells also differentiate into skeletal muscle in the absence of chemical inducers of myogenesis after MyoD transfection (unpublished observations). We conclude that forced expression of MyoD can activate markers indicative of muscle differentiation in cell lines that represent derivatives of each of the three germ layers.

For rat neuroblastoma and for B16 melanoma cells, 10–20% of the cell population will activate both MHC and desmin 3 days after retrovirus infection and subsequent induction of the muscle phenotype by growth in low serum. For both of these cell types, expression of MHC and myosin light chain 2 is dependent on serum withdrawal (see Fig. 4B), indicating that the signaling system for this aspect of muscle induction is not cell type specific. G418-resistant clones of MyoD-positive, MHC-positive neuroblast or B16 melanoma cells were propagated. Northern analysis of early passage polyclones (Fig. 4 A and B) demonstrates that after induction by serum withdrawal, MHC and myosin light chain 2 genes are expressed. In addition, two cDNA markers, MyoA and MyoH (2), which are induced by MyoD in proliferating myoblasts in the presence of serum, are also expressed in the MyoD-activated neuroblastoma and melanoma cell lines in the presence of serum (Fig. 4 A and D). Exogenous MyoD also activates endogenous MyoD in some (Fig. 4C, lane 2), but not all, transfected or infected cell types (7). With MyoD retrovirus-infected melanoma or neuroblastoma cells, activation of the endogenous MyoD could not be detected (Fig. 4C). With continued passage, these melanoma and neuroblastoma clones lost their ability to produce MyoD as assayed by immunostaining and concomitantly lost their ability to activate muscle markers; however, they remained G418 resistant. We do not know the basis for this effect and, in particular, whether it is a selection for cells that happen to turn off MyoD (MyoD is known to inhibit colony formation in 10% transfecants; ref. 2) or whether these differentiated cells have an active mechanism for inhibiting MyoD expression. We also do not know whether loss of MyoD is also accompanied by loss of MyoD DNA or RNA or neither. In this regard, we have also transfected and infected MyoD into a number of other cell types (Ca-Co2, colon carcinoma; GH3, rat pituitary; MEL, murine erythroleukemia; and P388, mouse macrophage), and in each case forced expression of MyoD from the retroviralLTR was not observed.

Expression of Differentiation Markers in Single Cells. In long-term cultures and in (metastable) clones, most B16 melanoma cells remain pigmented, and about half express MHC when induced. At the single cell level, over 90% of MHC-positive cells also contain large numbers of pigment granules (Fig. 5 A and B). Because the pigment cell markers might be quite stable, this type of assay does not address whether both programs are actively being expressed at the transcriptional level. However, in cultures that are not induced for myogenesis, all cells are pigmented, and by Northern analysis, considerable levels of the MyoD-induced myoblast markers, MyoA and MyoH, are expressed (Fig. 4D). After induction of MyoD-activated rat neuroblasts with cAMP (for neuroblast differentiation) and low serum (for muscle differentiation), most cells send out axon-like pro-

![Fig. 4. Expression of muscle-specific RNA in differentiated cell lines converted by MyoD. MHC and myosin light chain 2 (MLC) are induced markers for differentiated muscle. MyoA, MyoH, and MyoD are markers present in proliferating myoblasts as well as in induced myotubes. v-MyoD is the RNA product from the retrovirus LMDSN. (A) Activation of MHC and MLC by LMDSN after serum withdrawal. F3, azayoblasts; NB, neuroblastoma B-30 cells; B16, melanoma cells; NB-V-MD, neuroblastoma polyclones infected with the viral MyoD retrovirus; B16-V-ML, melanoma polyclones infected with viral MyoD. (B) Induction of MHC and MLC by serum withdrawal. M, growing cells; D, cells incubated in differentiation medium; NB-MD and B16-MD, polyclones derived from infection with the MyoD retrovirus. (C) Endogenous MyoD is not activated in melanoma or neuroblastoma cells. 10% serum was replaced with LMDSN, and the RNA was prepared after 3 days of induction in serum-free medium. Both the exogenous (v-MyoD) and the endogenous (MyoD) gene products are observed. (D) Activation of the myoblast markers MyoA and MyoH in growing (G) B16-V-MD and NB-V-MD cells. Eth Br, ethidium bromide.](image-url)
functions (22, 23). We found it committed we myocytes programs from both (26, 27). MyoD hepatoma cells of MyoD either fat types cell muscle. Expression of MHC. The failure of M-CPK-CAT with MyoD, and CV1 cells. MyoD and B16 melanomas, B16 myoblasts, or B-30 neuroblastoma cells. Coexpression of MHC. The failure of M-CPK-CAT with MyoD, and cells that do not convert to muscle do not trans-activate M-CPK-CAT with MyoD.

Three cell lines tested express MyoD but fail to activate MHC or desmin. These lines are CV1 (an African green monkey kidney-derived line), HeLa (human cervical carcinoma), and HepG2 (human hepatoma). Attempts to activate stable MyoD-expressing CV1 lines by treatment with azacytidine or butyrate or both were not successful. MyoD-expressing CV1 cells grow slowly, elongate, and become multinucleate (properties of MyoD-transfected fibroblasts); however, they did not express any of the tested molecular markers for myogenesis.

**Trans-Activation by MyoD.** A construction containing the 5' controlling region (3.3 kilobases of upstream sequence) of the M-CPK gene driving CAT expression (12) was used as a target for trans-activation by LTR-driven MyoD. Cotransfection of the two plasmids into 10T½ cells followed by muscle cell induction in serum-free medium resulted in high levels of CAT expression (Fig. 6A). M-CPK-CAT alone or with a control vector DNA gave no detectable activity. Coexpression of MyoD also activated a desmin-CAT construction (13) (Fig. 6A). SV2-CAT was also activated, but only 2-3 fold (data not shown). Activation of the enhancerless SV1-CAT plasmid could not be detected (data not shown). These results show that in the context of transfected 10T½ cells, MyoD can trans-activate, either directly or indirectly, expression from the M-CPK- or desmin-controlling region.

To test whether other cell types were also permissive for MyoD trans-activation, these same protocols were used to transfect B16 melanoma cells and rat neuroblastoma cells. In both cases, activation by MyoD was observed (Fig. 6B). These results suggest that if additional regulatory components are needed for this activity, these cells as well as 10T½ cells seem to express them.

In contrast, trans-activation of M-CPK-CAT by cotransfection with MyoD was not observed in HeLa or CV1 cells (Fig. 6C), cells that do not convert readily to muscle after forced expression of MyoD. Thus, cell types that can be converted to muscle trans-activate M-CPK-CAT with MyoD, and cells that do not convert to muscle do not trans-activate M-CPK-CAT with MyoD.

The failure of HeLa and CV1 to support MyoD-mediated activation by these two assays could reflect an absence of an essential positive factor or the presence of a negative factor. The putative negative factor could act in cis at sites in the M-CPK-controlling region or in trans, either directly on the MyoD protein or with an element that interacts with MyoD.
To further explore the reasons for the failure of CV1 cells to activate myogenesis, a MyoD-expressing CV1 line (CV1-MD) was fused to 10T½ cells. CV1-MD cells express MyoD, elongate when serum is removed, contain a high level of multinucleated cells, and grow slowly. They do not express MHC or a number of other muscle markers as assayed by Northern analysis. 10T½ cells convert to muscle at high frequency after infection with a MyoD retrovirus. The two cell types (CV1-MD and 10T½) were fused to form heterokaryons and then induced for muscle by growth in serum-free medium. Three days later they were stained for MHC and desmin, and mixed heterokaryons were scored for muscle markers. The results clearly showed activation of MHC and desmin in heterokaryons. As controls, fusion of CV1-MD cells with CV1 cells or CV1 cells with 10T½ cells or HeLa cells failed to activate muscle markers. These results suggest that the failure to activate the myogenic program is not because CV1 cells express a dominantly acting negative signal; instead, it is possible that either 10T½ cells are supplying a positive factor missing in CV1-MD cells or that 10T½ cells are supplying a factor needed to turn off a negative factor expressed in CV1 cells.

It is now clear that a number of potential oncogenes can inhibit myogenesis and that indeed in two cases—fos and ras—there is a decrease in MyoD expression (29). Cotransfection of a fos expression vector with MyoD and M-CPK-CAT or des-CAT results in a decrease in MyoD-activated CAT expression (29), and it is also clear that for myogenesis removal of serum is required for terminal differentiation—a procedure known to lead to decreased fos levels.

To test whether fos might be involved in inhibiting the trans-activation of MyoD in CV1 and HeLa cells, LTR-MyoD and M-CPK-CAT were cotransfected with either an antisense fos construct (covering the first 119 bases of human fos) or, instead, a vector, with the same construct in the sense orientation containing a fos deletion (Δfos). This antisense fos construct has been used successfully to show that fos mediates some of the effects of a variety of transforming oncogenes (11). For both HeLa and CV1 cells, although attempts to activate endogenous MHC with MyoD were not successful, cotransfection with antisense fos led to a significant increase in M-Park-CAT activity that was dependent on MyoD expression (Fig. 6D). These results raise the possibility that failure to express the myogenic program in CV1 and HeLa cells might be secondary to excessive levels of fos and/or other growth-related factors. It is possible that in heterokaryons between 10T½ cells and CV1-MD cells, an inhibitory signal from CV1 cells is recessive.

**DISCUSSION**

Forced expression of MyoD can convert a large number of differentiated cell types to muscle. These include liver, melanoma, and neuroblastoma lines as well as fat and fibroblast lines. Our results are compatible with heterokaryon experiments demonstrating the activation of muscle genes in a variety of cell types fused to myotubes (30, 31). Primary chicken, rat, and human fibroblasts are also converted to muscle.

The fact that differentiated cell lines such as melanoma, neuroblastoma, fat, and liver can activate muscle-specific markers when infected or transfected by MyoD suggests that the activation of these markers by MyoD does not require additional tissue-specific factors since it would be unlikely that all of these cell types contain these factors. On the other hand, it is likely that MyoD does require additional constitutively expressed factors [and possibly MyoD-induced factors such as myogenin (7) or Myf-5] for the activation of downstream muscle markers. In addition, it is likely that tissue-restricted gene products are involved in turning on MyoD itself. Thus, in terms of cell type-specific gene expression, we view the expression of MyoD as a nodal point along the pathway to muscle cell differentiation. We postulate that the endogenous MyoD gene could be activated by specific combinations of inductive, spatial, temporal, and lineage cues that would define the time and position in the embryo (i.e., in the somites) where muscle cells should be determined. Once this temporal and positional information is established, master genes (like MyoD) would be activated to encode cell type. Confirmation of this view will require a detailed analysis of the regulation of the endogenous MyoD gene in combination with an analysis of the trans-acting elements responsible for the initial activation of MyoD during development.

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