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Differential *trans* Activation Associated with the Muscle Regulatory Factors MyoD1, Myogenin, and MRF4

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Expression of the mammalian muscle regulatory factors MyoD1, myogenin, and MRF4 will convert C3H10T1/2 fibroblasts to stable muscle cell lineages. Recent studies have shown that MyoD1 and myogenin also *trans*-activate expression of a number of cotransfected contractile protein genes, suggesting that these muscle regulatory factors are involved in controlling terminal differentiation events. The extent and specificity of *trans* activation by the muscle regulatory factors, however, have not been compared directly. In this study, we found that MyoD1, myogenin, and MRF4 exhibited different *trans*-activation capacities. In contrast to MyoD1 and myogenin, MRF4 was inefficient in *trans*-activating most of the genes tested, although conversion of C3H10T1/2 fibroblasts to a myogenic lineage was observed at similar frequencies with all three factors. Addition of basic fibroblast growth factor to cells expressing exogenous muscle regulatory factors inhibited the transcriptional activation of cotransfected genes, demonstrating that MyoD1, myogenin, or MRF4 proteins alone are not sufficient to produce a terminally differentiated phenotype. In all cases, *trans* activation was dependent on signal transduction pathways that are regulated by fibroblast growth factor. Our observations, coupled with previous studies showing differences in the temporal expression and protein structure of MyoD1, myogenin, and MRF4, suggest that the individual members of the muscle regulatory factor family have distinct biological roles in controlling skeletal muscle development.

Skeletal muscle differentiation is an ideal model system in which to examine gene regulation during development. Myogenic stem cells isolated from embryonic tissues or from established cell lines can be cultured in vitro and induced to differentiate by depleting growth factors from the medium. Myogenic differentiation is characterized by the fusion of postmitotic myoblasts into multinucleated muscle fibers and the transcriptional activation of the contractile protein gene set. Recently, a number of laboratories have identified specific DNA regulatory sequences that control the expression of several contractile protein genes, including those encoding α-actin (36), muscle creatine kinase (MCK) (12, 27, 28, 44) myosin light chain (MLC) 1/3 (20), MLC 2 (11), troponin I (TnI) (54), and troponin T (32). In addition, protein factors have been identified that interact with the contractile protein genes to potentiate their muscle-specific expression (13, 25, 39).

Although the transition from proliferating myoblast to differentiated muscle fiber has been extensively studied, little is known about the molecular mechanisms by which multipotential stem cells commit to a myogenic lineage. Recently, a number of gene products have been identified that appear to be involved in this determination process. Expression of the mammalian muscle regulatory factors MyoD1 (19), myogenin (22, 52), Myf-5 (10), and MRF4 (9, 34, 42) in C3H10T1/2 fibroblasts leads to the conversion of these cells to determined myoblasts, which, after growth factor depletion, fuse and express the contractile protein genes. MyoD1, myogenin, Myf-5, and MRF4 proteins are structurally related, sharing both a common basic region and a proposed helix-loop-helix (HLH) amino acid domain that are required for the protein oligomerization and DNA-binding properties associated with MyoD1 (18, 30, 45). The basic and HLH domains also are common features of other gene products that are involved in developmental decisions. Among these are the *Drosophila* regulatory proteins twist (48), daughterless (14, 17), and the achaete-scute complex (1) as well as the immunoglobulin enhancer-binding proteins E12, E47 (37) and ITF-1, and ITF-2 (26).

The initial classification of MyoD1, myogenin, Myf-5, and MRF4 as muscle regulatory factors was based on their ability to convert fibroblasts to stable muscle cell lineages. However, MyoD1, myogenin, and Myf-5 also regulate expression of a number of contractile protein genes (10, 22, 31, 37, 50). For example, expression of MyoD1 or myogenin in C3H10T1/2 fibroblasts leads to the *trans* activation of a cotransfected MCK gene (22, 50). Lassar et al. (30), using in vitro DNA-binding assays, demonstrated that MyoD1 binds directly to the MCK enhancer region. Similar results showing myogenin binding to the MCK enhancer also have been reported (12), indicating that MyoD1 and myogenin interact with specific DNA regulatory sequences to control the tissue-specific expression pattern of the MCK gene.

We previously showed that exogenously supplied growth factors inhibit expression of the muscle regulatory factor genes as well as the contractile protein genes (42, 53). These studies, along with the *trans* activation and protein-DNA binding studies described above for the MCK gene, strongly suggest that MyoD1, myogenin, Myf-5, and MRF4 are intimately linked to the regulation of myogenic differentiation events. Unfortunately, very little is known about how these factors operate during terminal differentiation. The extent of *trans* activation by each muscle regulatory factor has not been compared directly. In addition, it is unclear whether the muscle regulatory factors exhibit unique specificities for individual contractile protein genes or whether functional redundancies exist between MyoD1, myogenin, Myf-5, and MRF4.

To compare directly the *trans*-activation potentials of the muscle regulatory factor gene family, we cotransfected MyoD1, myogenin, and MRF4 with a variety of chloramphenicol acetyltransferase (CAT) reporter genes and deter-

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mined the extent of CAT expression induced by each muscle regulatory factor. Our results indicate that MyoD1 and myogenin *trans*-activate contractile protein genes, including those encoding MCK, TnI, and α-actin, as well as a number of nonmuscle genes. Surprisingly, MRF4 does not efficiently *trans*-activate most of the genes tested, although MRF4 readily converts C3H10T1/2 fibroblasts to a differentiated muscle phenotype. In all cases, *trans* activation by each muscle regulatory factor is inhibited by basic fibroblast growth factor (FGF), indicating that MyoD1, myogenin, and MRF4 rely on an FGF signal transduction system to modulate their activity. This study demonstrates that members of the muscle regulatory factor gene family exhibit different biological activities, suggesting that these regulatory factors have distinct roles in controlling myogenic determination and differentiation events.

MATERIALS AND METHODS

Experimental gene constructions. The MyoD1, myogenin, and MRF4 cDNAs were cloned adjacent to the Moloney sarcoma virus long terminal repeat promoter in the expression vector pEMSVscribeα2 (19). The contractile protein gene plasmids used were TnICAT1, which contains nucleotides −530 to +1604 of the quail TnI gene (54); −3300MCKCAT, which contains nucleotides −3300 to +7 of the mouse MCK gene (27, 28); and pHCA177CAT, which contains nucleotides −177 to +68 of the human cardiac α-actin gene (36), each linked to the bacterial CAT gene.

To delete the respective regulatory elements associated with each contractile protein gene, pHCA177CAT was digested with *Apa*I and treated with S1 nuclease, and *Eco*RI linkers were added. This plasmid then was digested with *Eco*RI and religated to remove the proximal and distal CArG regulatory elements located between −177 and −100 (36). Deletion of the troponin I internal regulatory element (IRE) region was accomplished by digesting TnICAT1 with *Kpn*I and *Sst*I to remove nucleotides +240 to +920. After S1 nuclease treatment, *Bgl*II linkers were added to generate plasmid TnICAT36. The enhancerless MCK gene, p80MCKCAT, contains −80 to +7 of the mouse MCK gene ligated to the CAT reporter gene as described by Jaynes et al. (28).

The nonmuscle reporter genes tested included TKCAT, which contains nucleotides −109 to +54 of the herpes simplex virus thymidine kinase (TK) gene linked to a CAT reporter plasmid (28, 33); pSV1CAT, containing the simian virus 40 (SV40) early-region promoter, and pSV2CAT, containing the early-region promoter plus the core enhancer of SV40 (24, 28); and β-actin, containing ~2.5 kilobases of 5′-flanking DNA, the first exon, and the entire first intron of the chicken β-actin gene ligated to CAT (23). Plasmid 250NPYCAT contains nucleotides −250 to +1 of the rat neuropeptide Y (NPY) gene (35), and pBxSST contains nucleotides −750 to +50 of the rat somatostatin gene, each ligated to a CAT reporter plasmid (2).

Cell culture, DNA transfections, and CAT assays. C3H10T1/2, NIH 3T3, HeLa, and COS1 cells were obtained from the American Type Culture Collection. The myogenic cell line 23A2 and the adipogenic cell line 30A5 were derived from 5-azacytidine-treated C3H10T1/2 cells as described previously (29). All cell lines were maintained in growth medium containing basal medium Eagle (GIBCO Laboratories) supplemented with 10% fetal bovine serum plus penicillin (100 U/ml) and streptomycin (100 μg/ml), with the exception that 23A2 myoblasts were maintained in 15% fetal bovine serum and NIH 3T3 fibroblasts were maintained in Dulbecco modified Eagle medium (GIBCO) supplemented with 10% calf serum.

Transient DNA transfections were performed as described previously (54). DNA precipitates containing 5 μg of a CAT reporter gene and 5 μg of a muscle regulatory factor cDNA in the pEMSVscribeα2 vector (MyoD1, myogenin, or MRF4) or containing the expression vector without an insert were added to 10^6 cells per 100-mm dish. Four hours after the addition of DNA, cultures were glycerol shocked for 2 min in serum-free medium containing 20% glycerol and fed basal medium Eagle supplemented with 15% fetal bovine serum. Twenty-four hours later, cells were refed growth medium; 2 days after transfection, they were fed differentiation medium plus or minus purified human basic FGF (R and D systems, Inc.). The differentiation medium (ITS) consisted of low-glucose Dulbecco modified Eagle medium supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), and selenium (5 ng/ml) (54). Cells treated with basic FGF (10 ng/ml) were fed ITS containing heparin (7.5 μg/ml) and 5% horse serum as described previously (53). Two days after induction, cells were harvested for CAT assays or fixed for immunocytochemistry.

CAT assays were performed as described by Yutzey et al. (54) and were normalized on the basis of protein concentration, which was determined by the modified Bradford assay (Bio-Rad Laboratories); 1 to 50 μg of protein was used for each assay. CAT activity was calculated as the percent acetylated chloramphenicol per microgram of protein. Dilutions of each extract were tested to ensure that each value remained within the linear range of the CAT assay. Each value represents the average of two to four independent transfections per experiment.

Immunocytochemistry. Differentiated cell cultures were rinsed twice in cold phosphate-buffered saline and fixed for antibody staining. Cells to be stained with an antimyosin mouse monoclonal antibody (MF-20) (3) were fixed in a 20:2:1 solution of 70% ethanol–Formalin–acetic acid for 1 min at 4°C. Cells to be stained with rabbit polyclonal anti-CAT serum (5 prime-3 prime, Inc.) were fixed in cold methanol for 10 min at −20°C. After incubation with a primary antibody, cells were rinsed twice in phosphate-buffered saline and treated with the appropriate biotinylated immunoglobulin G secondary antibody. Immunoreactivity was visualized by using a Vectastain ABC reagent (Vector Laboratories, Inc.).

Isolation of RNA and Northern hybridizations. Total RNA was isolated from transfected cells by the method of Chomzcynski and Sacchi (15). Equivalent amounts of RNA for each experimental group were electrophoresed through 1.0% agarose-formaldehyde denaturing gels and transferred to GeneScreen membranes (Dupont, NEN Research Products). Membranes were hybridized to ^{32}P-labeled, random-primed cDNA probes (specific activity, ~1×10^9 cpm/μg) at 65°C in 6× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate), 2 mM EDTA, 20 mM Tris chloride (pH 7.6), 0.5% sodium dodecyl sulfate, 5× Denhardt solution, and 100 μg of denatured salmon sperm DNA per ml. Hybridized blots were washed at 65°C in 0.3× SSC, 0.2% sodium dodecyl sulfate, and 2 mM EDTA for 2 h and exposed to Kodak XAR film. Northern blots were subsequently rehybridized to radiolabeled pAL15 probes (6, 7). The control pAL15 probe detects an RNA that is expressed constitutively in most cultured cell lines (6, 7, 53).

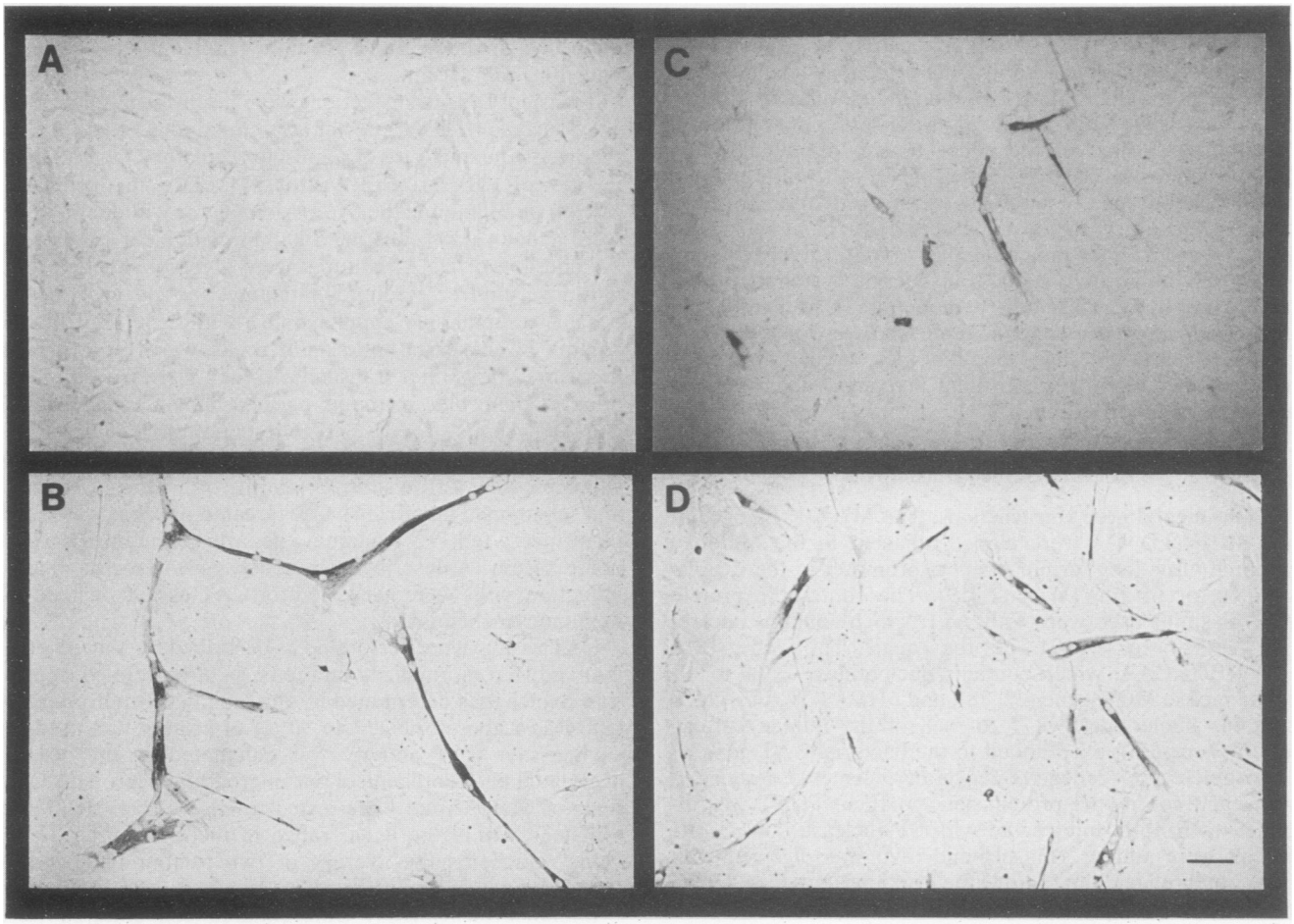

FIG. 1. Conversion of C3H10T1/2 fibroblasts to a muscle phenotype following the expression of muscle regulatory factor cDNAs. C3H10T1/2 cultures were transiently transfected with the control pEMSVscribeα2 expression vector (A) or with MyoD1 (B), myogenin (C), and MRF4 (D) expression vectors and induced to differentiate. Differentiated cells were detected by immunocytochemistry using the MF-20 antibody, which recognizes sarcomeric myosin heavy chains (3). Bar = 100 μm.

RESULTS

Conversion of C3H10T1/2 fibroblasts by MyoD1, myogenin, and MRF4. To compare the individual roles of MyoD1, myogenin, and MRF4 in muscle determination and differentiation, C3H10T1/2 fibroblasts were transfected independently with each muscle regulatory gene and induced to differentiate (see Materials and Methods). As expected, expression of each muscle regulatory factor cDNA in these cells led to the production of differentiated, elongated muscle fibers that synthesized myosin heavy chains (Fig. 1). The degree of myogenic conversion differed for each regulatory factor; MyoD1 and MRF4 converted ~2 to 3% of the C3H10T1/2 cells to a differentiated muscle phenotype, whereas myogenin converted fewer than 1% of the cells. Although MRF4 and MyoD1 exhibited similar myogenic frequencies, MyoD1-induced fibers often contained more nuclei. Myogenin-induced cultures consisted mainly of differentiated, mononucleated myocytes (Fig. 1). Similar observations were made when multiple plasmid preparations were tested, suggesting that these regulatory genes, although capable of converting C3H10T1/2 fibroblasts to a muscle lineage, exhibit different biological activities.

***trans* activation of skeletal muscle contractile protein genes by MyoD1, myogenin, and MRF4.** Previous studies have shown that MyoD1 and myogenin *trans*-activate expression of cotransfected contractile protein genes (22, 31, 50). Preliminary studies from our laboratory also suggested that MRF4 is capable of *trans*-activating specific genes (42), although a detailed comparison of the *trans*-activation capacity for each regulatory factor has not been reported. To begin characterizing the activities of MyoD1, myogenin, and MRF4, we examined the ability of each muscle regulatory factor to *trans*-activate a CAT reporter gene linked to the *cis*-acting DNA regulatory sequences that control expression of the TnI (54) and MCK (28) contractile protein genes. Expression of the TnI gene is controlled by an internal regulatory element (IRE) contained within the first intron, whereas expression of the MCK gene is regulated by a distal upstream enhancer element. CAT reporter gene constructs containing the TnI promoter and IRE or the MCK promoter and enhancer element are not expressed in nonmuscle cells but are transcriptionally activated during muscle differentiation (28, 54).

MyoD1, myogenin, and MRF4 expression plasmids were cotransfected into C3H10T1/2 fibroblasts with the TnI and MCK muscle gene CAT constructs. After transfection, the cells were fed a defined medium to induce terminal differentiation (53, 54). The level of *trans*-activation for each CAT gene then was determined by comparing CAT expression in cultures cotransfected with the control pEMSVscribeα2

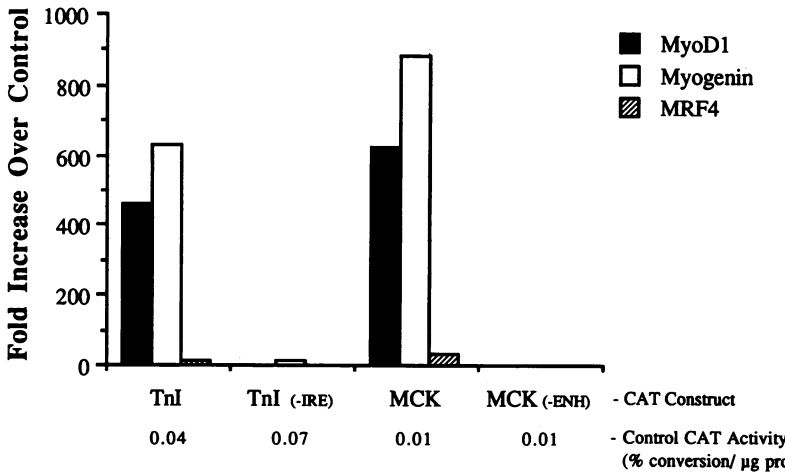

FIG. 2. Expression of the muscle regulatory factor cDNAs in C3H10T1/2 fibroblasts leads to the *trans* activation of cotransfected contractile protein genes. Shown are results of assays of *trans* activation by MyoD1, myogenin, and MRF4 (left to right) of control TnI and MCK genes, each containing their respective muscle-specific regulatory elements, or of mutated contractile protein genes in which the TnI IRE (−IRE) and MCK enhancer (−ENH) regulatory sequences were deleted (see Materials and Methods for details). CAT activity was calculated as the percent acetylated chloramphenicol per microgram of protein; relative values are presented as the fold increases over expression of the reporter gene construct when cotransfected with the control pEMSVscribeα2 expression vector. The basal level of CAT expression in the control pEMSVscribeα2 transfections is indicated below each gene construct. Values represent the average of multiple independent transfections.

expression vector. Expression of the MyoD1 or myogenin cDNA in C3H10T1/2 fibroblasts led to the transcriptional activation of the TnI and MCK cotransfected genes (Fig. 2). Unlike MyoD1 and myogenin, however, MRF4 cotransfections produced very low levels of TnI and MCK CAT expression. Similar results were obtained when a rat MLC 1/3 enhancer element (20) was tested, with MyoD1 and myogenin again producing high levels of CAT activity and MRF4 producing very low levels (data not shown). The inability of MRF4 to *trans*-activate the TnI, MCK, and MLC gene constructs efficiently was particularly surprising since a high percentage of cells converted to a myogenic lineage in the MRF4 transfected cultures (Fig. 1). Interestingly, MRF4 was capable of *trans*-activating some contractile protein genes, since MRF4, MyoD1, and myogenin equivalently *trans*-activated cotransfected cardiac α-actin CAT gene constructs approximately 200-fold (data not shown). Therefore, the relative levels of activation by each muscle regulatory factor are different for each contractile protein gene. In addition, the ability of the regulatory proteins to *trans*-activate contractile protein genes is not indicative of their capacity to convert C3H10T1/2 fibroblasts to a muscle phenotype.

Recent studies have reported that MyoD1 (18) and myogenin (12) bind to a specific DNA sequence located within the MCK enhancer, suggesting that *trans* activation of cotransfected genes by the muscle regulatory factors requires appropriate muscle-specific regulatory elements. Since the TnI IRE also contains a potential MyoD1 and myogenin binding site (H. Lin and S. Konieczny, unpublished results), we examined whether contractile protein reporter genes lacking their respective regulatory sequences could be activated under these experimental conditions. C3H10T1/2 fibroblasts were cotransfected with a MyoD1, myogenin, or MRF4 expression vector and a TnI gene minus the IRE or an MCK gene in which the upstream enhancer element was deleted (see Materials and Methods). The TnI and MCK genes lacking their muscle-specific enhancers were not significantly activated by the regulatory genes (Fig. 2), demonstrating that the TnI and MCK enhancers are required to obtain full expression in the MyoD1 and myogenin transfections. However, in parallel transfections we found that each muscle regulatory factor (MRF4, MyoD1, and myogenin) efficiently *trans*-activated cardiac α-actin CAT genes from which the muscle-specific CArG regulatory elements (36) had been removed (data not shown). From our results, we conclude that the major *trans*-activation responses from the TnI and MCK genes require their respective muscle-specific enhancer elements, whereas *trans* activation of the α-actin gene does not involve the upstream CArG regulatory sequences.

MyoD1 and myogenin can *trans*-activate nonmuscle genes. The dramatic activation of the TnI and MCK CAT genes by MyoD1 and by myogenin implies that a muscle-specific *cis-trans* regulatory system is responsible for this increased expression. We were surprised, however, to find that MRF4, MyoD1, and myogenin equally *trans*-activated expression of an α-actin promoter from which the muscle-specific CArG boxes had been removed, suggesting that the muscle regulatory factors may be able to potentiate transcription of nonmuscle promoter elements as well. To determine whether MyoD1, myogenin, and MRF4 *trans*-activate only contractile protein genes, we cotransfected each muscle regulatory gene with nonmuscle gene constructs containing general, constitutive promoters linked to the CAT reporter gene. The nonmuscle promoters were derived from the herpes simplex virus TK gene (33), the chicken β-actin gene (23), and the SV40 early promoter with and without the 72-base-pair repeat enhancer elements (49). The TK and SV40 genes contain relatively weak promoters, whereas the β-actin and SV40 promoter-enhancer genes are expressed at very high levels in most cell types. Cotransfection assays with the TK and SV40 promoters showed that expression of these constitutive genes could be increased by MyoD1 and myogenin but not by MRF4 (Fig. 3). As with the TnI and MCK genes, myogenin-transfected cells consistently exhibited the highest level of CAT expression, whereas MRF4 elicited an extremely weak activation response.

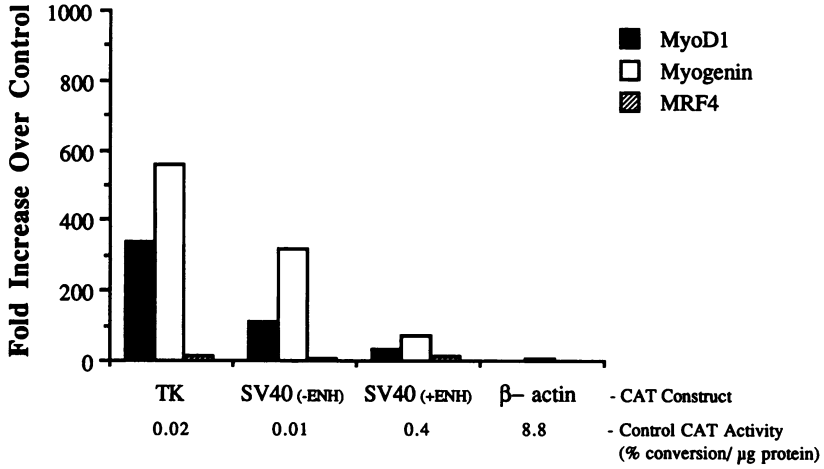

FIG. 3. *trans* activation of nonmuscle regulatory elements by MyoD1, myogenin, and MRF4. DNA plasmids containing the TK promoter, the SV40 promoter (−ENH), the SV40 promoter plus enhancer (+ENH), or the β-actin promoter were transiently cotransfected with each of the muscle regulatory factor expression vectors. CAT activity was calculated as the percent acetylated chloramphenicol per microgram of protein; relative values represent the fold increases over expression in parallel pEMSVscribeα2 control cotransfections. Basal CAT activity in the control pEMSVscribeα2 transfections is indicated below each gene construct. Each value represents the average of multiple independent transfections.

Although expression of the TK and SV40 promoters was enhanced by MyoD1 and by myogenin, we found that genes which normally are expressed at very high levels in C3H10T1/2 fibroblasts were minimally affected by these muscle regulatory factors. In control transfected C3H10T1/2 cells, the SV40 promoter-enhancer gene was expressed at a 40-fold-higher level than the SV40 gene lacking the enhancer element. Similarly, the β-actin gene was expressed at a 440-fold-higher level than the TK gene. When MyoD1, myogenin, or MRF4 was cotransfected with these genes, only low levels of *trans* activation were observed (Fig. 3). The initial high rates of transcription from the SV40 promoter-enhancer gene and from the β-actin gene apparently limits these promoters from being significantly enhanced by MyoD1, myogenin, or MRF4 under these experimental conditions.

To study further the range of DNA regulatory elements that are susceptible to *trans* activation, two tissue-specific genes that normally are not expressed in the myogenic lineage were tested. Somatostatin (2) and neuropeptide Y (NPY) (35) genes are expressed exclusively in neuronal and endocrine cell types. Gene constructs containing the DNA elements responsible for their respective tissue-specific expression were ligated to the CAT reporter gene and tested for *trans* activation as described above (2; C. Minth and J. Dixon, personal communication). These genes were not expressed when transfected into C3H10T1/2 cells. Interestingly, the NPY gene was activated to very high levels (~400-fold) by MyoD1 and myogenin but not by MRF4. Transcription of the somatostatin gene, on the other hand, was unaffected by the presence of each muscle regulatory factor (data not shown). Our results suggest that nonmuscle genes can be activated by MyoD1 and by myogenin. However, specific DNA regulatory sequences likely are required, since not all genes responded similarly to these muscle regulatory factors.

***trans*-activation responses are cell type specific.** The specific cells in which muscle regulatory factors are expressed may affect the capacity of these factors to *trans*-activate contractile protein genes. Previous studies have shown that MyoD1 converts many cell types, including several primary and established fibroblast cell lines as well as cell lines committed to different cell lineages (50). However, HeLa and COS1 cells, which are transformed cell lines, are resistant to myogenic conversion by each muscle regulatory factor (50; K. Yutzey and S. Konieczny, unpublished data). Although these cells do not convert to a myogenic phenotype, we reasoned that *trans* activation of a cotransfected reporter gene may occur independently of endogenous cellular factors if the muscle regulatory proteins *trans*-activate transfected TnI and TK genes directly. To investigate this question further, NIH 3T3 fibroblasts, 30A5 preadipocytes, and HeLa and COS1 cells were cotransfected with the TnI and TK genes plus the MyoD1, myogenin, or MRF4 gene. As reported previously, MyoD1, myogenin, and MRF4 convert NIH 3T3 fibroblasts to a muscle phenotype, but the myogenic conversion is at least 3 to 10 times less efficient than observed in C3H10T1/2 cells (22, 42). The levels of *trans* activation for the TnI and TK genes also were correspondingly lower (Fig. 4). Cotransfections in the 30A5 adipogenic cell line, which is a derivative of C3H10T1/2 cells, also resulted in very low levels of *trans* activation for the TnI and TK gene constructs. As expected, fewer than 0.2% of the 30A5 preadipocytes converted to a muscle phenotype. In each case, MyoD1 and myogenin were more efficient than MRF4 in activating cotransfected genes in these cells.

Expression of the muscle regulatory factor cDNAs in HeLa and COS1 cells resulted in less than a twofold increase in TnI or TK CAT expression over the level for control transfected cells (Fig. 4). In addition, cotransfection of MyoD1 plus myogenin or MyoD1 plus MRF4 in COS1 cells failed to produce a *trans*-activation response. Immunocytochemistry using the MF-20 myosin heavy-chain antibody also failed to identify any differentiated cells in the HeLa and COS1 cultures transfected with MyoD1 or MRF4 (data not shown). These studies demonstrate that the ability of the muscle regulatory factors to activate transfected genes correlates directly with their ability to convert various cell types to a differentiated muscle phenotype. Cells that do not convert to a myogenic lineage do not *trans*-activate cotransfected genes, even when presented with a constitutively expressed muscle regulatory factor cDNA.

FGF inhibits *trans* activation. The depletion of specific growth factors, such as FGF, is required to induce skeletal

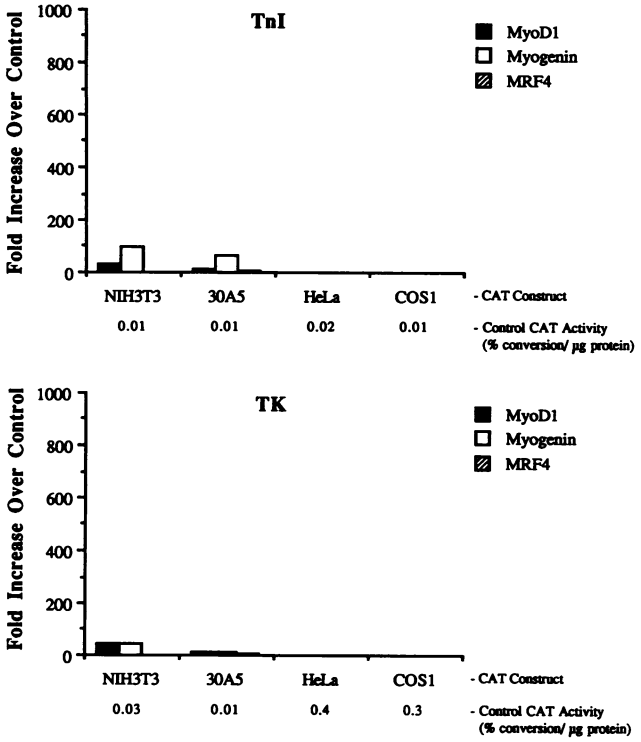

FIG. 4. Cell type-specific *trans* activation by MyoD1, myogenin, and MRF4. The TnI gene containing the IRE and the nonmuscle TK gene were cotransfected independently with MyoD1, myogenin, or MRF4 (left to right) cDNAs. *trans* activation was measured in NIH 3T3 fibroblasts, 30A5 preadipocytes, and HeLa and COS1 cells. Relative CAT activity was calculated as for Fig. 2 and 3. The basal level of CAT expression in control pEMSVscribeα2 transfections is shown below each cell type.

myoblasts to withdraw from the cell cycle, fuse, and transcriptionally activate the contractile protein gene set (16, 40, 51, 53). In order to examine whether the FGF signal transduction pathway regulates the ability of MyoD1, myogenin, and MRF4 to activate exogenously supplied genes, we examined the expression of various CAT genes in cotransfected C3H10T1/2 fibroblasts maintained in the presence or absence of purified basic FGF. C3H10T1/2 fibroblasts expressing a transfected MyoD1, myogenin, or MRF4 cDNA and cultured in medium containing FGF were inhibited from expressing the endogenous myosin heavy-chain contractile protein genes (Table 1). FGF also inhibited the *trans* activation of the TnI gene when cotransfected with MyoD1, myogenin, and MRF4. Surprisingly, FGF similarly inhibited the *trans* activation of the nonmuscle TK gene, implying that the *trans* activation of nonmuscle genes by each muscle regulatory factor is controlled through an FGF signal transduction pathway (Table 1). Similar results were observed when these cultures were maintained in high concentrations of fetal bovine serum (data not shown).

To characterize the inhibition of *trans* activation by FGF more precisely, transfected cultures were immunocytochemically stained with antisera directed against the CAT protein to detect expression of the transfected TnI and TK gene constructs. Quantitation of the cells exhibiting CAT antibody reactivity revealed a 68 to 100% reduction in the number of cells expressing CAT protein when the cultures were maintained in FGF (Table 1; Fig. 5). In addition to the total number of cells staining with the CAT antiserum, the relative level of staining detected for each CAT gene construct paralleled the degree of *trans* activation produced by MyoD1, myogenin, and MRF4, with MRF4 only weakly *trans*-activating the TnI and TK CAT genes (Fig. 5). We conclude that FGF inhibits the myogenic conversion of C3H10T1/2 fibroblasts and blocks the *trans*-activation properties of each muscle regulatory factor, regardless of whether muscle or nonmuscle reporter genes are tested.

Endogenous muscle regulatory genes are activated in cells expressing transfected muscle regulatory factor cDNAs. The MyoD1, myogenin, and MRF4 genes are capable of regulating their own expression as well as the expression of other muscle regulatory genes (8, 22, 34, 42, 47). For example, expression of a transfected MRF4 gene leads to activation of the endogenous MRF4, MyoD1, and myogenin genes, although neither the MyoD1 nor myogenin gene activates expression of the C3H10T1/2 MRF4 gene (34, 42). The feedback regulation of these genes may play a direct role in how each muscle regulatory factor *trans*-activates a cotransfected CAT gene construct.

In these studies, the extent of endogenous regulatory factor gene activation was assessed posttransfection, after the cells had been in differentiation medium for 2 days. RNA from the transfected cultures was electrophoresed and hybridized with labeled DNA probes for each of the myogenic factors. Under these conditions, autoregulation is not detected because the endogenous mRNA is obscured by the expression signal from the transfected gene. However, we were able to determine whether other muscle regulatory

TABLE 1. Inhibition by FGF of the differentiation and *trans*-activation properties of MyoD1, myogenin, and MRF4 in transfected C3H10T1/2 fibroblasts

Transfected gene constructs	Myosin expression[a]		CAT activity[b]		CAT expression[a]	
	−FGF	+FGF	−FGF	+FGF	−FGF	+FGF
TnI + MyoD1	100 (169)	26 (44)	100 (12.1)	20 (2.4)	100 (104)	32 (33)
TnI + myogenin	100 (20)	5 (1)	100 (22.8)	9 (2.0)	100 (82)	16 (13)
TnI + MRF4	100 (131)	20 (26)	100 (0.4)	50 (0.2)	100 (176)	0 (0)
TK + MyoD1	100 (132)	30 (39)	100 (5.4)	39 (2.1)	100 (130)	22 (29)
TK + myogenin	100 (37)	8 (3)	100 (12.0)	10 (1.2)	100 (59)	25 (15)
TK + MRF4	100 (92)	11 (10)	100 (0.7)	43 (0.3)	100 (38)	31 (12)

[a] Antibody reactivity to demonstrate expression was calculated as the percentage of cells that stained positively with the myosin or CAT antibodies. +FGF values are expressed as a percentage of the corresponding −FGF value, which was arbitrarily set at 100% for each transfection. The values in parentheses indicate the number of stained cells detected out of 5,000 total cells for each experimental group.

[b] Calculated as the percent acetylated chloramphenicol per microgram of protein, shown in parentheses, for both −FGF and +FGF cultures. +FGF values are expressed as a percentage of the corresponding −FGF value, which was arbitrarily set at 100% for each transfection.

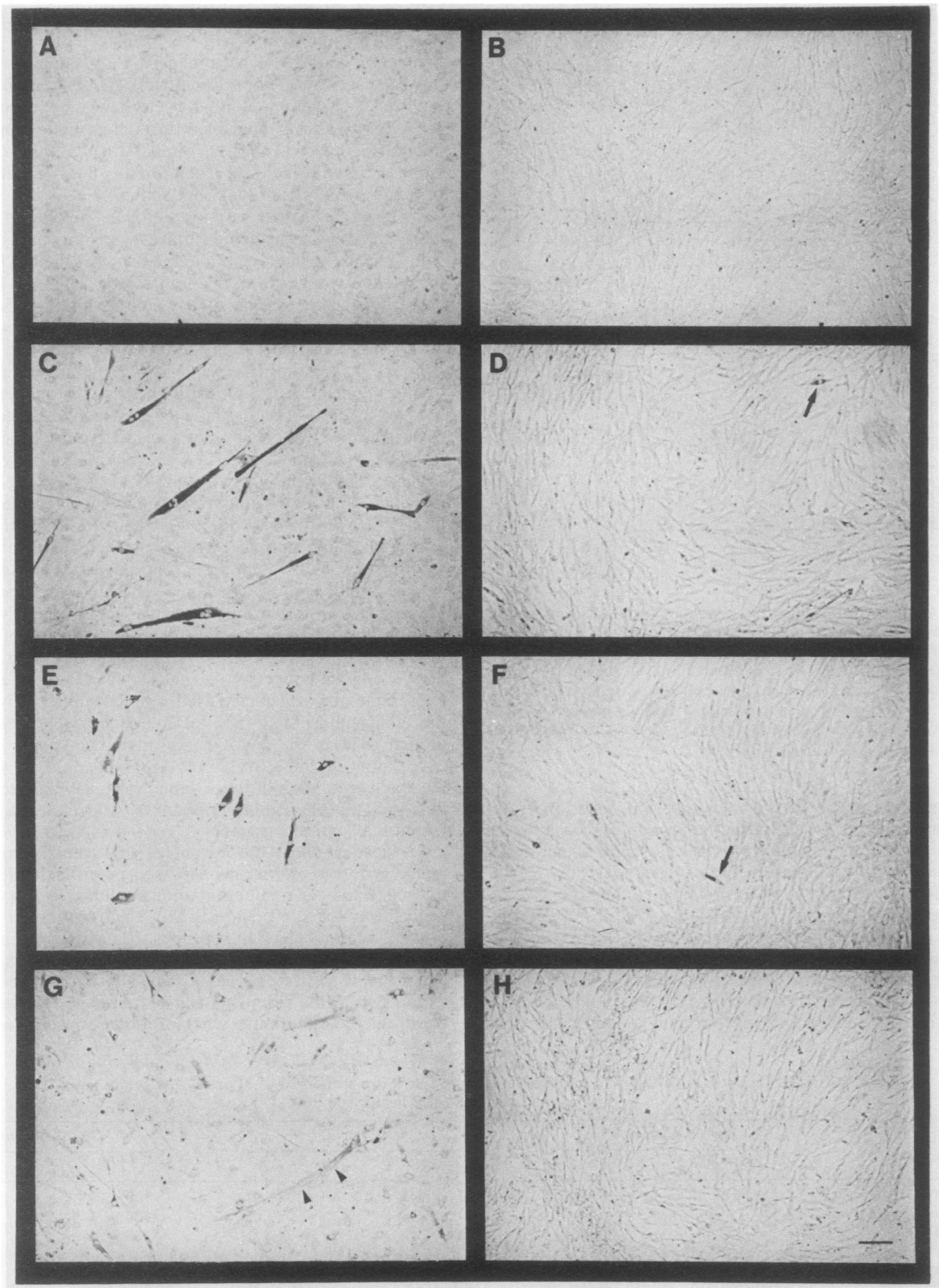

FIG. 5. FGF inhibits MyoD1, myogenin, and MRF4 from converting C3H10T1/2 fibroblasts to a myogenic phenotype. C3H10T1/2 fibroblasts transiently cotransfected with pEMSVscribeα2 (A and B), MyoD1 (C and D), myogenin (E and F), or MRF4 (G and H) and a TnI CAT gene containing the IRE were induced to differentiate in the absence (A, C, E, and G) or presence (B, D, F, and H) of FGF and immunocytochemically stained with anti-CAT rabbit serum. Arrows in panels D and F indicate rare cells that have differentiated despite the presence of FGF. The arrowheads in panel G indicate a multinucleated muscle fiber that expresses very low levels of CAT protein as a result of the inability of MRF4 to *trans*-activate the cotransfected TnI gene (see Fig. 2). Bar = 100 μm.

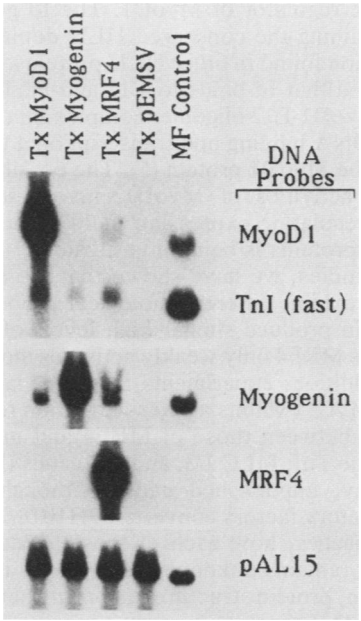

FIG. 6. Northern analyses of RNA isolated from C3H10T1/2 cultures transiently transfected with MyoD1, myogenin, or MRF4. Total RNA was isolated from differentiated cultures independently transfected (Tx) with each muscle regulatory factor or transfected with the control expression vector pEMSVscribeα2 (pEMSV). Equivalent amounts of RNA from each experimental culture (40 μg) and RNA isolated from 23A2 control muscle fibers (15 μg) (MF Control) were electrophoresed through agarose-formaldehyde gels, transferred to nylon membranes, and probed independently for MyoD1, fast TnI, myogenin, MRF4, and pAL15. As reported previously (42), MRF4 is not expressed in the 23A2 myogenic cell line and also is not detected in the MyoD1 and myogenin transfected cultures. The control pAL15 probe, which detects a constitutively expressed RNA, was used to ensure that equivalent amounts of RNA were bound in each test lane. (Longer exposures of the MyoD1 blot revealed a weak MyoD1 signal in the myogenin transfected cells.)

genes were activated. Each muscle regulatory factor cDNA was expressed to equivalent high levels in the transfected C3H10T1/2 fibroblasts (Fig. 6). As expected, cultures transiently transfected with MyoD1, myogenin, and MRF4 expressed the endogenous mouse TnI gene. The level of TnI expression reflected the number of differentiated muscle cells produced in each experiment, with MyoD1 and MRF4 converting a higher percentage of the cells to a myogenic lineage than did myogenin (Fig. 1). As reported previously, expression of the endogenous myogenin gene is detected in MyoD1 and MRF4 transfected cells, and expression of the endogenous MyoD1 gene is detected in MRF4 and myogenin-transfected cultures (42). Neither MyoD1 nor myogenin-transfected cells expressed detectable levels of the endogenous MRF4 gene (42; Fig. 6). Similar expression patterns for the endogenous muscle regulatory genes occurred when NIH 3T3 fibroblasts were transfected with each muscle regulatory factor cDNA (S. Rhodes and S. Konieczny, unpublished results). In all cases, the levels of activation for the endogenous genes were very low compared with the expression detected in normal myogenic cell lines and with the levels of expression from the transfected muscle regulatory genes. Since MRF4 cultures expressed MyoD1 and myogenin but failed to *trans*-activate most CAT gene constructs, we conclude that the minimal expression of the endogenous muscle regulatory genes is not sufficient for the high levels of *trans* activation observed in these studies.

DISCUSSION

The observation that 5-azacytidine converts C3H10T1/2 fibroblasts to skeletal myoblasts led to the hypothesis that expression of a single genetic locus was sufficient to establish stable muscle cell lineages (29). Subsequently, five distinct mammalian genes, *myd* (41), MyoD1 (19), myogenin (22, 52), Myf-5 (10), and MRF4 (9, 34, 42), have been described that independently convert C3H10T1/2 fibroblasts to a muscle phenotype. MyoD1, myogenin, Myf-5, and MRF4 belong to a family of DNA-binding proteins that contain basic and HLH amino acid domains. An additional property of this muscle regulatory factor gene family is that each is capable of *trans*-activating cotransfected contractile protein genes in C3H10T1/2 cells (10, 22, 31, 42, 46, 50). Although previous studies have reported the *trans* activation of contractile protein genes by individual muscle regulatory factors, the extent of *trans*-activation by these proteins in the presence or absence of growth factors has not been compared directly. In this study, we found that members of the muscle regulatory factor gene family rely on an FGF signal transduction pathway and exhibit different *trans* activation capacities, implying that these regulatory factors have different roles in controlling myogenic determination and differentiation events.

The mechanisms by which cotransfected genes become transcriptionally activated in cells expressing MyoD1, myogenin, and MRF4 are not well characterized. *trans* activation of the TnI, MCK, and α-actin contractile protein genes suggested that muscle-specific DNA regulatory elements are required to achieve a *trans*-activation response. However, an α-actin gene in which the CArG regulatory sequences were deleted, as well as nonmuscle genes such as the TK and NPY genes, are *trans*-activated by the muscle regulatory factors. The *trans*-activation properties of MyoD1, myogenin, and MRF4 are dependent directly on the cell phenotype as well as on the presence or absence of serum or purified FGF, indicating that a suitable cell and growth factor environment is critical in modulating the activity of these muscle regulatory factors.

Activation of gene expression by the muscle regulatory factors may involve an indirect mechanism by which muscle-specific transcription factors are induced or ubiquitously expressed transcription factors are modified. Alternatively, the muscle regulatory factors may interact directly with the DNA regulatory sequences associated with the contractile protein genes. Sequence specificity clearly is important in the ability of the muscle regulatory factors to *trans*-activate some cotransfected genes. Lassar et al. (30) have shown that MyoD1 binds directly to specific regions within the MCK enhancer. Similar binding of myogenin to the MCK enhancer also has been demonstrated (12). In addition, experiments from our laboratory have shown that MyoD1 and myogenin bind to a related DNA sequence found within the TnI IRE (H. Lin and S. Konieczny, unpublished results). Whether MRF4 also binds to the TnI and MCK regulatory elements remains to be determined. These experiments will be particularly important since MRF4 does not *trans*-activate the TnI and MCK genes. As expected, MCK gene constructs lacking the MCK enhancer and TnI gene constructs lacking the IRE are not activated by MyoD1 or by myogenin. Contrary to these results, removal of the CArG box sequences from the α-actin promoter had little effect on the ability of the

promoter to be *trans*-activated by MyoD1, myogenin, and MRF4. Interestingly, sequence analysis of the deleted α-actin gene has revealed a potential MyoD1 and myogenin binding site at position −50, suggesting that this DNA region may be responsible for the activation of the α-actin gene in these experiments. A similar MyoD1/myogenin DNA consensus sequence also is contained within the TK promoter.

Although MyoD1 and myogenin bind to the MCK and TnI enhancer sequences, other DNA regulatory elements or protein factors may be important in controlling the specificity of MyoD1, myogenin, and MRF4. For example, the SV40 and NPY promoter regions do not contain identifiable MyoD1 or myogenin DNA-binding consensus sequences, although they are strongly activated by cotransfected MyoD1 and myogenin genes. Sequence analyses and protein-DNA binding studies have shown that the SV40 and NPY promoters contain several Sp1-binding sites (21; C. Minth and J. Dixon, personal communication). In our studies, we found that DNA regulatory regions that do not contain Sp1-binding sites, such as the somatostatin (2), TnI (4), and MCK (27) promoters, are not activated by MyoD1, myogenin, or MRF4. Since Sp1 functions as a transcription factor, it is conceivable that the muscle regulatory proteins interact with Sp1 to enhance expression of the SV40 and NPY promoters. In support of this hypothesis, the TnI IRE contains a potential Sp1 DNA-binding site as well as a MyoD1 and myogenin DNA-binding site. Both recognition sequences are required to achieve the proper muscle-specific expression of the TnI gene (H. Lin, K. Yutzey, and S. Konieczny, unpublished data). Whether ubiquitously expressed Sp1 DNA-binding proteins are involved directly in modulating the *trans*-activation properties of the muscle regulatory factors is being investigated.

The ability of MyoD1, myogenin, and MRF4 to induce terminal differentiation and the activation of contractile protein genes depends on the presence of growth factors. Cells maintained in high serum concentrations or in FGF are inhibited from differentiating and from expressing cotransfected muscle and nonmuscle genes, even when high levels of MyoD1, myogenin, and MRF4 continue to be expressed. The response of the muscle regulatory factors to FGF also is cell type specific, since HeLa and COS1 cells are nonpermissive for *trans* activation under any of the tested growth conditions. These results imply that MyoD1, myogenin, and MRF4 are not sufficient by themselves to produce a terminally differentiated cell. Instead, the muscle regulatory factors require additional gene products, specific modifications, or the removal of negative factors to produce a myogenic phenotype. In support of this model, recent studies by Schäfer et al. (43) have shown that other regulators, which are present in C3H10T1/2 and NIH 3T3 fibroblasts but not in other cell types, are required for the MyoD1-induced activation of muscle-specific genes.

Additional gene products clearly are involved in regulating some of the activities of MyoD1. In vitro binding to the MCK enhancer requires a hetero-oligomer complex consisting of MyoD1 or myogenin and the immunoglobulin-binding protein E12 (12, 38). Studies from our laboratory have shown that MRF4-E12 complexes also can be produced in vitro (H. Lin and S. Konieczny, unpublished data). Other hetero-oligomers consisting of E47 and MyoD1, E12 and achaete-scute, E47 and achaete-scute, and aschaete-scute and daughterless also have been identified in vitro (38). All of these proteins share a common basic region and a proposed HLH amino acid domain. Recently, Benezra et al. (5) isolated a new member of the HLH gene family, Id, that acts as a negative regulator of MyoD1. The Id gene encodes a protein containing the conserved HLH domain but lacking the basic region found in other HLH proteins. These authors have proposed that Id binds to E12 and inhibits the formation of the MyoD1-E12 oligomer complex that is required for the normal DNA binding and transcriptional activities associated with the MyoD1 protein (5). The possibility that FGF inhibits the activities of MyoD1, myogenin, and MRF4 directly by regulating expression of Id or other negatively acting HLH proteins is being investigated.

In these studies, we have shown that the muscle regulatory factors exhibit different biological properties. MyoD1 and myogenin produce similar high levels of CAT expression, whereas MRF4 only weakly activates most of the genes tested. In addition, experiments from our laboratory have shown that Myf-5 exhibits a *trans*-activation response that is intermediate between those of MRF4 and myogenin when tested with the TnI, MLC 1/3, and TK genes (K. Yutzey and S. Konieczny, unpublished data). Although each of the muscle regulatory factors converts C3H10T1/2 fibroblasts to myogenic lineages, how each of these genes is utilized in development remains unknown. Differences in their temporal expression, protein structure, and *trans*-activation capacities suggest that these regulatory genes have distinct biological roles. Determining the evolutionary and functional relationships of these gene products will be essential in understanding the molecular regulatory mechanisms that control skeletal muscle development.

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LITERATURE CITED

1. **Alonso, M. C., and C. V. Cabrera.** 1988. The *achaete-scute* complex of *Drosophila melanogaster* comprises four homologous genes. EMBO J. **7**:2585–2591.
2. **Andrisani, O. M., T. E. Hayes, B. Roos, and J. E. Dixon.** 1987. Identification of the promoter sequences involved in the cell specific expression of the rat somatostatin gene. Nucleic Acids Res. **15**:5715–5728.
3. **Bader, D., T. Masaki, and D. A. Fischman.** 1982. Immunochemical analysis of myosin heavy chain during avian myogenesis *in vivo* and *in vitro*. J. Cell Biol. **95**:763–770.
4. **Baldwin, A. S., E. W. Kittler, and C. P. Emerson, Jr.** 1985. Structure, evolution and regulation of a fast skeletal muscle troponin I gene. Proc. Natl. Acad. Sci. USA **82**:8080–8084.
5. **Benezra, R., R. L. Davis, D. Lockshon, D. L. Turner, and H. Weintraub.** 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell **61**:49–59.
6. **Bernlohr, D. A., C. W. Angus, M. D. Lane, M. A. Bolanowski, and T. Kelly, Jr.** 1984. Expression of specific mRNAs during adipose differentiation: identification of an mRNA encoding a homologue of myelin P2 protein. Proc. Natl. Acad. Sci. USA **81**:5468–5472.
7. **Bernlohr, D. A., M. A. Bolanowski, T. J. Kelly, Jr., and M. D. Lane.** 1985. Evidence for an increase in transcription of specific mRNAs during differentiation of 3T3-L1 preadipocytes. J. Biol. Chem. **260**:5563–5567.
8. **Braun, T., E. Bober, G. Buschhausen-Denker, S. Kotz, K.-H. Grzeschik, and H. H. Arnold.** 1989. Differential expression of

myogenic determination genes in muscle cells: possible autoactivation by the Myf gene products. EMBO J. **8**:3617–3625.
9. **Braun, T., E. Bober, B. Winter, N. Rosenthal, and H. H. Arnold.** 1990. Myf-6, a new member of the human gene family of myogenic determination factors: evidence for a gene cluster on chromosome 12. EMBO J. **9**:821–831.
10. **Braun, T., G. Buschhausen-Denker, E. Bober, E. Tannich, and H. H. Arnold.** 1989. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. EMBO J. **8**:701–709.
11. **Braun, T., E. Tannich, G. Buschhausen-Denker, and H. H. Arnold.** 1989. Promoter upstream elements of the chicken cardiac myosin light-chain 2-A gene interact with *trans*-acting regulatory factors for muscle-specific transcription. Mol. Cell. Biol. **9**:2513–2525.
12. **Brennan, T. J., and E. N. Olson.** 1990. Myogenin resides in the nucleus and acquires high affinity for a conserved enhancer element on heterodimerization. Genes Dev. **4**:582–595.
13. **Buskin, J. N., and S. D. Hauschka.** 1989. Identification of a myocyte nuclear factor that binds to the muscle-specific enhancer of the mouse muscle creatine kinase gene. Mol. Cell. Biol. **9**:2627–2640.
14. **Caudy, M., H. Vassin, M. Brand, R. Tuma, L. Y. Jan, and Y. N. Jan.** 1988. *daughterless*, a *Drosophila* gene essential for both neurogenesis and sex determination has sequence similarities to *myc* and the *achaete-scute* complex. Cell **55**:1061–1067.
15. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156–159.
16. **Clegg, C. H., T. A. Linkhart, B. B. Olwin, and S. D. Hauschka.** 1987. Growth factor control of skeletal muscle differentiation: commitment to terminal differentiation occurs in G_1 phase and is repressed by fibroblast growth factor. J. Cell Biol. **105**:949–956.
17. **Cronmiller, C., P. Schedl, and T. W. Cline.** 1988. Molecular characterization of *daughterless*, a *Drosophila* sex determination gene with multiple roles in development. Genes Dev. **2**:1666–1676.
18. **Davis, R. L., P.-F. Cheng, A. B. Lassar, and H. Weintraub.** 1990. The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. Cell **60**:733–746.
19. **Davis, R. L., H. Weintraub, and A. B. Lassar.** 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell **51**:987–1000.
20. **Donoghue, M., H. Ernst, B. Wentworth, B. Nadal-Ginard, and N. Rosenthal.** 1988. A muscle-specific enhancer is located at the 3′ end of the myosin light-chain 1/3 gene locus. Genes Dev. **2**:1779–1790.
21. **Dynan, W. S., and R. Tjian.** 1983. The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. Cell **35**:79–87.
22. **Edmondson, D. G., and E. N. Olson.** 1989. A gene with homology to the *myc* similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. Genes Dev. **3**:628–640.
23. **Fregien, N., and N. Davidson.** 1986. Activating elements in the promoter region of the chicken β-actin gene. Gene **48**:1–11.
24. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
25. **Gossett, L. A., D. J. Kelvin, E. A. Sternberg, and E. N. Olson.** 1989. A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes. Mol. Cell. Biol. **9**:5022–5033.
26. **Henthorn, P., M. Kiledjian, and T. Kadesch.** 1990. Two distinct transcription factors that bind the immunoglobulin enhancer μE5/κE2 motif. Science **247**:467–470.
27. **Jaynes, J. B., J. S. Chamberlain, J. N. Buskin, J. E. Johnson, and S. D. Hauschka.** 1986. Transcriptional regulation of the muscle creatine kinase gene and regulated expression in transfected mouse myoblasts. Mol. Cell. Biol. **6**:2855–2864.
28. **Jaynes, J. B., J. E. Johnson, J. N. Buskin, C. L. Gartside, and S. D. Hauschka.** 1988. The muscle creatine kinase gene is regulated by multiple upstream elements, including a muscle-specific enhancer. Mol. Cell. Biol. **8**:62–70.
29. **Konieczny, S. F., and C. P. Emerson, Jr.** 1984. 5-Azacytidine induction of stable mesodermal stem cell lineages from 10T1/2 cells: evidence for regulatory genes controlling determination. Cell **38**:791–800.
30. **Lassar, A. B., J. N. Buskin, D. Lockshon, R. L. Davis, S. Apone, S. D. Hauschka, and H. Weintraub.** 1989. MyoD is a sequence-specific DNA binding protein requiring a region of *myc* homology to bind to the muscle creatine kinase enhancer. Cell **58**:823–831.
31. **Lin, Z.-Y., C. A. Deschene, J. Eldrige, and B. M. Paterson.** 1989. An avian muscle factor related to MyoD1 activates muscle-specific promoters in nonmuscle cells of different germ-layer origin and in BrdU-treated myoblasts. Genes Dev. **3**: 986–996.
32. **Mar, J. H., and C. P. Ordahl.** 1988. A conserved CATTCCT motif is required for skeletal muscle-specific activity of the cardiac troponin T gene promoter. Proc. Natl. Acad. Sci. USA **85**:6404–6408.
33. **McKnight, S. L., and R. Kingsbury.** 1982. Transcriptional control signals of a eukaryotic protein-coding gene. Science **217**:316–324.
34. **Miner, J. H., and B. Wold.** 1990. Herculin, a fourth member of the MyoD family of myogenic regulatory genes. Proc. Natl. Acad. Sci. USA **87**:1089–1093.
35. **Minth, C. D., P. C. Andrews, and J. E. Dixon.** 1986. Characterization, sequence, and expression of the cloned human neuropeptide Y gene. J. Biol. Chem. **261**:11974–11979.
36. **Miwa, T., and L. Kedes.** 1987. Duplicated CArG box domains have positive and mutually dependent regulatory roles in expression of the human α-cardiac actin gene. Mol. Cell. Biol. **7**:2803–2813.
37. **Murre, C., P. Schonleber McCaw, and D. Baltimore.** 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, *daughterless*, *MyoD*, and *myc* proteins. Cell **56**:777–783.
38. **Murre, C., P. Schonleber McCaw, H. Vaessin, M. Caudy, L. Y. Jan, Y. N. Jan, C. V. Cabrera, J. N. Buskin, S. D. Hauschka, A. B. Lassar, H. Weintraub, and D. Baltimore.** 1989. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell **58**:537–544.
39. **Muscat, G. E. O., T. A. Gustafson, and L. Kedes.** 1988. A common factor regulates skeletal and cardiac α-actin gene transcription in muscle. Mol. Cell. Biol. **8**:4120–4133.
40. **Olson, E. N., E. Sternberg, J. S. Hu, G. Spizz, and C. Wilcox.** 1986. Regulation of myogenic differentiation by type β transforming growth factor. J. Cell Biol. **103**:1799–1805.
41. **Pinney, D. F., S. H. Pearson-White, S. F. Konieczny, K. E. Latham, and C. P. Emerson, Jr.** 1988. Myogenic lineage determination and differentiation: evidence for a regulatory gene pathway. Cell **53**:781–793.
42. **Rhodes, S. J., and S. F. Konieczny.** 1989. Identification of MRF4: a new member of the muscle regulatory factor gene family. Genes Dev. **3**:2050–2061.
43. **Schäfer, B. W., B. T. Blakely, G. J. Darlington, and H. M. Blau.** 1990. Effect of cell history on response to helix-loop-helix family of myogenic regulators. Nature (London) **344**:454–458.
44. **Sternberg, E. A., G. Spizz, M. E. Perry, D. Vizard, T. Weil, and E. N. Olson.** 1988. Identification of upstream and intragenic regulatory elements that confer cell-type restricted and differentiation-specific expression on the muscle creatine kinase gene. Mol. Cell. Biol. **8**:2896–2909.
45. **Tapscott, S. J., R. L. Davis, M. J. Thayer, P.-F. Cheng, H. Weintraub, and A. Lassar.** 1988. MyoD1: a nuclear phosphoprotein requiring a myc homology region to convert fibroblasts to myoblasts. Science **242**:405–411.
46. **Tapscott, S. J., A. B. Lassar, R. L. Davis, and H. Weintraub.** 1989. 5-Bromo-2′-deoxyuridine blocks myogenesis by extinguishing expression of MyoD1. Science **245**:532–536.
47. **Thayer, M. J., S. J. Tapscott, R. L. Davis, W. E. Wright, A. B. Lassar, and H. Weintraub.** 1989. Positive autoregulation of the

myogenic determination gene MyoD1. Cell **58**:241–248.
48. **Thisse, B., C. Stoetzel, C. Gorostiza-Thisse, and F. Perrin-Schmidt.** 1988. Sequence of the *twist* gene and nuclear localization of its protein in endomesodermal cells of early *Drosophila* embryos. EMBO J. **7**:2175–2183.
49. **Weiher, H., M. Konig, and P. Gruss.** 1983. Multiple point mutations affecting the simian virus 40 enhancer. Science **219**: 626–631.
50. **Weintraub, H., S. J. Tapscott, R. L. Davis, M. J. Thayer, M. A. Adam, A. B. Lassar, and A. D. Miller.** 1989. Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. Proc. Natl. Acad. Sci. USA **86**:5434–5438.
51. **Wice, B., J. Milbrandt, and L. Glaser.** 1987. Control of muscle differentiation in BC3H1 cells by fibroblast growth factor and vanadate. J. Biol. Chem. **262**:1810–1817.
52. **Wright, W. E., D. A. Sassoon, and V. K. Lin.** 1989. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. Cell **56**:607–617.
53. **Vaidya, T. B., S. J. Rhodes, E. J. Taparowsky, and S. F. Konieczny.** 1989. Fibroblast growth factor and transforming growth factor β repress transcription of the myogenic regulatory gene MyoD1. Mol. Cell. Biol. **9**:3576–3579.
54. **Yutzey, K. E., R. L. Kline, and S. F. Konieczny.** 1989. An internal regulatory element controls troponin I gene expression. Mol. Cell. Biol. **9**:1397–1405.