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

(muscle regulatory gene/MyoD retrovirus)

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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.

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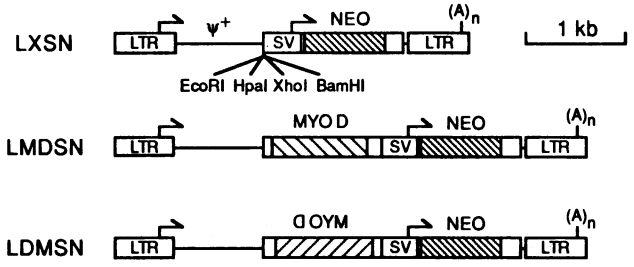

FIG. 1. Maps of the parent and MyoD retroviruses. $(A)_n$, poly(A) site; SV, simian virus 40 early promoter; NEO, neomycin phosphotransferase gene; ψ^+, the retroviral packaging signal (15); kb, kilobase. Hatching indicates protein coding regions, and arrows indicate the initiation site and direction of transcription.

EXPERIMENTAL PROCEDURES

Cells. All cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% (vol/vol) fetal calf serum. For muscle induction, cells were switched to DMEM with transferrin (10 μg/ml) and insulin (5 μg/ml) for 2 to 3 days. F3 is a myoblast clone derived from 5-azacytidine treatment of 10T½ cells (1). BNL liver cells were obtained from the American Type Culture Collection, and myogenic 3T3-L1 adipocytes were made by using the LTR-MyoD expression vector (2).

DNA Clones. LTR-MyoD (2) is a MyoD cDNA in the EMSV-scribe expression vector, which contains a Moloney murine sarcoma virus long terminal repeat (LTR) and a simian virus 40 poly(A) site. pSVΔfos contains a deletion of exons II, III, and IV that inactivates the *fos* gene; pSV-sof contains antisense fos sequences from base pair 175–292. They are described by Schöntal *et al.* (11). M-CPK-CAT contains 3300 base pairs of upstream M-CPK sequence containing an M-CPK-specific enhancer (12), Des-CAT contains 3400 base pairs of the upstream desmin-controlling region (13).

Virus Construction and Propagation. An 1156-base-pair *Rsa* I to *Xmn* I DNA fragment containing the *MyoD* coding region was excised from a cDNA clone of *MyoD* (2) and inserted into the *Hpa* I site of the murine leukemia virus-based retroviral vector pLXSN (Fig. 1). DNA clones containing the insert in either the forward (pLMDSN) or reverse (pLDMSN) orientation were isolated. Amphotropic retrovirus-producing cell lines were generated by using PA317 cells as described (14). Medium harvested after a 12-hr exposure to a confluent layer of cells producing the LMDSN or LDMSN viruses contained about 10^7 neomycin-resistant colony-forming units per ml when assayed on NIH 3T3 cells.

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

Virus-containing medium was stored at −70°C. Cells were infected by exposure to virus-containing medium overnight in the presence of Polybrene (4 μg/ml).

General Methods. Myosin heavy chain (MHC) and MyoD immunostaining (3, 16), Northern analysis (2), chloramphenicol acetyltransferase (CAT) assays (17), and transfections (3) were performed as described.

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. 2*A*), desmin-positive cells (Fig. 2*B*) 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. 2*C*) or rat (Fig. 2*D*) 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. 2*D 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. 2*C*) 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. 2*D Lower*) and human cells. These experiments clearly demonstrate that MyoD induces muscle gene expression in primary chicken, rat, and human cells.

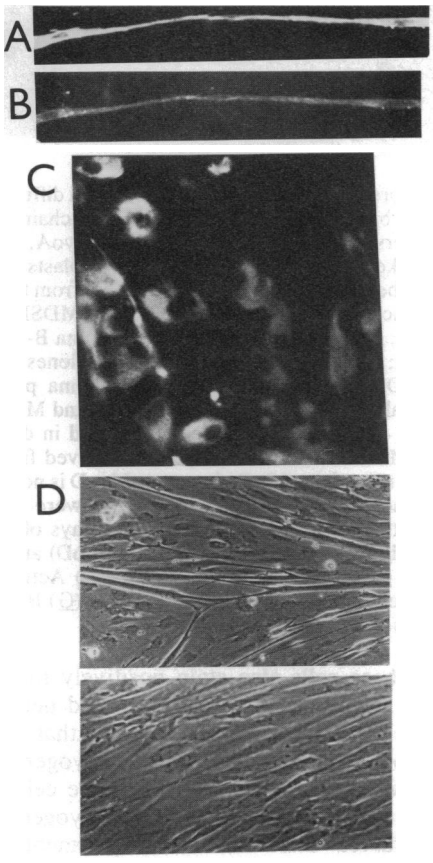

FIG. 2. Conversion of primary cells to muscle. Chicken dermal fibroblasts were transiently transfected with LTR-MyoD and stained for MHC (*A*) or desmin (*B*) after induction. (*C*) Primary human fibroblasts were infected with LMDSN, induced for muscle for 3 days, and then stained for MHC. (*D*) Primary rat fibroblasts were infected with a control virus expressing only *neo* (*Lower*) or *MyoD* virus (LMDSN) (*Upper*) and induced for muscle for 3 days. (×100.)

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

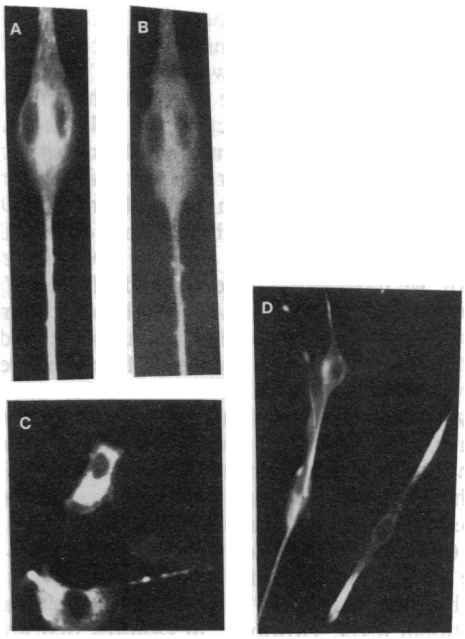

FIG. 3. Conversion of melanoma, liver, and neuroblastoma cell lines to muscle. B16 melanoma cells were transiently transfected with LTR-MyoD and stained for MHC (*A*) or desmin (*B*). (*C*) BNL liver cells were transiently transfected with LTR-MyoD and stained for MHC. MHC is used to assay myogenic conversion. (*D*) B-50 neuroblastoma cells were transfected and stained as in *C*. (×200).

(B50 clone 5) (Fig. 3*D*) (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. 3*C*). In experiments of others, baby hamster kidney cells can be converted to muscle by transfection with LTR-MyoD (R. Palmiter 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. 4*B*), 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. 4*C*, 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. 4*C*). With continued passage, these melanoma and neuroblastoma clones lost their ability to produce MyoD as assayed by immunostaining and concommitantly 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 10T½ transfectants; 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 P3881, mouse macrophage), and in each case forced expression of *MyoD* from the retroviral LTR 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. 4*D*).

After induction of *MyoD*-activated rat neuroblasts with cAMP (for neuroblast differentiation) and low serum (for muscle differentiation), most cells send out axon-like processes, and these cells also stain positively for MHC and desmin (Fig. 5*C*). Thus, for melanoma and neuroblastoma cell lines, the available evidence suggests that the endogenous developmental program and the myogenic program imposed by MyoD can coexist in the same cells. Whether pigment or neuronal programs inactivate myogenesis cannot be fully evaluated since in these experiments *MyoD* is expressed from a viral promoter. If such an inactivation occurs, it would appear to be directed at the genomic *MyoD* or at upstream genes that might control *MyoD*.

Stable, myotube-forming adipocyte lines transfected with *MyoD* (2) have been derived. When plated at clonal density and induced simultaneously for both muscle and fat, most colonies yield cells or patches of cells expressing either

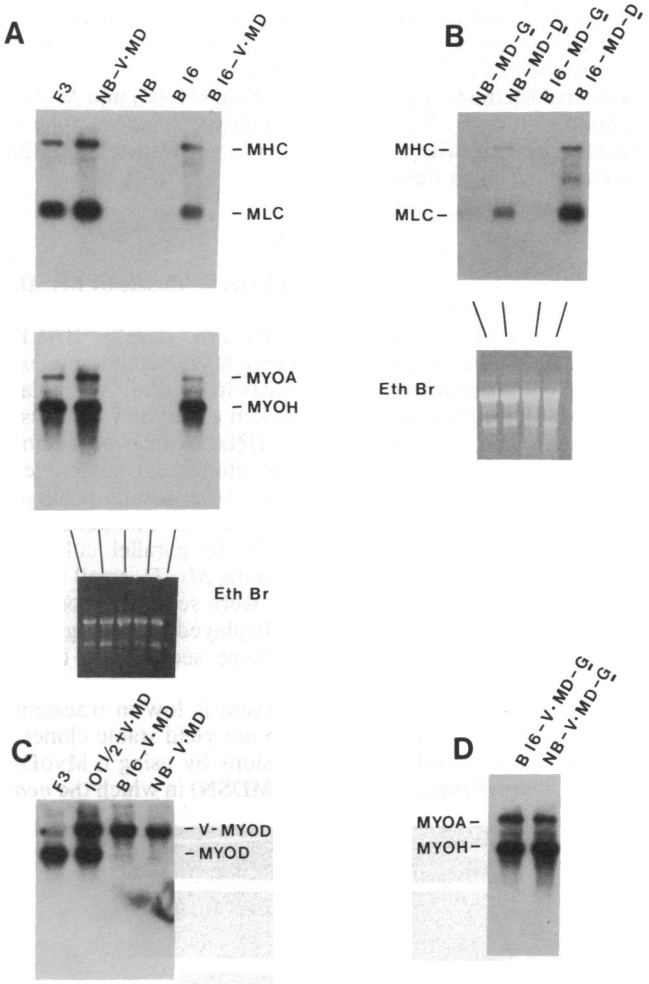

FIG. 4. Expression of muscle-specific RNA in differentiated cell lines converted by MyoD. MHC and myosin light chain 2 (MLC) are induced markers for differentiated muscle. MyoA, MyoH, and MyoD are markers present in proliferating myoblasts as well as in induced myotubes. v-MyoD is the RNA product from the retrovirus LMDSN. (*A*) Activation of MHC and MLC by LMDSN after serum withdrawal. F3, azamyoblasts; NB, neuroblastoma B-50 cells; B16, melanoma cells; NB-V·MD, neuroblastoma polyclones infected with the viral MyoD retrovirus; B16-V·MD, melanoma polyclones infected with viral MyoD. (*B*) Induction of MHC and MLC by serum withdrawal. G, 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. 10T½ cells were infected 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.

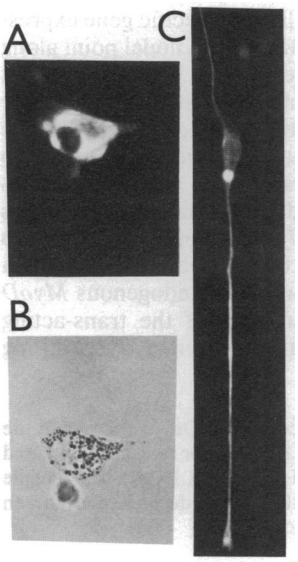

FIG. 5. Expression of the myogenic program in individual pigment and nerve cells. An individual melanocyte from B16-V·MD polyclones induced for muscle and then stained for MHC (*A*) or photographed by phase contrast (*B*). (*C*) An individual neuron from NB-V·MD polyclones induced simultaneously for muscle and nerve (10^{-3} M cAMP) and stained for MHC. (×100.)

muscle as assayed by fusion and staining with MHC antibody or fat as assayed by the presence of multiple lipid droplets (data not shown). Individual cells also tended to express either fat or muscle. Thus, expression of fat seems to be incompatible with expression of MHC. Because we have found it impossible to reliably stain fat cell nuclei for MyoD, we cannot be certain that MyoD remains present in the committed adipocytes in the population.

Our conclusion from these studies is that forced expression of *MyoD* does not turn off the endogenous pigment or neuronal pathway; muscle differentiation does seem to be mutually incompatible with adipogenesis. In heterokaryons between hepatoma cells and myotubes, liver-specific functions are extinguished (21). Fibroblasts also extinguish liver functions (22, 23). In heterokaryons between nerve cells and myocytes or between adrenal cells and myocytes, both programs from both fusion partners can coexist (24, 25), and in early Ascidian embryos, inhibition of cell cleavage results in cells coexpressing markers of several different lineages (26, 27). In *Caenorhabditis elegans*, the same experiment results in cells expressing only a single program (28). As more cell types are studied in this type of analysis, perhaps a clear set of rules will emerge, possibly relating to embryological lineage; for example, perhaps MyoD turns off an endogenous program of differentiation only in lineages closely related to muscle.

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. 6*A*). 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. 6*A*). SV2-CAT was also activated, but only 2- or 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. 6*B*). 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. 6*C*), 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.

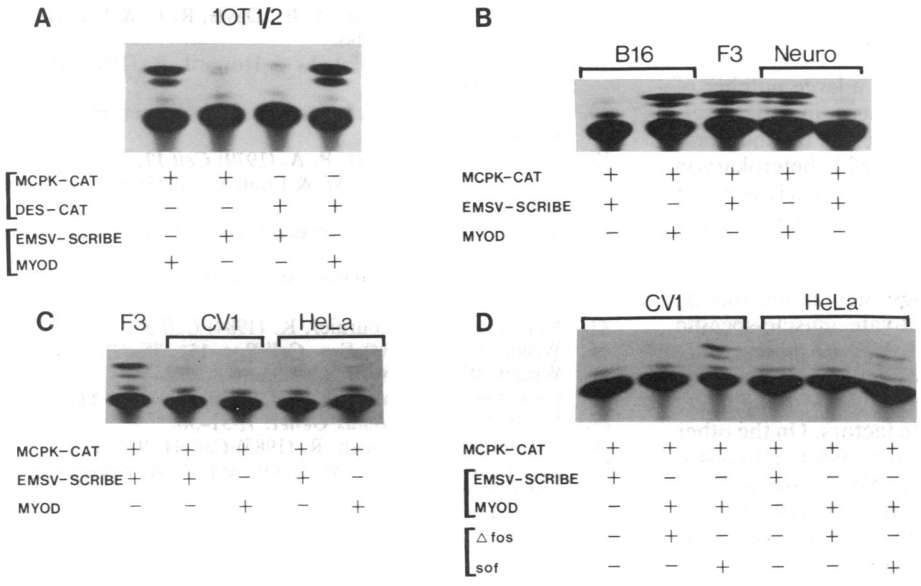

FIG. 6. Trans-activation of M-CPK-CAT by cotransfection with *MyoD*. Cells were cotransfected with the indicated vectors. They were then induced for muscle for 3 days. (*A*) M-CPK-CAT or des-CAT were at 5 μg per dish while *MyoD* or the parent expression vector EMSV-scribe were at 15 μg per dish. (*B*) Same conditions as in *A*, except transfection was into B16 melanomas, F3 myoblasts, or B-50 neuroblastoma cells. (*C*) Same conditions as in *A*, except transfection was into F3, CV1, or HeLa cells. (*D*) M-CPK-CAT was at 2 μg per dish, EMSV-scribe or *MyoD* was at 8 μg per dish, and SV-Δfos (a control *fos* deletion) or SV-sof (antisense *fos*) was at 15 μg per dish.

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 at least 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, as a control, 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-CPK-CAT activity that was dependent on MyoD expression (Fig. 6*D*). 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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