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A Combination of MEF3 and NFI Proteins Activates Transcription in a Subset of Fast-Twitch Muscles

FRANÇOIS SPITZ, MARJO SALMINEN,† JOSIANE DEMIGNON, AXEL KAHN, DOMINIQUE DAEGELEN, AND PASCAL MAIRE*

Institut Cochin de Génétique Moléculaire, INSERM U129, Université René Descartes, 75014 Paris, France

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The human aldolase A pM promoter is active in fast-twitch muscles. To understand the role of the different transcription factors which bind to this promoter and determine which ones are responsible for its restricted pattern of expression, we analyzed several transgenic lines harboring different combinations of pM regulatory elements. We show that muscle-specific expression can be achieved without any binding sites for the myogenic factors MyoD and MEF2 and that a 64-bp fragment comprising a MEF3 motif and an NFI binding site is sufficient to drive reporter gene expression in some but, interestingly, not all fast-twitch muscles. A result related to this pattern of expression is that some isoforms of NFI proteins accumulate differentially in fast- and slow-twitch muscles and in distinct fast-twitch muscles. We propose that these isoforms of NFI proteins might provide a molecular basis for skeletal muscle diversity.

Adult skeletal muscle is composed of different types of myofibers. Each myofiber exhibits specific biochemical and physiological properties, which are determined mainly by the different isoforms of contractile proteins and metabolic enzymes expressed. The four major types of mammalian skeletal muscle fibers (determined by their content in myosin heavy-chain [MHC] isoforms) comprise one slow-twitch oxidative type (type I) and three fast-twitch types, oxidative (type IIA), glycolytic (type IIB), and glycolytic-oxidative (type IIX) (12, 40). Although several factors including hormonal influence (21), innervation, and muscle activity (reviewed in reference 40) seem to take part in the modulation of the fiber type pattern of adult muscles, the molecular mechanisms involved in the establishment of the diversity of skeletal muscle fibers remain poorly understood. It has been suggested that distinct cell lineages could exist—at least in chicken—and preferentially give rise either to fast or slow fibers (10, 56). However, no definitive evidence for the existence of fast or slow precursors in mammals has been obtained. Moreover, in addition to fiber type diversity, each muscle is unique by its position, its shape, its function, and the nature of the motoneurons which innervate it; the mechanisms responsible for the specification of such muscle diversity are still unknown (reviewed in reference 12).

Several transcriptional factors involved in muscle-specific gene expression have been characterized; among them, the most extensively studied ones are the myogenic bHLH proteins (MyoD, Myf5, myogenin, and MRF4), which activate transcription through an E-box motif, and the MEF2/RSRF factors, which bind to AT-rich sequences (reviewed in reference 5). However, none of the known transcriptional factors has been proven to account for muscle diversity.

Characterization of the *cis*-acting sequences involved in the expression of fiber type-specific genes and identification of the corresponding *trans*-acting proteins should shed light on the factors involved in the establishment of the different fiber types. A growing number of regulatory sequences involved in the pattern of expression of fast or slow muscle-specific genes have been characterized; these include myosin light chain fast (MLC1fast) (11, 42), MLC3fast (24), troponin I slow (TnIslow) (8, 36), TnIfast (3, 16, 36), MHCβ (25), and aldolase A (50, 52). However, so far, no single *cis*-acting element able to direct expression in a particular fiber type has been characterized.

We have previously shown that the proximal sequences of the human aldolase A muscle-specific promoter (pM) are sufficient to direct the expression of a chloramphenicol acetyltransferase (CAT) reporter gene in muscles composed mainly of fast-twitch glycolytic fibers in transgenic mice (50). This small regulatory region comprises binding sites for several families of transcription factors (51). Mutational analysis of these binding sites performed in transgenic mice revealed that three of them were necessary for a high level of activity (52). Mutation of either one of the two consensus MEF3 sites, of the M1 sequence (which interacts with unknown factors), or of the upstream stimulatory factor/major late transcription factor (USF/MLTF) binding site nearly abolished pM activity. In addition, an overlapping MEF2-NFI binding site displayed an activating function whose importance varied among individual muscles. Nevertheless, whichever binding site was mutated, at least one transgenic line displayed a correct tissue-specific pattern of expression. As a consequence, these results did not allow us to distinguish binding sites which dictate the pM pattern of expression from those which are required only for enhancing tissue-specific activity. Moreover, it could not be excluded that the elements which determine fast-twitch muscle specificity are localized elsewhere in our transgene (i.e., in the M exon or in the TATA box).

To find which DNA binding site(s) is sufficient to confer a specific expression to fast-twitch muscles, we decided to examine the ability of different subfragments from the pM regulatory region to convert a neutral promoter into a fast-muscle-specific one. Our findings demonstrate that muscle-specific expression can be achieved without any binding sites for the myogenic regulatory factors of the MEF2 and MyoD families.

* Corresponding author. Mailing address: ICGM, INSERM U129, 24 rue du Faubourg St Jacques, 75014 Paris, France. Phone: (33) 1 44 41 24 16. Fax: (33) 1 44 41 24 21. E-mail: maire@icgm.cochin.inserm.fr.

† Present address: Department of Molecular Cell Biology, Max Planck Institute for Biophysical Chemistry, D-37077 Göttingen, Germany.

We found that different combinations of binding sites can lead to a fiber type-specific expression and that the MEF3 motif is always required for such activity. Moreover, a small fragment comprising only the MEF3 and the overlapping MEF2-NFI binding sites is sufficient to confer a fast-muscle-specific activity to the herpes simplex virus thymidine kinase minimal promoter (tk-105), but, surprisingly, this occurs only in a subset of fast-twitch muscles. We also show that some NFI proteins are differentially expressed in skeletal muscles, with a pattern that correlates with the NFI binding-site-mediated activation of pM. These results show that transcriptional activity in fast-twitch muscles is regulated through distinct pathways and suggest that NFI transcription factors could provide a molecular basis for muscle identity in mammals.

MATERIALS AND METHODS

Plasmid constructions. The pM310CAT construct has been described previously (50). The dmMEF2-E construct was obtained by introducing the *Eco*NI-*Xma*I fragment from the mE construct instead of the corresponding fragment in the mMEF2 construct (51). Thus, the resulting construct carries two linker mutations (introduction of a *Bgl*II site), one in the MEF2 site and the other in the E box of pM310CAT (see Fig. 1). For the pM-thymidine kinase promoter chimeric constructs, different pM subfragments were inserted upstream from the tk-105 promoter in the pBLCAT2 plasmid (31). The M-tkCAT construct was obtained by introducing a blunt-ended *Age*I-*Alu*I fragment containing the whole pM regulatory region (from −235 to −35) upstream of the tk-105 promoter (see Fig. 2). MΔB-tkCAT was created by insertion of the *Bam*HI-*Bam*HI (pM sequence from −310 to −101) fragment from pM310CAT into the *Bam*HI site of pBLCAT2. ΔAB-tkCAT and ΔAT-tkCAT plasmids were constructed by deletion of the *Hin*dIII-*Apa*I (up to −164) fragment from MΔB-tkCAT and M-tkCAT, respectively. MΔ3-tkCAT construct was created by an insertion of the *Hin*dIII-*Bgl*II fragment (pM sequences from −310 to −120) from mMEF2 into an *Hin*dIII-*Bam*HI-digested pBLCAT2 vector. All constructs were verified by dideoxy sequencing and propagated in *Escherichia coli* B2-37 to avoid DNA methylation.

Cell culture. Primary chicken myoblasts were prepared from the hind limb muscles of 10-day-old embryos. Briefly, hind limb muscles were dissected into small pieces with scissors in minimal essential medium followed by mechanical dissociation through a fine pipette. Cells were plated on gelatin-coated 5-mm petri dishes (1.4 million cells when cultured to form myotubes, 500,000 for myoblasts). The culture medium was composed of 3 volumes of minimal essential medium for 1 volume of medium 199 (Gibco) complemented with 10% fetal calf serum. Transfection analyses were performed by the calcium phosphate coprecipitation method with 5 μg of test plasmid and 250 ng of Rous sarcoma virus luciferase vector (9) as an internal standard for transfection efficiency. Transfection was performed 4 h after plating. After incubation with the precipitate for 4 h, medium was removed, cells were washed twice with 0.9% NaCl, and fresh medium was added. Cells were harvested as myoblasts 20 h later or as myotubes 5 days later (the medium was changed on day 3). Luciferase and CAT activities were determined as previously described (51). For each construct, the CAT activity was calculated after normalization for transfection efficiency (measured by luciferase activity) and with pBLCAT2 activity set to 1 in myoblasts and myotubes.

Transgenic mice and CAT analysis. For creating transgenic mice, DNA fragments were purified as previously described (7). M-tkCAT and tk-105 fragments were isolated after *Hin*dIII and *Sst*I digestion of M-tkCAT and pBLCAT2 plasmids, respectively; MΔB-tkCAT and MΔ3-tkCAT were isolated after *Age*I and *Sac*I digestion of the corresponding plasmids; ΔAB-tkCAT and ΔAT-tkCAT fragments were isolated after *Bsp*120I (recognition site as *Apa*I) and *Sac*I digestion of MΔB-tkCAT and M-tkCAT plasmids, respectively; the ΔPT-tkCAT fragment was isolated after *Ppu*MI and *Cla*I digestion of M-tkCAT plasmid.

Transgenic mice were generated, identified, and propagated as previously described (52). The transgene copy numbers were determined from F_1 transgenic mice as previously described (50), except that Southern blots were scanned with a PhosphorImager by using Imagequant V3.3 software (Molecular Dynamics). For CAT assays, various tissues were dissected from adult (at least 7-week-old) F_1 transgenic animals, except for the few founders analyzed, for which the transgene presence in each tissue was verified by Southern blotting. CAT activity was measured as previously described (50).

Nuclear extract preparation and gel mobility shift assays. Nuclear extracts from rat spleen and liver were prepared as previously described (32). Nuclear extracts from rat muscles were prepared as described in references 26, 32, and 51. In detail, muscles were homogenized (20 volumes of buffer per weight of muscle) in homogenization buffer (2.4 M sucrose, 10 mM HEPES [pH 7.6], 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 2 mM EDTA) in a Waring blender homogenizer. The homogenate was then diluted twofold in dilution buffer (10 mM HEPES [pH 7.6], 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 2 mM EDTA, 1% low-fat dry milk, 0.4% Triton X-100) and filtered through two layers of gauze on a stainless-steel screen. The homogenate was then centrifuged at 3,000 × *g* for 5 min. The pellet was resuspended in homogenization buffer containing 2.3 M sucrose (5 volumes of buffer/initial weight of tissue), homogenized in a Potter-Elvehjem glass Teflon homogenizer, and layered on top of prepared 10-ml cushions of homogenization buffer made with 2 M sucrose-10% glycerol. The tubes were placed in Beckman SW-27 buckets and spun at 22,000 rpm for 1 h. The following ingredients were added before use in the homogenization buffers and dilution buffer: 1 mM dithiothreitol, 0.1% aprotinin, 0.7 μg of pepstatin per ml, 0.7 μg of leupeptin per ml, 0.1 mM benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride. Nuclear proteins were extracted from the pelleted nuclei was done as described elsewhere (15). Gel mobility shift assays were performed as previously described (51).

Western blots. Western blotting was carried out essentially as described by Harlow and Lane (18). After electrotransfer, proteins bound on nitrocellulose were transiently stained with Ponceau S as a control. Rabbit antibodies raised against human CTF1, *Xenopus* NFI-B1, or *Xenopus* NFI-X3 were gifts from N. Tanese, M. Puzianowska-Kuznicka, and B. Corthesy and W. Wahli, respectively. Rabbit antibody against Sp1 was obtained from Santa Cruz Biotechnology Inc. These antibodies were used to detect corresponding proteins among the blotted nuclear proteins. Bound antibodies were detected by peroxidase-conjugated swine anti-rabbit immunoglobulin (Dako), and revealed with enhanced chemiluminescence reagent from Amersham. Contacts between the membrane and X-Omat films (Kodak) varied from 30 s (for Sp1 detection) to 10 min (for NFI-B).

RESULTS

pM is still active in fast-twitch muscles when both the E-box and the MEF2 binding site are mutated. The pM regulatory sequences contain putative binding sites for the MEF2 and MyoD families of myogenic factors. We have previously shown that these sites are poorly bound, if at all, by these proteins (51). Moreover, we have shown that the muscle-specific expression of pM is conserved in transgenic mice despite mutation of either the MEF2 site or the E box (52). However, since it has been shown that the MyoD and MEF2 factors could interact and activate transcription through either a MEF2 or MyoD binding site (35), it is possible that separate mutations do not prevent these factors from controlling the activity of pM in vivo.

Therefore, we mutated both the MEF2 site and E box in the pM310CAT construct (consisting of the bacterial CAT reporter gene under the control of pM regulatory sequences) (Fig. 1). In transient-transfection experiments, this construct (dm MEF2-E) was still activated 4- to 6-fold during the differentiation of avian myoblasts, while a 12-fold activation was measured for the wild-type promoter (data not shown). Four transgenic lines were generated with this mutant transgene, and CAT activity was analyzed in various tissues of the F_1 progeny (Table 1; Fig. 1). In three independent lines, the transgene was expressed in fast-twitch muscles, either the gastrocnemius or the vastus lateralis, but not (or to a much lesser extent) in the slow-twitch soleus. These results clearly demonstrate that muscle-specific expression can be achieved in cell culture and in vivo without binding sites for MEF2 and MyoD factors and suggest that alternate regulatory mechanisms can lead to muscle-specific expression.

To define the mechanisms underlying pM muscle-specific expression, we have determined the minimal fragment of pM regulatory regions sufficient to confer muscle specificity on a neutral promoter in cell culture and in transgenic mice.

The regulatory elements of pM are able to confer a muscle-specific expression to the tk-105 minimal promoter in cell culture. In transient-transfection assays, tk-105 is active in nearly every cell type. In transgenic mice, it displays activity only as a result of position effects (1). Various tissue-specific enhancers have been shown to activate the tk-105 promoter in the appropriate manner. In particular, chimeric constructs with the tk-105 promoter and muscle fiber-specific enhancers displayed the expected pattern of expression: slow type specific with the TnI-slow upstream enhancer (8), and fast type specific

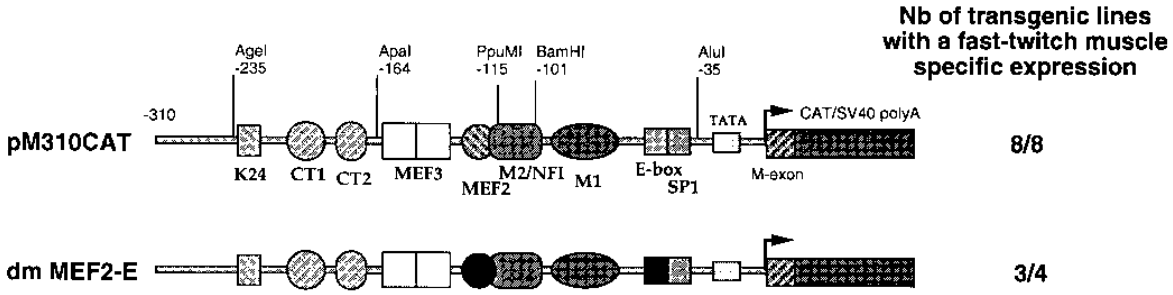

FIG. 1. Schematic representation of the pM310CAT and dmMEF2-E constructs. The protein binding sites detected by footprinting assays (49, 51) are indicated as boxes. The restriction sites used are indicated and localized from the pM transcription start site. The mutated E box and MEF2 binding site are shown in black. The number of transgenic lines analyzed and the number of those with a fast-muscle-specific expression are given. Fast-twitch specificity corresponds to at least a 10-fold-higher expression in either gastrocnemius or vastus lateralis compared to soleus. For pM310CAT, data are from reference 50.

with the MLC1 fast enhancer (33). Thus, tk-105 seemed to be suitable as a neutral promoter for our experiments.

We have cloned an *Age*I-*Alu*I fragment of pM regulatory sequences (from bp −235 to −35 relative to the transcription initiation site) upstream from the tk-105 minimal promoter with the CAT gene as a reporter gene. This fragment comprised all sequences that have been shown to be required for pM activity but the TATA box and M exon (Fig. 2). The resulting construct, named M-tkCAT, was transiently transfected in primary chicken myoblasts. In these myoblasts, the activity of the M-tkCAT construct was not very different from that of the control tk-105 construct. In contrast, when myoblasts differentiated into myotubes, the activity of M-tkCAT became ninefold higher than that of the tk-105 construct (Fig. 2). Thus, the pM regulatory sequences between −235 and −35 are able to confer a myotube-specific activation to the tk-105 promoter. Myotube-specific enhancer activity of pM sequences was conserved with the subfragment MΔB (fivefold stimulation between myoblasts and myotubes). In contrast, when the distal sequences were deleted, as in ΔAT or ΔAB, only a weak induction was observed in myotubes. However, these sequences were not by themselves responsible for the strong induction in myotubes, since the MΔ3-tkCAT construct was also induced poorly if at all. This is in agreement with the previous observations that the MEF2-NFI element is required for high-level pM activity in differentiated myotubes (51). Nevertheless, even if associated with the MEF3 motif (ΔAB), this element is not sufficient to induce myotube specificity, indicating that other cooperating elements are required.

The M exon and the most proximal pM sequences are not required for fast-twitch muscle-specific expression in transgenic mice. We tested whether the M-tkCAT construct displayed a fast-twitch glycolytic muscle-specific pattern in vivo, like the original pM310CAT construct (50). We generated four transgenic mice harboring the chimeric M-tkCAT construct and assayed CAT activity in muscles of the F_1 progeny of these mice (Fig. 3; Table 1). In all lines, the transgene was active in the gastrocnemius and vastus lateralis, two hind limb muscles composed mainly of fast-twitch glycolytic fibers; the transgene was far less active in the soleus, which is composed mainly of slow-twitch oxidative fibers and is devoid of type IIB fibers (11, 17). The ratio of CAT activity in the fast-twitch muscles to that in the soleus is similar in the M-tkCAT transgenic lines (at least 40-fold) and in the pM310CAT transgenic lines (from 20- to 450-fold) (50). No activity was found in other tissues tested, except rare ectopic expression varying from one line to another. In transgenic mice harboring the control tk-105CAT construct, no activity was detected in the muscles of nine different lines (whose copy number varied approximately from 5 to 100 [data not shown]). These results show that the pM sequences between −235 and −35 are sufficient to confer a fiber type-specific expression in transgenic mice to the tk-105 promoter.

It is also worth noting that, like the pM310CAT transgene, the M-tkCAT transgene was active in all the transgenic lines analyzed: this observation suggests that the pM regulatory elements are sufficient to avoid the complete repression of a transgene by the surrounding chromatin. However, while a correlation between the copy number and the level of activity was observed for the pM310CAT transgenes (50), this was not the case with the M-tkCAT transgenes (Table 1). Thus, it seems that pM regulatory sequences act more efficiently against chromatin-dependent repression when they are linked to their innate TATA box and transcription start site.

Two overlapping fragments can direct expression into fast-twitch muscles of transgenic mice. The capacity of the different subregions of pM regulatory sequences to confer fast-twitch muscle-specific expression to the tk-105 promoter was addressed by creating several transgenic lines harboring various pM/tk-105 chimeric constructs (Fig. 3; Table 1).

The ΔAT fragment was able to activate tk-105 in the fast-twitch muscles in 6 of 10 transgenic lines analyzed, but the level of activity varied widely from one line to another. However, when the transgene was active in skeletal muscles, the level of activity was always at least 100-fold higher in the gastrocnemius and the vastus lateralis than in the soleus. Therefore, the ΔAT fragment retains sufficient information for myofiber type-specific expression. However, ΔAT-tkCAT transgenes seem to be much more prone to position effects than M-tkCAT transgenes do. These results suggest that the distal region (bp −235 to −164) is required for establishment of an open chromatin structure and/or for a high level of activity, but not for fast-twitch muscle specificity. These results are in agreement with those reported previously (52) with the pM310CAT transgene: deletion of the sequences upstream of position −164 led to a 12-fold decrease in the expression of the transgene. This distal region also seems to be required for silencing the transgene in nonmuscle tissues, since ectopic expression was observed in the liver and brain of four independent ΔAT-tkCAT transgenic lines whereas ectopic activity was very rare in the M-tkCAT mice. In vitro DNase I footprinting with liver and muscle nuclear extracts revealed three protected regions within this distal sequence (49). One region (K24) comprises a consensus recognition sequence for Krox-like proteins (30); the other two (CT1 and CT2) correspond to CT-rich sequences.

A further 5′ deletion of the ΔAT fragment which removes the MEF3 motif and the putative MEF2 binding site (fragment ΔPT) led to a complete lack of expression in the three trans-

TABLE 1. CAT activity in tissues of transgenic mice

Transgenic mouse line	Copy no.	CAT activity (cpm/μg of protein/min)[b] in:							
		Liver	Kidney	Spleen	Heart	Brain	Gast.[c]	V.L.[c]	Soleus
dm MEF2-E									
1	3	<0.01	<0.01	<0.01	<0.01	<0.01	2.2	27.3	<0.01
20	3	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
52	16	<0.01	<0.01	<0.01	<0.01	0.35	<0.01	6.6	<0.01
27	18	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.6	<0.01
M-tkCAT									
8	2	<0.01	<0.01	<0.01	<0.01	<0.01	173.3	159.5	1.1
79	2	<0.01	<0.01	<0.01	<0.01	<0.01	1.8	2.1	<0.01
81	3	<0.01	<0.01	<0.01	<0.01	<0.01	0.2	2.7	<0.01
86	12	<0.01	<0.01	<0.01	<0.01	9.0	869	800.4	17.6
ΔAT-tkCAT									
41	2	ND[c]	ND	ND	ND	ND	<0.01	<0.01	<0.01
8	3	0.11	<0.01	<0.01	<0.01	<0.01	0.32	0.2	<0.01
19	7	<0.01	<0.01	<0.01	<0.01	0.49	2,476	2,073	2.91
13	12	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
20a	13	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
31a	14	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
20b	15	0.27	<0.01	<0.01	<0.01	2.8	110	109	0.3
46	15	<0.01	<0.01	<0.01	<0.01	<0.01	0.9	1.2	<0.01
51	20	0.15	<0.01	<0.01	<0.01	0.1	0.13	0.1	<0.01
31b	25	0.22	<0.01	<0.01	<0.01	0.27	12.9	6.29	0.07
ΔPT-tkCAT									
25	2	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
21	5	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
31	6	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
MΔB-tkCAT									
38a	3	0.34	<0.01	<0.01	<0.01	0.1	<0.01	<0.01	<0.01
38b	3	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
62	4	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
67	5	<0.01	1.0	<0.01	<0.01	2.3	6.8	5.4	0.6
MΔ3-tkCAT									
54	2	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
56(fd)[a]	2	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
60	3	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
58(fd)[a]	10	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
72	15	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
52	25	<0.01	<0.01	<0.01	<0.01	0.23	<0.01	<0.01	<0.01
ΔAB-tkCAT									
16	2	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
17a	2	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
22(fd)[a]	3	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
17b	3	<0.01	<0.01	<0.01	<0.01	<0.01	0.16	<0.01	<0.01
24	4	<0.01	<0.01	<0.01	<0.01	<0.01	0.15	<0.01	<0.01
9(fd)[a]	5	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
20	7	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
25	8	<0.01	<0.01	<0.01	<0.01	<0.01	0.15	<0.01	<0.01
27	12	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
13	14	<0.01	<0.01	<0.01	<0.01	<0.01	0.48	<0.01	<0.01
12	15	<0.01	<0.01	<0.01	<0.01	<0.01	0.06	0.07	<0.01
10	17	<0.01	<0.01	<0.01	<0.01	<0.01	0.56	<0.01	<0.01
30	17	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

[a] Only the founder mouse was analyzed.
[b] <0.01 means that no activity above background was detected under any condition of time and amount of proteins tested.
[c] Gast., gastrocnemius; V.L., vastus lateralis; ND, not done.

genic lines analyzed (Fig. 3; Table 1). Therefore, the MEF3 or MEF2 binding site, or both, are required for the enhancer activity of the ΔAT fragment.

The ΔPT fragment contains binding sites for USF and SP1 factors, as well as the M1 sequence, which binds unknown proteins (51). When these proximal sites were removed (fragment MΔB), the transgene was expressed in only one of four lines tested (Fig. 3; Table 1). However, the CAT activity in this

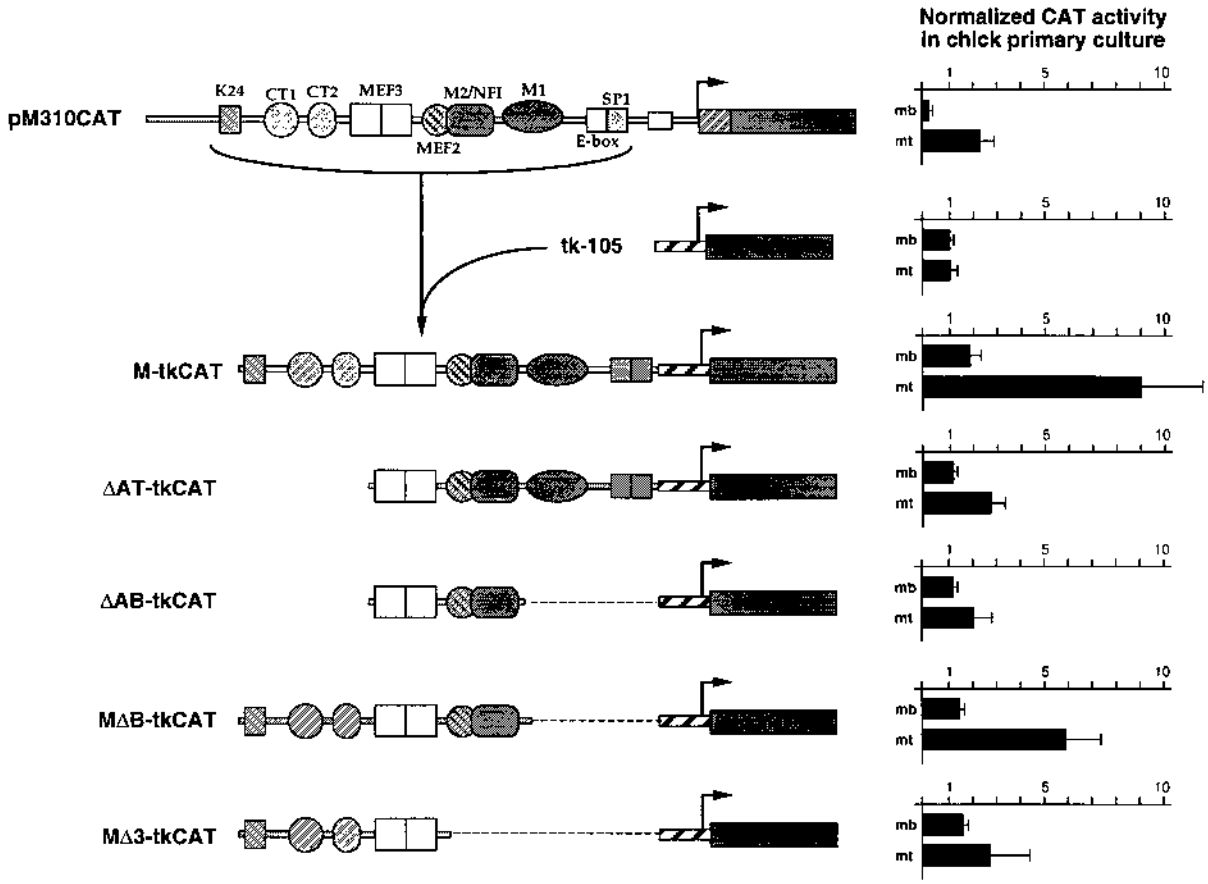

FIG. 2. Activity of pM310CAT and pM/tk-105 chimeric constructs in transient-transfection assays in chicken primary myoblasts or myotubes. Each value represents the mean of three to five experiments.

line is still 10-fold higher in fast muscles (gastrocnemius and vastus lateralis) than in slow muscles (soleus), suggesting that the pM proximal region (USF, SP1, and M1) is not absolutely required to obtain fast-muscle specificity.

When the proximal deletion was extended to involve the MEF2-NFI element (MΔ3-tkCAT), no expression was detected in muscles of the transgenic mice tested (four lines and two founders) (Fig. 3; Table 1). This result shows that the MEF3 sites together with the distal region are not sufficient to reproduce muscle-specific expression and demonstrates the importance of the MEF2-NFI element in this context.

A small fragment containing the MEF3 motif and the MEF2-NFI binding site is sufficient to confer expression in a subset of adult muscles. Since the ΔAT and the MΔB fragments could confer muscle specificity on the tk-105 promoter, we tested whether the region common to both fragments (ΔAB), containing only the MEF3 and MEF2-NFI binding sites, was sufficient for fast-twitch muscle specificity in vivo. We studied the expression of the ΔAB-tk transgene in 2 founders and in 11 different lines (Fig. 3; Table 1). In five independent transgenic lines, a low (but clearly detectable) activity was observed in the fast gastrocnemius muscle, whereas there was no activity in the slow soleus or, surprisingly, in the fast vastus lateralis, whose myofiber composition is very similar to that of the gastrocnemius (40). An inverse discrepancy for transgene expression between these two fast-twitch muscles was observed with mutations in pM310CAT which reduced (mMEF2, dmMEF2-E) or abolished (mM2) NFI binding (52) (Table 1): all these transgenes gave a high level of expression in the vastus lateralis and only a low level of expression in the gastrocnemius.

To find if the ΔAB-tkCAT transgene was able to function in muscles other than the gastrocnemius, we assayed CAT activity in additional muscles of ΔAB-tkCAT mice (line 24). Five of these muscles were composed mainly of type IIB fibers, like the gastrocnemius and vastus lateralis: tibialis anterior, extensor digitorum longus (EDL), psoas major, triceps brachii, and pectoralis major. We also analyzed activity in the soleus (types I and IIA) and the diaphragm (mixed IIA and IIX). As a control for variations due to the difference in fiber type composition, CAT activity was analyzed in the same muscles in M-tkCAT (line 8) transgenic mice (Fig. 4). For M-tkCAT-8 mice, CAT activities in the soleus and diaphragm were less than 5% of the activity found in the gastrocnemius. These results are consistent with pM regulatory sequences directing expression of the transgene essentially in type IIB fibers and not in type IIX or IIA (the predominant fibers in the diaphragm) or in type I. Furthermore, in M-tkCAT mice, CAT activity is very similar in all fast-twitch (mainly type IIB) muscles. However, for the ΔAB-tkCAT transgenic mice, CAT activity was detected in some fast-twitch muscles only: gastrocnemius, tibialis anterior, and triceps brachii. Several muscles (vastus lateralis, EDL, pectoralis major, and psoas major) that were also composed mainly of type-IIB fibers were surprisingly devoid of CAT activity. Therefore, fast versus slow specificity is retained in the

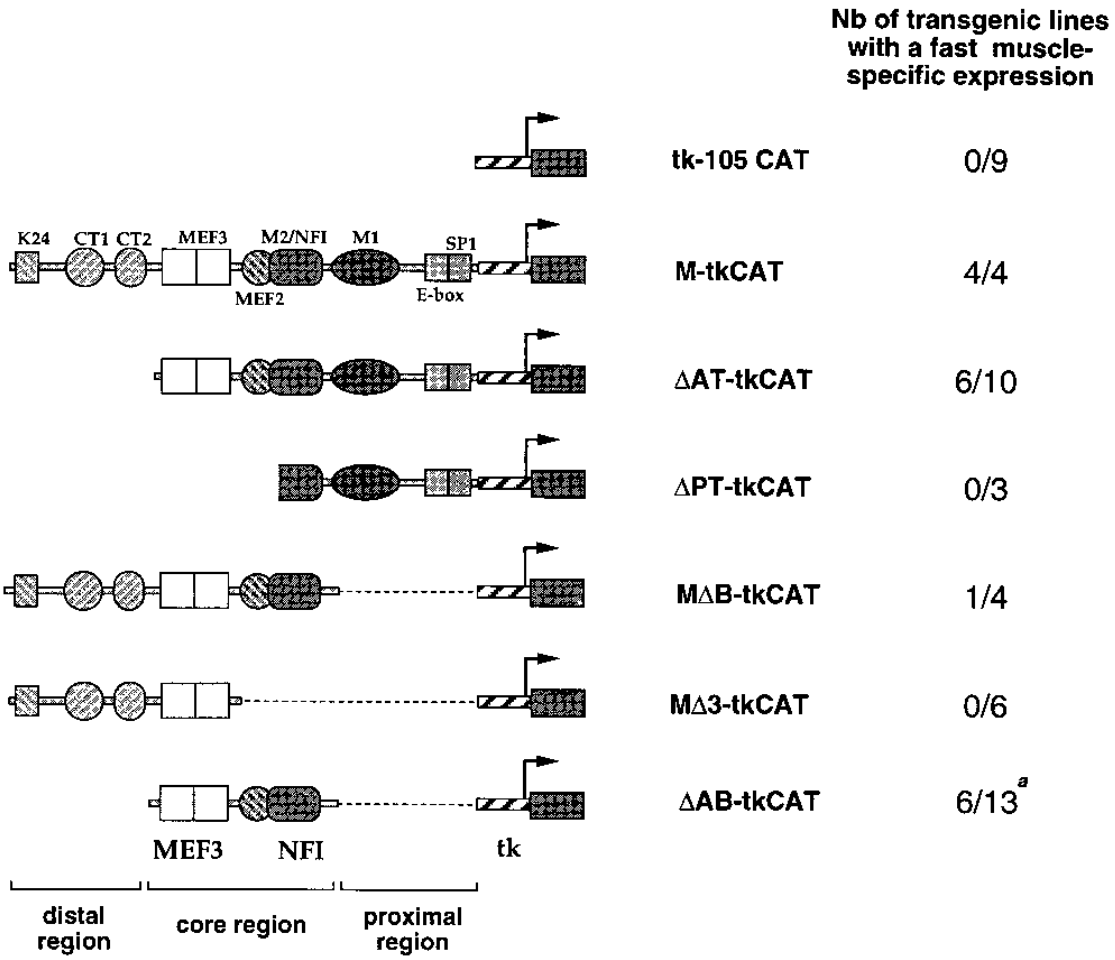

FIG. 3. Activity of pM/tk-105 hybrid constructs in fast-twitch muscles of transgenic mice. The various constructs are represented schematically, and the proportion of transgenic lines displaying a fast-twitch muscle-specific expression among the transgenic lines analyzed is given. [a]Five of six lines displayed activity in the gastrocnemius but not in the vastus lateralis.

ΔAB-tk mice, but expression is limited to a subset of fast-twitch muscles.

The ΔAB fragment is bound by ubiquitous MEF3 proteins and by NFI complexes. The ΔAB fragment contains a MEF3 motif and an overlapping MEF2-NFI site. Previous work has shown that the MEF3 site (consensus sequence, TCAGGTTT) forms complexes with proteins from liver and skeletal muscle (20, 38). We investigated the nature and amount of the proteins bound on this motif in various muscles by gel mobility shift assays. Nuclear extracts were prepared from soleus (slow twitch), gastrocnemius and triceps brachii (fast glycolytic and in which the ΔAB transgene was active), and vastus lateralis (fast glycolytic and in which the ΔAB transgene was inactive). MEF3 complexes were identical in amount and in mobility in all muscle nuclear extracts used (Fig. 5A). The amount of nuclear proteins in the various extracts was controlled with a consensus binding site for the ubiquitous NFY protein as a standard (44) (Fig. 5B). Consequently, despite their role in pM fast-twitch muscle-specific expression, MEF3 proteins seem to be equally expressed in all types of muscles.

For the MEF2-NFI sequence, in agreement with previous data (51), we detected binding mainly of NFI-like complexes (Fig. 6); only a very faint binding of MEF2 proteins could be detected, and this required using a large amount of nuclear proteins (data not shown). Complexes formed with muscle nuclear extracts migrated faster than those formed with liver nuclear extracts, which suggests that NFI proteins are different between these two tissues (Fig. 6A). These retarded complexes were identified as NFI proteins by competition with the NFI binding site of the adenovirus major late promoter and supershift by anti-NFI-C antibody. However, even with a large amount of this antibody, we observed a residual nonsupershifted band that we assumed to correspond to other types of NFI complexes devoid of the NFI-C subunit. The differential interaction of NFI proteins with the MEF2-NFI site in skeletal muscle and liver is reminiscent of our previous results (51) showing that a muscle-specific DNase I hypersensitive site located in the same region seemed to be related to the interaction of muscle, but not liver, NFI factors with its cognate DNA site. Taken together, these results strongly suggest that muscle-specific isoforms of the NFI family bind to the pM MEF2-NFI element. However, gel shift assays did not show any clear difference between NFI proteins in nuclear extracts from different slow or fast muscles (Fig. 6B).

NFI proteins are differentially expressed in different muscles. About 12 different isoforms of NFI proteins have been described (27). They are produced by alternative splicing of mRNAs issued from four distinct genes in vertebrates (A, B, C, and X), and have molecular masses from 45 to 65 kDa. These proteins bind DNA as dimers, and all combinations between

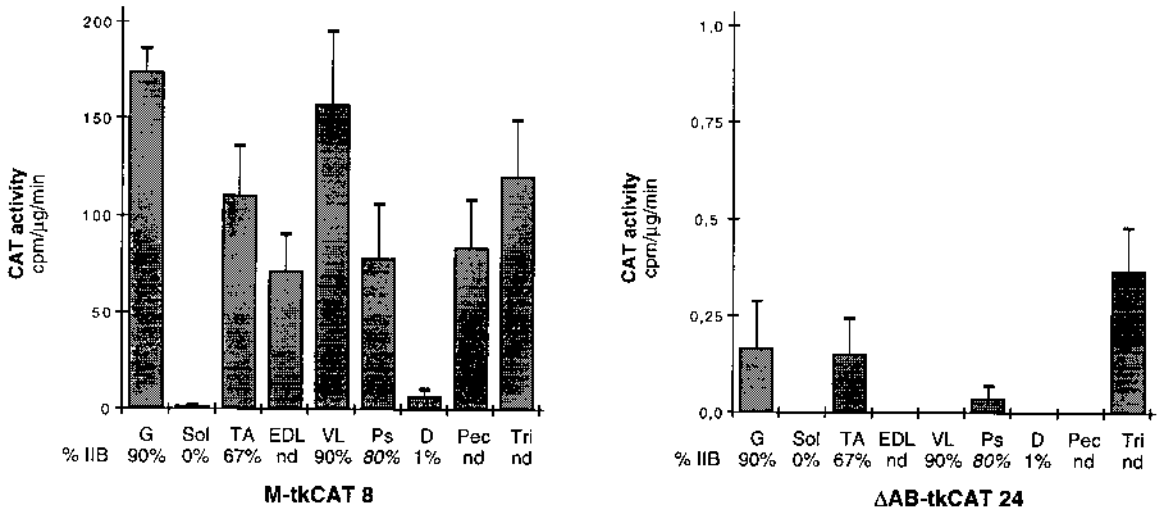

FIG. 4. CAT activity in various adult muscles of ΔAB-tkCAT (line 24) and M-tkCAT (line 8) transgenic mice. The different muscles used were gastrocnemius (G), tibialis anterior (TA), vastus lateralis (VL), EDL, soleus (Sol), psoas major (Ps), diaphragm (D), pectoralis major (Pec), and triceps brachii (Tri). The proportion of type IIB fibers for each muscle is indicated when known (nd, not determined; italic, data from rat muscles). Data were compiled from references 11, 17, and 40. Note that the scale is not the same for M-tkCAT and ΔAB-tkCAT.

the various isoforms are possible (28). This complexity may mask the presence of tissue-specific isoforms in gel shift experiments. Therefore, we performed a Western blot analysis to determine the pattern of expression of NFI proteins in various tissues and in different muscles. For this purpose, we used the following antibodies: an NFI-C antibody raised against the human CTF1 protein (54), one raised against the carboxy-terminal part of the *Xenopus* NFI-X3 protein (47), and one raised against the *Xenopus* NFI-B1 protein (41). As NFI proteins are extremely highly conserved among vertebrates (about 95% identity for NFI-B between *Xenopus* and rodents or for NFI-C between humans and rodents), these antibodies should recognize the cognate NFI proteins in rat nuclear extracts. In addition, the carboxy-terminal part of NFI-X3 is conserved in some NFI-A isoforms (92% identity on one-third of the 87-amino-acid antigenic peptide), such that the NFI-X3 antibody may recognize NFI-A isoforms as well.

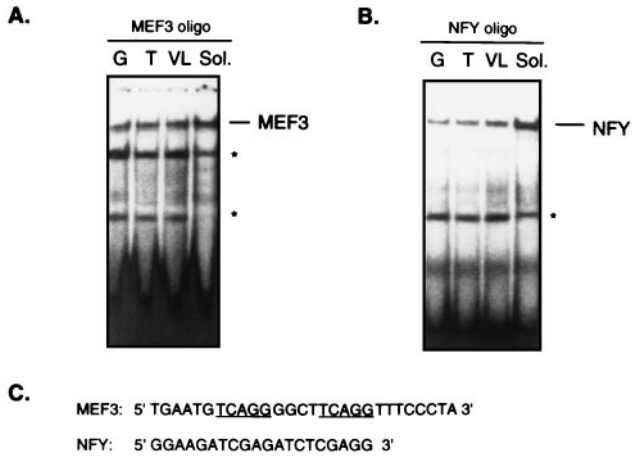

FIG. 5. (A and B) Gel mobility shift assays with the MEF3 sequence (A) or a NFY binding site (B). The end-labelled double-stranded oligonucleotides were incubated with nuclear extracts from rat gastrocnemius (G), triceps (T), vastus lateralis (VL), and soleus (Sol.) and analyzed on nondenaturing polyacrylamide gels. Nonspecific complexes are indicated by asterisks. These complexes were not competed by MEF3 or NFY oligonucleotides (data not shown). (C) Sequences of the oligonucleotides used.

As shown in Fig. 7A, NFI isoforms were not equally distributed in all tissues: both the relative amount and the nature of the bands revealed in spleen, liver, and skeletal muscle nuclear extracts were different. Moreover, striking differences were observed for NFI-B and NFI-C proteins in distinct muscles (Fig. 7B). In fast-twitch muscle (vastus lateralis and gastrocnemius) nuclear extracts, NFI-C antibody revealed a major band migrating at approximately 65 kDa and two faint minor bands (γ and γ′, at 48 and 53 kDa, respectively); in slow-twitch muscles (soleus), these two lower-migrating bands were as abundant as the higher one. The NFI-B antibody revealed two distinct bands (62 and 49 kDa) in muscle nuclear extracts which were not detected in liver or spleen: the upper one (β′) was detected in soleus, vastus lateralis, and gastrocnemius muscle nuclear extracts, while the lower band (β) was clearly more abundant in gastrocnemius than in vastus lateralis or soleus muscle nuclear extracts. It is worth noting that this NFI-B immunoreactive band is detected in muscles in which the ΔAB-tkCAT transgene is active and is far less common in muscles in which it is silent. Thus, we suggest that this NFI isoform could be involved in the differential activity of the ΔAB-tkCAT transgene in the gastrocnemius muscle. In addition, the amount of the NFI-X3 immunoreactive bands is inversely correlated with the transcriptional activation mediated by the pM NFI binding site (gastrocnemius > vastus lateralis > soleus). Therefore, NFI proteins vary qualitatively and quantitatively between different tissues, different muscle types, and even different fast-twitch muscles. These differences may provide a molecular basis for the relative importance of the NFI site in the activity of the aldolase A muscle promoter in distinct muscles.

DISCUSSION

Muscle-specific expression without MEF2 and MyoD binding sites. We have previously shown that the most proximal 280-bp region of pM is sufficient to give a high level of activity in adult fast muscle fibers (52). This region contains putative

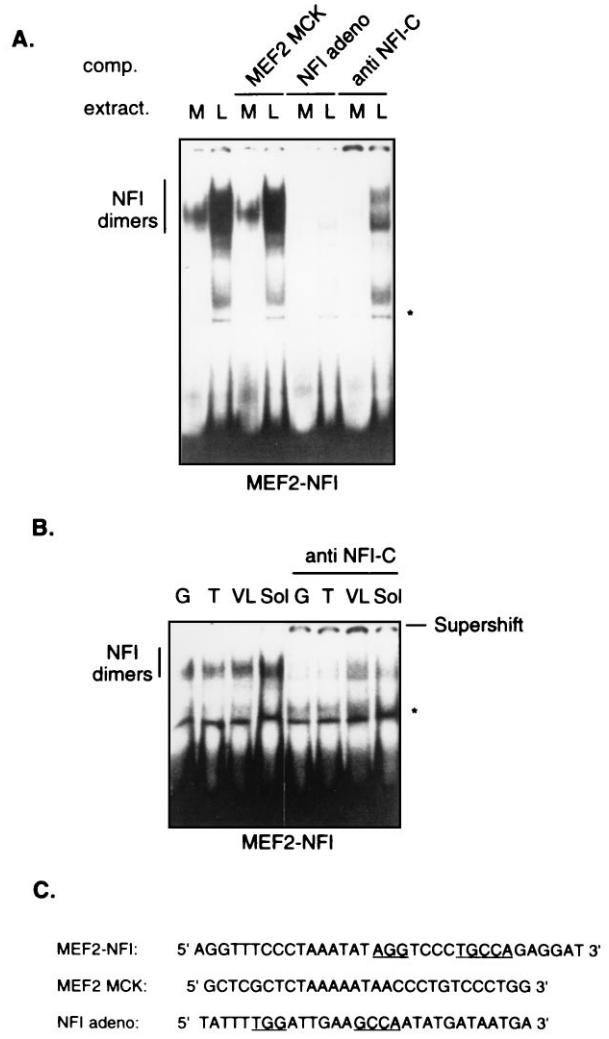

FIG. 6. Gel mobility shift assays with the MEF2-NFI sequence. (A and B) The end-labelled oligonucleotide was incubated with nuclear extracts from rat liver (L) and skeletal muscle (M) (A) or from various individual muscles, gastrocnemius (G), triceps (T), vastus lateralis (VL), and soleus (Sol) (B). A 50-fold molar excess of unlabelled oligonucleotide was added for competition experiments with the MEF2 binding site of the muscle creatine kinase enhancer (MEF2 MCK) or with the NFI site of the major late promoter of adenovirus (NFI adeno). Supershift experiments were done with a human CTF1 anti-serum (anti NFI-C). Nonspecific complexes are indicated by asterisks. (C) Sequences of the oligonucleotides used; the NFI binding sites are underlined.

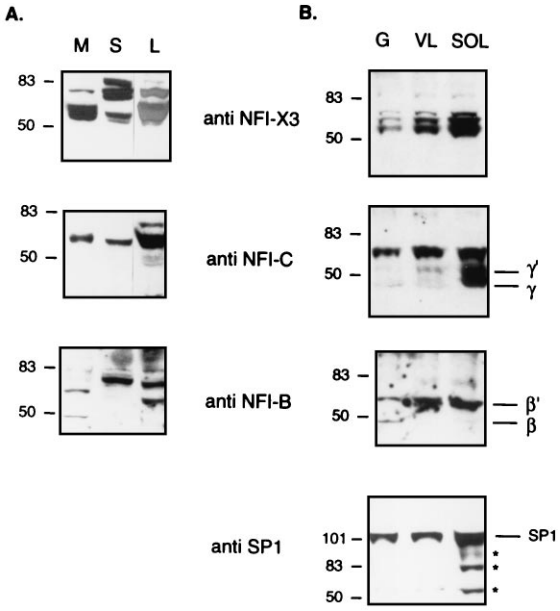

FIG. 7. Western blot analysis of NFI isoforms in different tissues. The antibodies used were raised respectively against human CTF1 (anti NFI-C) and *Xenopus* NFI-B1 (anti NFI-B) proteins; anti-NFI-X3 was raised against the X3-specific part of the *Xenopus* NFI-X3 protein (which is nevertheless conserved in some NFI-A isoforms). (A) Immunodetection of muscle-specific NFI isoforms. Western blots were performed with 100 μg of nuclear extracts from rat liver (L), spleen (S), and skeletal muscle (mainly hind limb) (M). The bands detected in nuclear extracts were not detected in whole tissue extracts (data not shown). (B) Distinct NFI isoforms in different types of muscles. Western blots were performed with 50 μg of rat soleus (SOL), gastrocnemius (G), and vastus lateralis (VL) nuclear extracts. The same results were obtained with two different preparations of nuclear extracts. The quality and relative amount of proteins were judged by gel shift assays (Fig. 6), by staining bound proteins on nitrocellulose with Ponceau S (data not shown), and by immunostaining with an SP1 antibody (additional bands marked by asterisks have been previously observed in Santa Cruz Inc. data). Muscle-specific bands (γ, γ′, β, and β′) were named with Greek letters to avoid confusion with known NFI proteins. The positions of protein size markers are indicated with their masses in kilodaltons.

binding sites for the myogenic factors of the MyoD and MEF2 families. We demonstrate here that a pM promoter carrying mutations in both the E box and MEF2 site still displays a fast-twitch muscle-specific expression in adult transgenic mice and is activated during avian myoblast differentiation. Several studies have previously suggested that E-box-independent pathways can establish muscle specificity (6, 13, 38), but in these examples, a MEF2 binding site was still present. Our results suggest that other transcription factors can establish and maintain a muscle-specific expression in cell culture and in adult skeletal muscles in vivo independently of a direct binding of MEF2 or MyoD family members. Nevertheless, we cannot exclude that the MyoD (or MEF2) protein may regulate pM expression through interactions with proteins bound on pM regulatory sequences, since they have been shown to be able to interact with multiple transcription factors; particularly, CASTING experiments have shown that an NFI binding site near a E box can favor myogenin binding on DNA, suggesting that myogenin could interact with NFI proteins (14).

The expression of many fiber type-specific genes has been shown to be E-box dependent (36, 46). Most of these genes are active in all muscle cell types during embryogenesis and become restricted to a certain type of fiber only later during postnatal development. Compared to these genes, pM is activated much later during rodent development and its expression increases in fast-twitch fibers with the maturation of adult muscles (7, 23). It is tempting to speculate that distinct transcriptional mechanisms correspond to genes that are activated at an early stage of development and whose expression is maintained in some fibers (TnI subclass) and those which are activated late in development in a restricted subset of fibers (pM subclass); this point is supported by the fact that the FIRE enhancer of TnIfast (36) and pM do not have common motifs.

Distinct elements for fast-twitch fiber-specific expression in different muscles. We demonstrate in this study that a core region (ΔAB) comprising two MEF3 motifs and an NFI binding site is sufficient to stimulate the expression of a CAT transgene directed by the neutral tk-105 promoter in a subset of fast muscles. The MEF3 motif is required for activity (52) (Fig. 3) but is not sufficient to drive expression in muscles of transgenic mice from the tk-105 promoter (see above; construct MΔ3-tkCAT). It was shown that mutation of the NFI

site reduced pM activity strongly in the gastrocnemius muscle yet only moderately in the vastus lateralis muscle (52). Indeed, the functions of the MEF3 and NFI sites could be different. The MEF3 site could be essential for the promoter activity in any type of fast-twitch muscles. In contrast, the NFI site should be required mainly in a subset of fast-twitch muscles, represented by gastrocnemius in the present study.

Nevertheless, several lines of evidence suggest that other fiber type-specific elements are present in the pM regulatory region. Mutation of the NFI binding site reduced activity in some fast-twitch muscles, but the fast-versus-slow specificity is conserved (52). Moreover, addition to the ΔAB core region of either the distal or proximal sequences both increase the activity and widen the specificity of ΔAB transgene to other fast-twitch muscles but still not to slow muscles (Fig. 3). Taken together, these results support the hypothesis that NFI and MEF3 are not the only fiber-specific elements in the pM regulatory region. Expression in all fast-twitch muscles results from cooperation with additional sequences localized in either the distal or proximal regions. Since these regions do not share similar DNA elements (and no putative NFI binding site), we suggest that distinct regulatory mechanisms lead to fast-twitch muscle specificity, even if their relative importance can vary from one muscle to another.

Ubiquitous MEF3 proteins for muscle specificity? As mentioned above, our data provide new insight into the role of MEF3 proteins in fiber type-specific expression. MEF3 was initially described as a ubiquitous binding activity on the promoter/enhancer of the slow/cardiac troponin C gene (cTnC) (38) and was found to be important for the activity of the cTnC and of the rat aldolase A muscle promoters in myogenic cells in transient-transfection assays (20, 38). We have previously shown that this sequence is required for high-level and position-independent expression of pM in fast skeletal muscles, since the mutation of the MEF3 motif caused a dramatic decrease in pM activity in muscles of transgenic mice (52). Here, we show that this motif is also required for fast-muscle-specific activation of the neutral tk-105 promoter (ΔPT-tkCAT, Fig. 3). However, the MEF3 motif even associated with the distal activating region of pM (MΔ3) is not sufficient to drive muscle-specific expression in vivo.

It is surprising that MEF3 proteins, which seem to be key regulatory factors for fast-muscle-specific expression, are expressed ubiquitously, as judged by gel shift assays. One possible explanation is that the MEF3 complex is involved in the establishment of an open chromatin structure on pM promoter, in order to allow the subsequent binding of fast-twitch muscle-specific proteins. Studies are in progress to determine whether mutation of the MEF3 motif alters the chromatin structure around pM. Nevertheless, Moch et al. demonstrated that deletion of the ΔAB fragment prevented expression from the pM promoter in transgenic mice despite its location in a transcriptionally active region (34). Thus, MEF3 does not behave only as a "chromatin opener" but is also required for transcriptional activation.

Fiber- and muscle-specific isoforms of NFI proteins. A combination of MEF3 and NFI binding sites is sufficient to activate the tk-105 promoter specifically in some fast-twitch muscles. While MEF3 proteins do not show any apparent fiber specificity, we found an unexpected diversity in the expression of NFI proteins in muscle.

NFI proteins are produced by alternative splicing from four different genes in vertebrates (27) and bind to palindromic or semipalindromic DNA sequences as homo- or heterodimers. We show here that the pattern of NFI isoform expression differs between skeletal muscle and other tissues and that some muscle-specific isoforms exist (NFI-Bβ and NFI-Bβ′). In slow muscles (soleus), two NFI-C-related proteins (γ and γ′) are expressed, in addition to the major muscle isoform detected in fast-twitch muscles (gastrocnemius and vastus lateralis), while an NFI-B isoform (β) is highly enriched in the fast-twitch gastrocnemius. Moreover, isoforms revealed by the NFI-X3 antibody are more abundant in slow muscles than in fast-twitch muscles. As NFI proteins bind DNA as heterodimers, slight variations in the ratio of the various isoforms could change the nature of the predominant heterodimer bound to pM. Fine tuning of gene expression by differential heterodimerization of transcription factors is a well-documented phenomenon (29, 43, 57). It has been shown that different NFI isoforms could either activate or repress transcription (2, 37, 58); as a consequence, modification of the ratio of the different NFI proteins could lead to the binding of complexes with different transcriptional properties. We suggest that the different ratio between fast- and slow-enriched NFI muscle isoforms could account for the pattern of expression of pM. Indeed, NFI-dependent activity is correlated with the specific accumulation of the β isoform in gastrocnemius, such that this β isoform could contribute to the promoter activity in this muscle.

NFI isoforms could arise either from differentially spliced mRNAs or from posttranslational modification (NFI proteins can be phosphorylated and glycosylated [22]). Several cDNAs coding for NFI proteins of a predicted molecular mass in accordance with the bands observed in Western blots have already been isolated (27). In particular, the NFI-B2 protein (predicted mass, 47 kDa) (48) could be the gastrocnemius-specific NFI-Bβ isoform. However, quantitative reverse transcription-PCR experiments showed that the corresponding mRNA was expressed at the same level in a large number of tissues including the muscle, liver, spleen, and brain (55); consequently, this mRNA is not likely to encode the specific β isoform. We are currently trying to identify which isoforms of NFI-B and NFI-C or which posttranslational modifications correspond to the observed muscle-specific NFI isoforms. Identification of these isoforms would allow us to test their effect on pM activity, to investigate whether they are expressed in distinct subsets of muscle fibers or distributed in whole muscle, and to find whether they could specify a muscle type.

NFI proteins and muscle identity. We have shown that NFI-mediated activity and NFI isoforms are not identical in muscles sharing the same fiber type composition. Mechanisms involved in muscle specification have been well documented in *Drosophila*; different transcription factors (S59, *apterous*, and *wingless*) and adhesion molecules are expressed in different subset of muscles (4, 12). In vertebrates, muscle identity has been illustrated by integration site-dependent expression in transgenic mice (39, 53): expression of the transgenes was restricted to various subset of muscles. More recently, a *cis*-acting element involved in neck-specific repression of a MLC1 transgene has been identified (42). It has also been shown that Engrailed-2 (19) and Hox11 (45) were expressed in some specific muscles of the brachial arch. However, no target gene has been identified for these transcription factors. Our results show that NFI proteins are differentially expressed in different hind limb muscles composed of the same MHC fiber type and are likely to be involved in the expression of the aldolase A gene in these muscles. Therefore, NFI factors could contribute to the specification of muscle diversity. Although confirmation of this hypothesis will require molecular and functional characterization of these muscle-specific NFI isoforms, this work provides a new insight on the possible molecular bases of muscle identity in mammals.

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