

Fast-Muscle-Specific DNA-Protein Interactions Occurring In Vivo at the Human Aldolase A M Promoter Are Necessary for Correct Promoter Activity in Transgenic Mice

MARJO SALMINEN,¹ SOLEDAD LÓPEZ,² PASCAL MAIRE,¹ AXEL KAHN,¹
AND DOMINIQUE DAEGELEN¹

*Institut Cochin de Génétique Moléculaire, Institut National de la Santé et de la Recherche Médicale U129,
Université René Descartes, 75014 Paris,¹ and Institut National de la Santé et de la Recherche
Médicale U30, 75015 Paris,² France*

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The human aldolase A tissue-specific M promoter (pM) has served as a model system for identifying pathways that lead to fast-muscle-specialized expression. The current study has delimited the sequences necessary and sufficient for fast-muscle-specific expression in transgenic mice to a short 209-bp fragment extending from bp –164 to +45 relative to the pM transcription start site. Genomic footprinting methods showed that in this proximal region, the same elements that bind muscle nuclear proteins in vitro are involved in DNA-protein interactions in intact muscle nuclei of transgenic mice. Furthermore, these experiments provided the first evidence that different DNA-binding activities exist between slow and fast muscles in vivo. Fast-muscle-specific interactions occur at an element named M1 and at a muscle-specific DNase I-hypersensitive site that was previously detected by in vitro methods. The formation of the muscle-specific DNase I-hypersensitive site reflects binding of proteins to a close element, named M2, which contains a binding site for nuclear factors of the NF1 family. Mutational analysis performed with transgenic mice confirmed the importance of the M1 element for high-level fast-muscle-specific pM activity and suggested that the M2/NF1 element is differently required for correct pM expression in distinct fast muscles. In addition, two other protein binding sites, the MEF3 motif and the USF site, seem to act as stage-specific activators and/or as participants in the establishment of an active chromatin configuration at pM.

Mature adult skeletal muscle is a highly specialized and heterogeneous tissue composed of different types of multinucleated muscle fibers that form through the fusion of mononucleated myoblasts. In mammalian muscles, four major fiber types are generally distinguished on the basis of their expression of distinct isoforms of contractile proteins and metabolic enzymes. Slow-twitch fibers express myosin heavy chain (MHC) type I and use principally oxidative metabolism. Fast-twitch fibers express either type IIA, IIX, or IIB MHC and employ mainly oxidative (IIA) or glycolytic (IIB and IIX) metabolism (48; reviewed in reference 39). How and when this myofiber diversity arises are not completely understood, but it has been suggested that a heterogeneous population of myoblasts that differ in their gene expression potentials provide the basis for muscle diversification (47; reviewed in references 13, 31, and 51). Neural stimulation and hormones can then modify to some extent the myofiber-specific gene expression later in development (3, 24, 37, 39, 41, 49, 56).

Relatively little is known of the transcription factors that participate in the establishment and maintenance of myofiber-specific gene expression. Studies of the myogenic determination factors, including MyoD, Myf-5, myogenin, and MRF4, have led to a large amount of information on the molecular mechanisms underlying early skeletal myogenesis (recently reviewed in references 9, 14, 35, and 55). Furthermore, their unequal expression in different populations of early myoblasts (50) and in different adult muscle fibers (23, 54) could provide the basis for the formation of distinct myofiber phenotypes.

However, no direct evidence of the role of these factors in fiber-type-specific gene expression has been presented so far. More recently, several members of another family, namely, the MEF2/RSRF transcription factors, have also been implicated in early muscle differentiation (reviewed in reference 8), but as with the MyoD family, their role later during muscle maturation remains obscure.

The understanding of the molecular mechanisms underlying adult fiber-type-specific gene regulation will require characterization of critical *cis*-regulatory elements and corresponding *trans*-acting factors. Because of the lack of suitable muscle cell culture models for adult-specific muscle phenotypes, the characterization of gene regulatory mechanisms operating in mature muscle must involve in vivo approaches such as transgenic mice. Although some fast- or slow-muscle-specific gene constructs have been analyzed in transgenic mice (4, 12, 19, 25, 26, 43), no individual fiber-type-specific *cis*-acting elements or *trans*-acting factors have been identified so far.

We have previously shown that the proximal sequences of the human aldolase A muscle-specific promoter, pM, are necessary and sufficient to target the expression of a chloramphenicol acetyltransferase (CAT) reporter gene to fast-twitch, glycolytic muscles of transgenic mice (43). In addition, we observed that the promoter is active at high levels in all transgenic lines, irrespective of the integration site in mouse chromatin. Because of the relatively small and integration site-independent regulatory region (located between bp –235 and +45), pM provides an excellent model system to study the molecular mechanisms involved in the fiber-type-specific activation of transcription.

In a first approach towards understanding the mechanisms underlying the fiber-type-specific pM activity, we used nuclear

* Corresponding author. Mailing address: Institut Cochin de Génétique Moléculaire, 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.

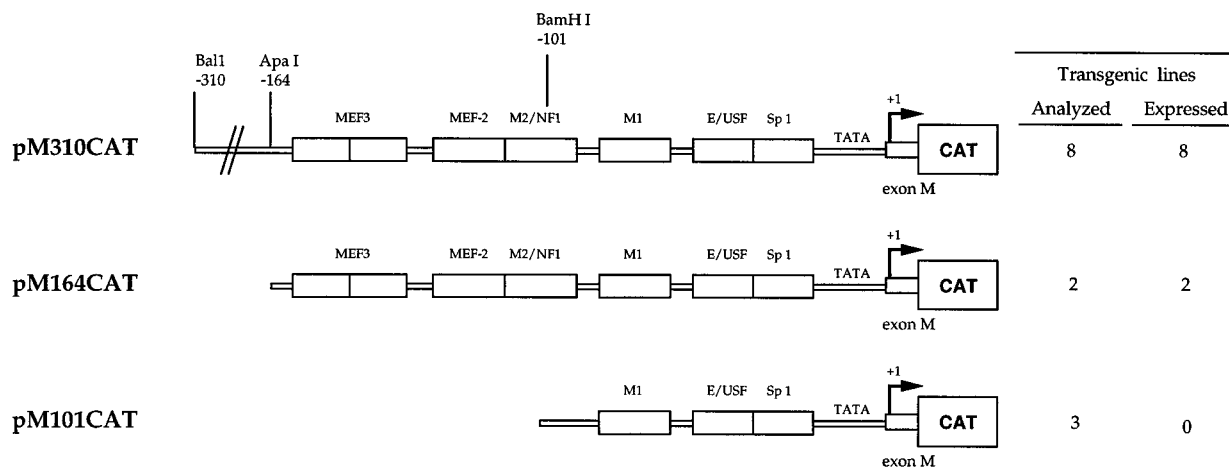


FIG. 1. Schematic representation of the parental pM310CAT transgene and two 5' deleted constructs. The protein binding DNA elements detected previously by *in vitro* binding assays between bp -164 and +1 are indicated as open boxes (not drawn to scale). The restriction sites used to create the 5' deletions are indicated above the pM310CAT transgene. The number of independent transgenic lines analyzed for each construct and the number of lines in which the promoter was active in fast muscles are given on the right.

extracts prepared from adult skeletal muscles to localize protein binding DNA sequences within the pM proximal region (45). The identified sequences include binding sites for several families of transcription factors, such as the Sp1, USF, NF1, and MEF2/RSRF families (see Fig. 1). In addition, two pM elements, namely, a MEF3 motif and M1 sequence, were shown to interact with still unidentified nuclear proteins *in vitro*. When the functional role of these elements was tested in myogenic cell cultures, overlapping binding sites for MEF2 and NF1 factors in addition to the M1 sequence proved to be the most important elements for high pM activity in differentiated muscle cells (45).

In the present work, the significance of the proximal pM elements and the corresponding *trans*-acting factors was investigated in the context of the whole organism. The minimum pM sequences capable of fast-muscle-specific activity in transgenic mice were further delimited to a 209-bp fragment extending from bp -164 to +45. With genomic footprinting approaches we showed that in this region the DNA-protein interactions occur at the same pM elements in muscle nuclei that were previously identified *in vitro* (45). Moreover, two elements were involved in fast-muscle-specific DNA-protein interactions. The contribution of the individual nuclear protein binding sites in fast-muscle-specific expression was evaluated by generating transgenic mice harboring chimeric pMCAT constructs in which these binding sites were mutated. We show that the USF binding site and, above all, the M1 and MEF3 elements are necessary for high-level pM activity in transgenic mouse muscles. Furthermore, mutations in the overlapping MEF2 and NF1 binding sites reveal striking differences in the gene expression potentials among individual fast muscles.

MATERIALS AND METHODS

Genomic footprinting. Genomic footprinting was performed using tissues from a transgenic mouse line (EAccl-3) that carries 40 copies of the EAccl aldolase A- β -globin transgene (11, 43). This transgene contains 4.3 kb of aldolase A 5' sequences including the three alternative promoters, pN, pM, and pH, linked to the 3' sequence from the β -globin gene (see Fig. 2A). For *in vivo* dimethylsulfate (DMS) footprinting, liver and two muscle tissues, gastrocnemius and soleus, were dissected. Liver was homogenized without breaking the cells with an electric Teflon glass homogenizer in buffer A, and the intact cells were washed as described previously (27). Muscle fibers were slightly separated with a needle in Dulbecco's modified Eagle's medium to ensure an equal penetration of DMS to all nuclei. The methylation of the hepatocytes and muscle fibers as well as the

naked, protein-free DNA was performed with 0.1% DMS as described by Lacroque et al. (27). Methylated DNA samples were then subjected to a cleavage reaction with 1 M piperidine at the modified guanine and adenine residues after sodium hydroxide treatment as described by Strauss et al. (52). For nuclear DNase I footprinting, nuclei from EAccl-3 transgenic mouse liver and hind limb skeletal muscles were prepared and treated with DNase I as described before for the hypersensitivity analysis (43).

Genomic DNA sequencing by ligation-mediated PCR method was performed essentially according to the method of Mueller and Wold (33) using 1 μ g of either DMS or DNase I-treated DNA. To exponentially amplify both strands of the proximal pM region, ligation-mediated PCR was performed with two sets of oligonucleotides as primers in the Sequenase extension reaction (MR1 or M1), in the PCR amplification reaction consisting of 25 to 27 cycles (MR2 or M2), and in the final labeling reaction employing a single amplification cycle (MR3, MR4, or M3). The linker primer was identical to that described by Mueller and Wold (33). The pM-specific primers for the noncoding strand analysis are as follows: MR1, 5'-CCACGTGATCCGAGTCCCG-3'; MR2, 5'-TCCCCTACCCCTTTCCTTCC-3'; MR3, 5'-TTCCTTCCCACAGGTGGCC-3'; and MR4, 5'-CTCTGAAGCACCGGTGAGTGGCC-3'. The following primers were used for the coding strand analysis: M1, 5'-CAGAGCTGGTACGAGCG-3'; M2, 5'-GTCAGCAGCGAGTGAGCTAGGG-3'; and M3, 5'-GCGAGTGAGCTAGG GGTCCCG-3'.

Production of transgenic mice and CAT assays. The transgene fragments were prepared from pM310CAT-derived deletion and *Bgl*II substitution mutation constructs (45). The construct mM2A was used to create mM2 transgenic mice. The transgenic mice were generated and identified as previously described (11), and the founder mice were bred with nontransgenic cohorts to generate stable transgenic lines. The transgene copy numbers were determined from F₁ transgenic mice as described before (43) except that the Southern blots were scanned with a PhosphorImager using Imagequant V3.3 software (Molecular Dynamics). For CAT assays, various tissues were dissected from adult (at least 6-week-old) F₁ transgenic animals. Protein concentration and CAT activity were measured as previously described (43). To evaluate the significance of the effects of the different mutations, we used a nonparametric Mann-Whitney U test. The EAccl-3 and the different pM310CAT transgenic lines have been already described in previous papers (11, 43).

RESULTS

The pM proximal region from bp -164 to +45 is sufficient to activate the promoter in fast muscles. We showed previously that a 310-bp sequence immediately upstream of the pM transcription start site together with the noncoding M exon (pM310CAT in Fig. 1) confers high levels of fast-muscle-specific expression to a linked CAT reporter gene in transgenic mice.

To further delimit the sequences responsible for the fast-muscle-specific pM expression, we created transgenic mice with two 5' deleted constructs, pM164CAT and pM101CAT, in which 164 or 101 bp of upstream sequences were present,

respectively, together with the noncoding exon and the CAT reporter gene (Fig. 1). In both lines created with the pM164CAT construct, pM was specifically active in two hind limb muscles composed of fast muscle fibers such as the vastus lateralis and gastrocnemius, whereas in the three pM101CAT lines, no activity could be detected in any of the tissues studied (Table 1). These results confirm a previous observation that the most proximal 100-bp sequence is not sufficient to activate pM transcription (43). Instead, the presence of an additional 60-bp fragment seems to be sufficient to establish and maintain specific pM activity in fast muscles.

The same pM proximal elements that bind muscle nuclear proteins in vitro are involved in DNA-protein interactions also in intact muscle nuclei. Several DNA elements within the small 209-bp regulatory region (from bp -164 to +45) have been shown to interact with nuclear factors extracted from different tissues in vitro (45) (Fig. 1). Four sequence elements, the MEF3 motif, the USF binding E box, the Sp1 binding site, and the M1 footprinted sequence, seem to bind factors from various tissues, and an AT-rich sequence binds with low affinity to members from the MEF2/RSRF protein family. A sequence named M2/NF1 (containing an NF1 binding half-site) interacts with proteins from various tissues but nevertheless forms specific DNA-protein complexes with nuclear extracts prepared from adult skeletal muscles (45). To investigate whether these different DNA elements are really bound by nuclear proteins in the chromatin of living cells, we applied two genomic footprinting methods for mouse tissues dissected from the previously described EAccl-3 transgenic mice (transgene diagrammed in Fig. 2A) in which pM is specifically active in skeletal muscles (11, 43).

For the nuclear DNase I footprinting study, nuclei were isolated from transgenic mouse hind limb muscles and liver tissue and treated with increasing amounts of DNase I. As seen in Fig. 2B, the DNase I digestion patterns in muscle and liver nuclei differed considerably. In liver nuclei, the protected regions were much more extensive than those observed in vitro with liver nuclear extracts (45). Instead, in skeletal muscle nuclei the protected regions and hypersensitive (HS) sites seemed to correspond closely (but not exactly) to those observed in vitro with muscle nuclear extracts (45) (Fig. 2B). In the coding strand, muscle-specific HS sites were detected at both sides of the protected regions corresponding to the M1 and M2/NF1 in vitro-footprinted sequences as well as near the MEF3 motif (Fig. 2B). While no obvious footprints were detected over the USF or Sp1 binding sites in the coding strand, these sequences were clearly protected from DNase I digestion in the noncoding strand, as were the M1, M2/NF1, and MEF3 elements (Fig. 2B and C). Furthermore, the only muscle-specific HS site (MHS) detected with DNase I in vitro was also detected in muscle nuclei. Since we have demonstrated that the formation of the MHS is due to factors that bind to the nearby M2/NF1 sequence (45), our present result strongly suggests that muscle-specific interactions occur at M2/NF1 in vivo also. No convincing footprints could be detected at the MEF2 site, except for the HS sites detected at both ends of the AT-rich consensus sequence (Fig. 2C).

It is noteworthy that the MEF3, M1, USF, and Sp1 elements, which interact with ubiquitous factors in vitro, are not protected from DNase I digestion in the same way in muscle and liver nuclei. In addition, the HS sites seen in the ends of these footprints differ considerably between muscle and liver. Furthermore, the region around the TATA box seems to be clearly protected only in muscle nuclei, reflecting promoter inactivity in liver.

In vivo DMS footprinting identifies two DNA elements in

the pM proximal sequence that interact specifically with nuclear proteins present in a fast skeletal muscle. Tissues from the EAccl-3 transgenic mice were also used to perform an in vivo DMS footprinting analysis which permits study of the DNA-protein interactions in the intact cells of individual mouse skeletal muscles. In EAccl-3 mice, pM is highly active in muscles composed of fast-twitch fibers such as gastrocnemius, much less active in muscles that contain a high proportion of slow-twitch fibers such as soleus, and inactive in liver. These three tissues were treated with DMS, which methylates guanine residues as well as adenine residues but with a weaker efficiency (30). Proteins bound at or near these purine residues can cause protection or enhancement of DMS methylation compared to unbound DNA (recently reviewed in reference 22).

Differences in the methylation pattern between in vitro-methylated genomic DNA and in vivo-methylated DNA from either liver, gastrocnemius, or soleus tissue were repeatedly detected in two pM regions. Interestingly, the methylation patterns in both of these regions were different in the three tissues studied. In the coding strand, the alteration localized to the guanine residues at bp -83 and -81 relative to the pM transcription start site (Fig. 3). Although cytidine residues are only occasionally methylated by DMS (46), an alteration was repeatedly observed on a cytidine residue at bp -73. All these residues are included in the M1 footprint (45). The guanine at bp -83 was enhanced in liver and soleus but not in gastrocnemius muscle, while the guanine at bp -81 and the cytidine at bp -73 were clearly enhanced only in gastrocnemius and slightly enhanced in soleus. In the noncoding strand, methylation modifications occurred at three adjacent guanines at bp -123, -124, and -125 (Fig. 3). These bases are among those that form the MHS in vitro (45). Two guanine residues (at bp -124 and -125) seemed to be protected from methylation in all three tissues compared to in vitro-methylated DNA. However, in gastrocnemius and soleus muscles, the guanine at bp -124 was less protected than in liver, whereas an additional guanine residue (at bp -123) was clearly protected only in gastrocnemius muscle (Fig. 3). In addition, weaker modifications could be observed in the M2/NF1 footprint region, especially at the guanine at bp -105, which seemed to be slightly protected in gastrocnemius compared to liver and soleus muscle. These modifications in methylation suggest that nuclear factors bind to the M2/NF1 sequence in vivo also.

Taken together, the results from in vivo DMS footprinting confirm that in a chromatin context, different DNA-protein interactions occur at M1 and M2/NF1 sequences in liver and gastrocnemius muscle. Most interestingly, the methylation pattern in soleus muscle differs from that observed in gastrocnemius.

A binding site for ubiquitous USF factors is a required component for the high-level muscle-specific pM activity. To study the potential role of the individual protein binding sequences in pM control, we created transgenic mice carrying substitution mutations in the proximal promoter region. The mutations were introduced into the context of the pM310CAT construct (Fig. 4), which produces high levels of CAT activity in transgenic mouse muscles (43). Importantly, this construct seems to escape from some of the position effects generally observed in transgenic mice, since pM is tissue specifically active in all independent pM310CAT transgenic lines.

In all four lines carrying a transgene in which the pM Sp1 binding site was mutated (mSp1 in Fig. 4), pM was active in a tissue-specific manner. CAT activities were detected in two fast muscles, vastus lateralis and gastrocnemius, whereas no activity could be observed in soleus, a slow muscle (Table 1). The Sp1

TABLE 1. Effects of different 5' deletions and substitution mutations on pM-driven CAT activities in transgenic mouse tissues

Transgenic mouse line ^a	Copy no.	CAT activity (cpm/μg of protein/min) ^b in:								pM activity/copy in vastus lateralis ^c	Mean pM activity/copy in vastus lateralis	pM activity/copy in gastrocnemius ^c	Mean pM activity/copy in gastrocnemius
		Brain	Heart	Vastus lateralis	Gastrocnemius	Liver	Kidney	Spleen	Soleus				
pM310CAT 88	2	0	ND	836.3	995.6	ND	ND	ND	1.8	418.2	100.1	497.8	105.2
pM310CAT 82A	3	ND	ND	34.6	27.3	ND	ND	ND	1.4	11.5		9.1	
pM310CAT 106	6	ND	ND	90.3	119.2	ND	ND	ND	2.8	15.1		19.9	
pM310CAT 24	6	ND	0	196.0	80.9	0	0	0	3.1	32.7		13.5	
pM310CAT 26	10	55.4	0	815.3	489.5	0	0	0	17.6	81.5		49.0	
pM310CAT 82B	18	0	ND	1,387.8	1,385.8	0	0	0	58.3	77.1		77.0	
pM310CAT 20	20	0	0	1,838.2	1,745.6	0	0	0	138.6	91.9		87.3	
pM310CAT 98	22	0	0	1,604.4	1,929.6	0	0	0	95.0 ^d	72.9		87.7	
pM164CAT 10	2	0	0	7.1	24.0	0	0	0	0	3.6	6.3	12.0	8.0
pM164CAT 28	2	0	0	18.0	7.7	0	0	0	0	9.0		3.9	
pM101CAT 11	4	0	0	0	0	0	0	0	0	0	0	0	0
pM101CAT 34	8	0	0	0	0	0	0	0	0	0		0	
pM101CAT 33	15	0	0	0	0	0	0	0	0	0		0	
mSp1 6	1	0	0	24.6	13.7	0	0	0	0	24.6	15.0	13.7	9.0
mSp1 38	2	0	0	31.7	27.5	0	0	0	0	15.9		13.8	
mSp1 16	2	0	0	16.4	18.0	0	0	0	0	8.2		8.2	
mSp1 4	2	0	0	22.4	0.9	0	0	0	0	11.2		0.45	
mE 17	10	0	0	0	0	0	0	0	0	0	0.51	0	0.50
mE 8	21	16.7	0	17.6	17.0	0	0	0	0	0.83		0.81	
mE 12	29	0	0	20.1	19.6	0	0	0	0	0.7		0.7	
mM1 16	2	0	0	0	0	0	0	0	0	0	0.012	0	0.00096
mM1 20	9	0	0	0.4	0	0	0	0	0	0.04		0	
mM1 48	45	0	0	0.4	0.3	0	0	0	0	0.009		0.0067	
mM1 41	46	1.6	0	0	0	0	0	0	0	0		0	
mM2 36	1	0	0	1.0	0.5	0	0	0	0	1.0	10.7	0.5	0.35
mM2 56A	10	0	0	31	3.5	0	0	0	0	3.1		0.35	
mM2 41	16	0	0	398	16.1	0	0	0	ND	24.9		1.0	
mM2 46	17	0	0	2.9	2.3	0	0	0	0	0.2		0.14	
mM2 47	20	0	0	2	2.7	0	0	0	0	0.1		0.14	
mM2 57	25	0	0	419.1	4.4	0	0	0	34.4	16.8		0.2	
mM2 56B	40	8.5	0	1,140	5.9	0	0	0	12.9	28.5		0.15	
mMEF2 23	1	0	0	5.9	2.9	0	0	0	0	5.9	143.7	2.9	16.7
mMEF2 42A	6	0	0	40.0	17.9	0	0	0	0	6.7		3.0	
mMEF2 40A	15	2.5	0	3,204	125.8	0	0	0	106	213.6		8.4	
mMEF2 3	20	3.3	0	4,848	52.2	0	0	0	6.7	242.4		2.6	
mMEF2 40B	21	0	0	6,608	1,812	0	0	0	65.8	314.7		86.3	
mMEF2 42B	23	0	0	2,714	294.5	0	0	0	31.6	118		12.8	
mMEF2 14	28	12.3	0	2,920	30.2	0	0	0	0.8	104.3		1.1	
mMEF3B 31	1	0	0	0	0	0	0	0	0	0	0.013	0	0
mMEF3B 1	2	0	0	0	0	0	0	0	0	0		0	
mMEF3B 53	2	0	0	0	0	0	0	0	0	0		0	
mMEF3B 5	15	0	0	2.0	0	0	0	0	0	0.1		0	
mMEF3B 20	27	0	0	0	0	0	0	0	0	0		0	
mMEF3A 8	2	0	0	0	0	0	0	0	0	0		0	
mMEF3A 13	3	0	0	0	0	0	0	0	0	0		0	
mMEF3A 21	8	0	0	0	0	0	0	0	0	0		0	

^a Transgenic lines indicated with the same number and marked with an A or a B have been derived from a common founder animal.

^b 0, no CAT activity could be detected under any conditions tested, which included the use of 200 μg of protein extract and incubation times of up to 1 h; ND, not determined.

^c Mann-Whitney probability values for the significance of differences between wild-type pM310CAT and the various mutant transgenes were calculated for the CAT activities observed in vastus lateralis and gastrocnemius muscles. mSp1, $P < 0.05$ in vastus lateralis and gastrocnemius; mM1, $P < 0.000001$ in vastus lateralis and gastrocnemius; mM2, $P < 0.02$ in vastus lateralis and $P < 0.001$ in gastrocnemius; mMEF2, not significantly different in vastus lateralis and $P < 0.02$ in gastrocnemius; mMEF3, $P < 0.000001$ in vastus lateralis and gastrocnemius. Because of the low number of lines obtained with the pM164CAT and mE constructs, this test could not be used for them.

^d A corrected value compared to a previously given result (43).

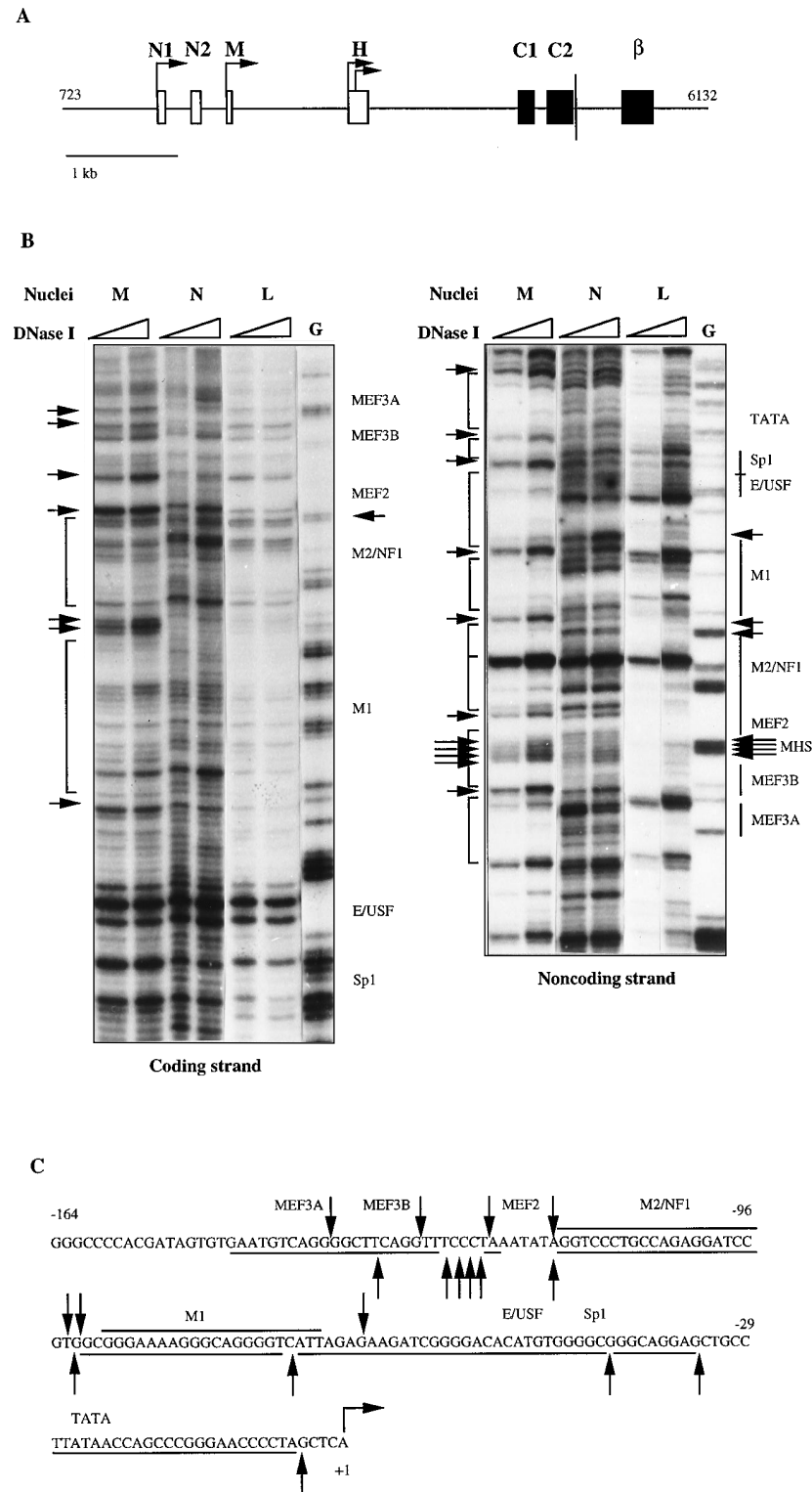


FIG. 2. Analysis of DNA-protein interactions at the pM proximal sequence with nuclear DNase I footprinting. (A) The EAccl transgene used to create the mice with which the footprinting analysis was performed. The three alternative aldolase A transcription start sites are shown with arrows, and the corresponding noncoding exons (N1, N2, M, and H) are indicated as open boxes. The two first aldolase A coding exons (C1 and C2) as well as the last exon from the human β -globin gene (β) are indicated as black boxes. (B) Naked genomic DNA (N) and nuclei prepared from EAccl-3 transgenic mouse skeletal muscle (M) or liver (L) tissues were treated with DNase I. The protected regions detected in muscle nuclei compared to naked DNA are indicated as brackets on the left side of the panels. The arrows point to the HS sites detected in muscle nuclei but not in liver nuclei. The locations of the nuclear protein binding sites (vertical lines) and HS sites (arrows) observed previously with muscle nuclear extracts in *in vitro* DNase I footprinting assays are marked on the right side of the panels. The G lanes correspond to the G-specific cleavage products of *in vitro* DMS-treated DNA which served as markers. (C) Summary of the DNA-protein interactions observed at pM in muscle nuclei. The DNase I protections are indicated as horizontal lines, and the HS sites are shown as arrows. The footprints observed in the coding strand are summarized above the sequence and in the noncoding strand under the sequence.

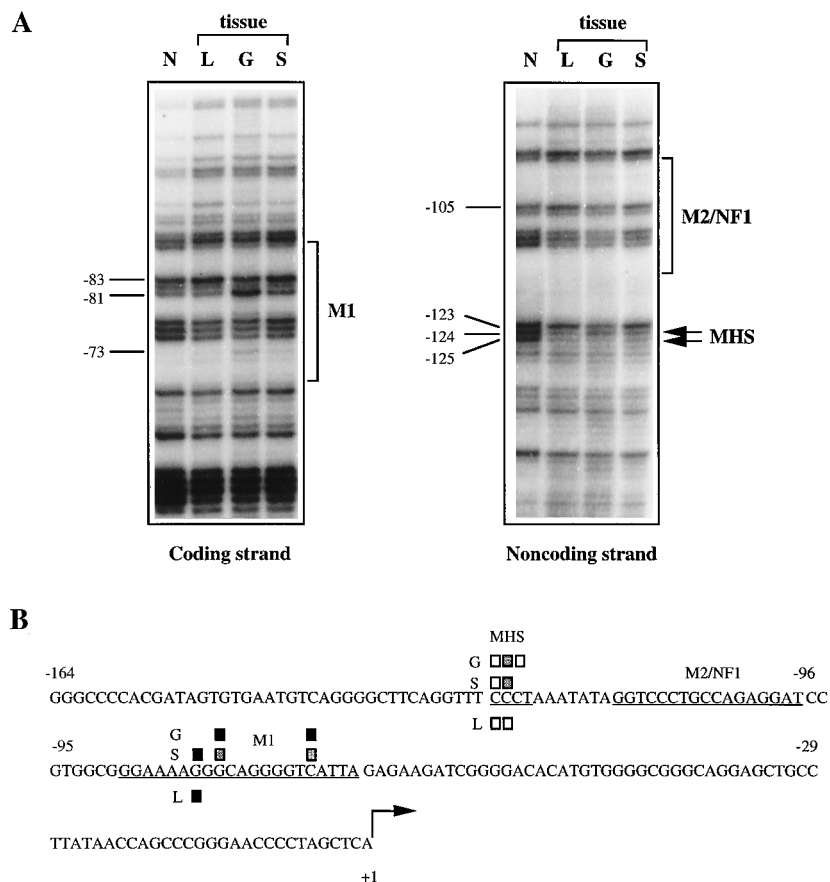


FIG. 3. In vivo DMS footprinting analysis of the pM proximal region in transgenic mouse tissues. (A) DMS methylation pattern generated with naked genomic DNA (N), liver (L), gastrocnemius muscle (G), and soleus muscle (S) at the regions surrounding the M1 sequence in the coding strand (left panel) and the M2/NF1 and MHS sequences in the noncoding strand (right panel). Modified guanine and cytosine residues are indicated on the left side of the panels, and their positions relative to the pM transcription start site are given. The locations of the M1 and M2/NF1 footprinted regions as detected with DNase I footprinting *in vitro* are marked with open bars on the right side of the panels. The arrows point to the regions that become HS to DNase I digestion *in vitro* (MHS). (B) The proximal pM sequence from bp -164 to +1 in which the M1 and M2/NF1 sequences and the bases involved in the formation of the MHS are underlined. The modifications in the methylation patterns in different tissues compared to *in vitro*-methylated DNA are illustrated by open (protection), gray (slight protection), or black (enhanced methylation) squares above the sequence for gastrocnemius and soleus and under the sequence for liver.

mutation resulted in a sixfold decrease in the mean CAT activity per transgene copy. However, all the mSp1 mice still expressed CAT, suggesting that the Sp1 element has a minor role in pM control.

Contrary to mSp1, substitution of the adjacent E box sequence (mE in Fig. 4) resulted in a remarkable drop in CAT activity in all three lines obtained. Detectable activity was present only in the two fast muscles in lines which carried the highest number of transgenes, and the mean expression level per copy in these mice had decreased 200-fold (Table 1). The pM E box is not a high-affinity binding site for myogenic basic helix-loop-helix factors (6, 57); instead, it interacts with ubiquitous USF proteins from the basic helix-loop-helix leucine zipper family (45). Our results indicate that the USF binding site functions as an important element for pM activity.

mM1 and mMEF3 mice show strongly impaired promoter activity. Unidentified nuclear proteins from various tissues interact *in vitro* with the GA-rich pM M1 element (45). However, fast-muscle-specific DNA-protein interactions seem to occur at this element *in vivo*. Remarkably, the M1 mutation severely impaired the promoter activity in four transgenic lines analyzed (Table 1). In two lines, no CAT activity could be observed, and in two additional lines, only a very faint activity

(8,000-fold weaker than the wild-type activity) was detected in fast muscles. This result suggests an important role for the M1 element in ensuring high promoter activity in mature fast muscles. However, the very low level of transgene expression detected in two mM1 lines remained tissue specific.

The human pM MEF3 motif consists of two tandem 5-bp core repeats (5'-TCAGG-3') that bind nuclear proteins from cultured muscle cells and different rat tissues, including adult skeletal muscle (45). As shown in Table 1, substitution of either of the two MEF3 core sequences (mMEF3A or mMEF3B in Fig. 4) totally shut down transgene expression in seven of eight transgenic lines analyzed, demonstrating for the first time that the MEF3 motif can play a critical role in muscle-specific gene expression *in vivo*. In one line (mMEF3B 5), very weak CAT activity could be detected in fast vastus lateralis muscles, indicating that here too, the weak transgene expression was regulated in a tissue-specific manner.

Mutations in the MEF2-M2/NF1 region have distinct effects in different muscles. We have previously shown that the MEF2-M2/NF1 footprinted sequence consists of overlapping binding sites for MEF2/RSRF and NF1 factors (45). Here we have analyzed the pM activity in seven independent transgenic lines for both MEF2 and M2/NF1 mutations (mMEF2 and

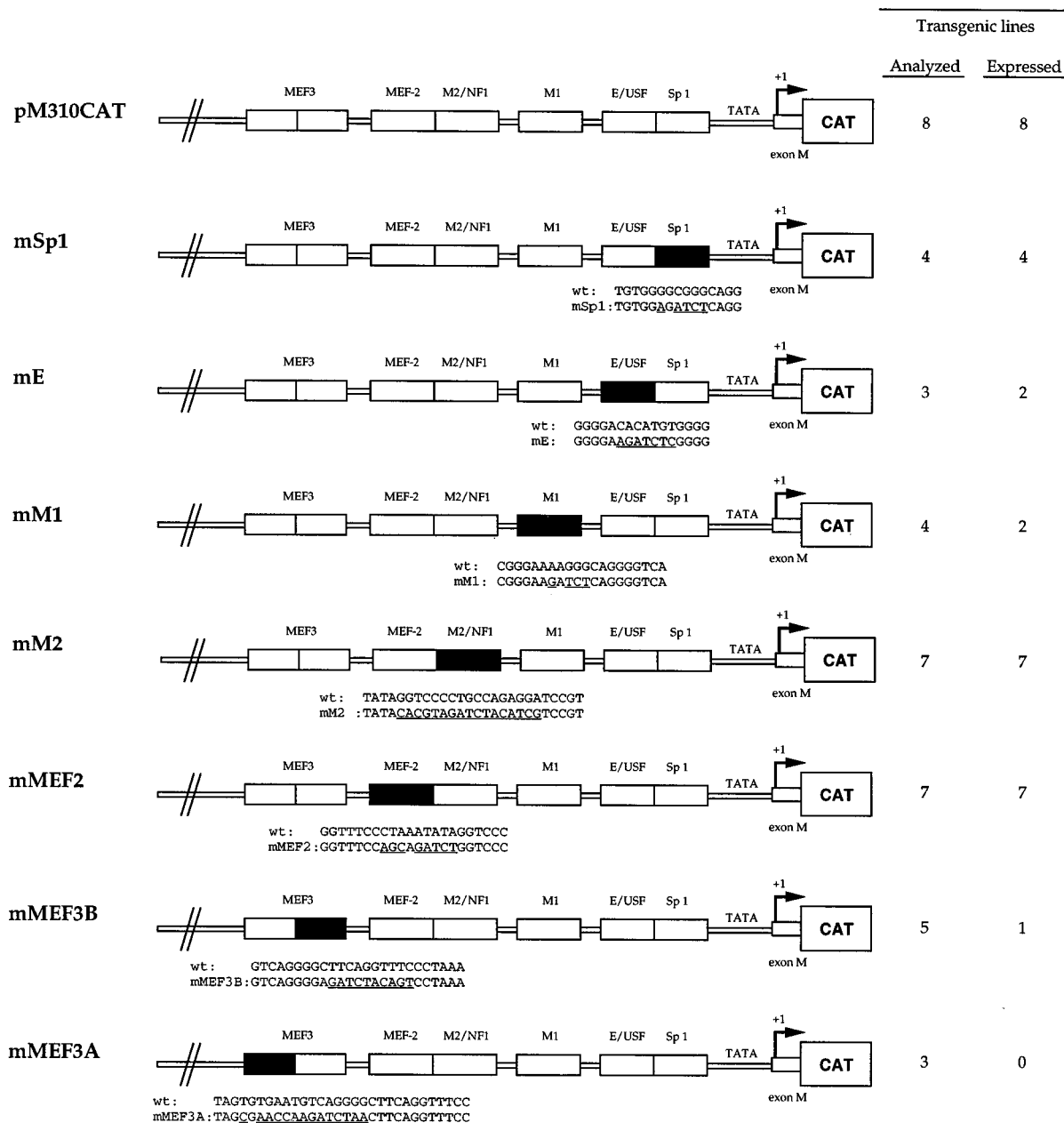


FIG. 4. Mutation analysis in the pM proximal region. *Bgl*II substitution mutations were introduced to different protein binding DNA elements. The intact protein binding sites are indicated as open boxes, whereas the mutated elements are indicated as black boxes. Wild-type and mutated nucleotide sequences are provided below the corresponding transgene, and the changed bases are underlined in the mutant sequence. The number of independent lines analyzed for each mutant construct as well as the number of lines in which pM was active are given on the right.

mM2A, respectively, in Fig. 4). In all these lines, pM remained active in fast muscles (Table 1), suggesting that these elements are dispensable for the overall pM specificity and for the creation of an active chromatin domain at pM. Nevertheless, the M2/NF1 mutation resulted in a remarkable loss of activity in two lines that carried a high number of copies (mM2 46 and 47). Such weak activity in lines carrying high copy numbers has not been observed with transgenic lines in which the proximal pM region is intact. This result could be due to increased sensitivity of the mutant promoter to the negative effects of the surrounding chromatin.

In wild-type pM310CAT mice, very similar CAT activities

are generally detected in the two fast muscles (Table 1), whereas in the slow soleus muscle only about 3% of this activity is observed (43). The M2/NF1 mutation resulted in remarkable changes in this typical muscle expression pattern. The mean pM activity decreased 10-fold in vastus lateralis muscle and approximately 300-fold in the other fast muscle, gastrocnemius, in which the activity fell in some lines under the level detected in slow soleus muscle (Table 1). Mutation in the weak-affinity MEF2 binding site did not result in significant changes in the pM expression levels in vastus lateralis muscle (Table 1). However, here too, an important decrease in pM activity could be detected in gastrocnemius. As for the mM2

lines, the difference between the activity levels in the two fast muscles was particularly striking in lines which exhibited the highest expression levels.

The results from the functional analysis of the various DNA-binding elements show that pM tissue specificity is very strictly controlled, since no ectopic expression could be detected in heart, liver, kidney, or spleen in any of the 38 lines carrying deleted or mutated pM constructs, even when high protein levels (up to 200 μ g) were used (Table 1). However, low ectopic expression could sometimes be detected in brain as well in the wild-type pM310CAT lines, as in some mutant lines (Table 1). This ectopic expression was more often detected in the mMEF2 lines, suggesting a slight relaxation of the promoter specificity in these mice.

DISCUSSION

Aldolase A is a glycolytic enzyme that is particularly abundant in skeletal muscle tissues. The human aldolase A gene is regulated by the fast-muscle-specific pM and two ubiquitously active promoters (29). We have previously shown that a 4.3 kb-fragment of the human aldolase A 5' sequences including the three promoters is able to reproduce correct tissue-specific and developmental regulation of the gene in transgenic mice (11). Furthermore, the most proximal 280-bp region of pM was shown to be sufficient for the autonomous activity of this promoter in adult fast skeletal muscles (43). Here we present a detailed report of the individual elements that play important roles in confining pM expression to mature fast muscle fibers.

The M1 and M2/NF1 elements are involved in fast-muscle-specific DNA-protein interactions. Transgenic mice have provided us a useful approach to study the human aldolase A promoters in an *in vivo* context (11, 43). Here we have used mouse tissues to demonstrate that it is possible to study the nuclear protein occupancy in adult skeletal muscles with different genomic footprinting methods. With nuclear DNase I footprinting assays we show that the same pM proximal elements that bind nuclear proteins *in vitro* are also involved in DNA-protein interactions in intact muscle nuclei. Although *in vivo* DNase I footprinting assays have generally indicated that factor binding sites are occupied only in cell types in which the genes are functional (5), we could detect DNA-protein interactions in liver tissue in which pM is inactive. However, these interactions differed remarkably from those that occur in muscle nuclei. Since in the analyzed transgene, pM is located between the two other ubiquitous promoters, pM regulatory elements are situated in a ubiquitously transcribed region (43), so that some factors may have access to DNA in liver nuclei. If this is the case, the difference in the digestion pattern between liver and muscle nuclei could be due to the presence of tissue-specific factors in muscle which rearrange the binding pattern and thus create the specific HS sites observed both *in vivo* and *in vitro*.

Interestingly, the DMS methylation pattern detected in fast gastrocnemius muscle differed from that observed in slow soleus muscle at M1, M2/NF1, and MHS sequences; the modifications at the MHS reflect fast-muscle-specific DNA-protein interactions at the M2/NF1 sequence. The fact that the M1 mutation, in which only four nucleotides, including the two guanines differently modified by DMS (Fig. 3A and 4), have been altered, leads to an important loss in pM activity suggests that the observed modifications have functional relevance. However, we cannot totally exclude the possibility that some of the modifications are due to the presence of multiple unequally methylated transgenes. The methylation modifications observed at both M1 and MHS in soleus muscle seemed to share

some characteristics with liver and some others with gastrocnemius. These results could be due to the heterogeneity of the soleus tissue. While the gastrocnemius is highly homogeneous in its fiber content (100% fast type II fibers) (20), the mouse soleus muscle contains roughly equal proportions of slow type I and fast type II fibers (12). By staining serial sections of muscles from pM310CAT transgenic mice, either with different MHC antibodies (48) or histochemically for CAT activity (12), pM activity has been shown to be restricted to the most glycolytic IIB fibers (44), which are abundant in gastrocnemius but absent from soleus. Therefore, the intermediate modification pattern of the soleus muscle could reflect differences in the quantity or quality of nuclear factors that bind to pM in these different fiber types. Nevertheless, no qualitative or quantitative differences in the DNA-binding activities can be observed at the M1 or M2/NF1 elements between adult rat soleus and gastrocnemius nuclear extracts in gel shift assays (42), even if the rat soleus is composed of a greater proportion of type I fibers than the mouse soleus is (1). This observation suggests that the DNA binding factors themselves are not fiber type specific. Instead, promoter specificity may be created by a specific combination of DNA binding factors and/or by protein-protein interactions including fiber-type-specific coactivators that do not directly bind DNA.

The MEF3, M1, and USF elements are required together for high-level, position-independent expression of pM in fast skeletal muscles. The MEF3 motif is a recently identified nuclear protein binding site that has been shown to be functionally important for the rat aldolase A pM and rat cardiac/slow muscle troponin C promoters in differentiated muscle cells in culture (21, 36). On the other hand, the mutations in the human pM MEF3 motif did not cause any major changes in promoter activity in transient transfection analysis of differentiated muscle cells (45). However, here we show that the mutations in either of the two pM MEF3 core motifs cause a dramatic decrease in the pM activity in transgenic mouse muscles, demonstrating that this element constitutes an essential part of the fast-muscle-specific regulatory mechanism of pM. The observation that no activity could be detected in most of the mMEF3 lines, in addition to the fact that the same mutations have little effect on pM activity in transient transfection assays, suggests that the MEF3 motif has a regulatory role that is mainly associated with a chromatin context. However, the possibility that this motif interacts with stage-specific factors required for elevated promoter activity in mature adult muscles cannot be excluded.

The M1 element is necessary for high pM induction during myoblast differentiation into myotubes (45). The differences in the DNA-protein interactions at the M1 element in fast and slow muscles suggest that this element plays an important role in the fast-muscle-specific promoter activity. Indeed, the substitution in the M1 sequence of four nucleotides results in a dramatic drop in the pM activity in transgenic mouse muscles. Interestingly, a highly homologous DNA element has been shown to be important in cultured muscle cells for the activity of the AMP deaminase (AMPD1) promoter, which in adult rats is particularly active in fast, glycolytic muscles (32). We have found M1-like elements in several other muscle-specific genes as well (45), but their roles in these contexts have not been studied yet.

The third element that is required for high-level pM activity in mature adult muscles is the USF binding E box. However, the mutation of this element causes a less dramatic drop in promoter activity than the MEF3 or M1 mutation. Since the E box is not required for high pM induction during myogenic differentiation in cell culture, it is likely that this element forms

a powerful stage-specific activating element. However, because it is very difficult to distinguish between a strong enhancing activity and a chromatin opening function, we cannot exclude the possibility that the USF binding site participates in pM control also at the chromatin level. Interestingly, a USF binding E box motif is present in one of the four DNase I HS sites that design the human β -globin locus control region, and it is supposed to participate there in the chromatin opening process (7). Moreover, USF factors have been shown to bind to E boxes that are important for the expression of the cardiac-muscle-specific myosin light-chain 2 gene in transgenic mice (34). Our results provide evidence that USF binding sites can be important also for skeletal-muscle-specific expression *in vivo*.

Although clearly required for the establishment and/or maintenance of important promoter activity in transgenic mice, none of the three protein binding sites, MEF3, M1, or USF element, alone or in pairs appear to be sufficient to confer high-level skeletal-muscle-specific expression to pM. Furthermore, it seems that the pM tissue specificity is not directly linked to any single promoter element, since all mutant constructs contribute to at least one transgenic line in which the promoter is tissue specifically active. In addition, none of the mutations result in a high frequency of ectopic expression. These results indicate that a specific combination of *cis*-acting elements is required to form a powerful fast-muscle-specific activating region.

Mutations in the MEF2-M2/NF1 region reveal striking differences in the gene expression potentials among individual muscles. A number of NF1 binding sites have been identified within 5'-flanking regions of various genes with different tissue distributions (10, 17, 28). Importantly, NF1 binding sites are present in some muscle-specific regulatory regions (2, 15, 18), e.g., in the human MyoD gene (16). In pM, an NF1 binding site overlaps with a weak-affinity binding site for the MEF2/RSRF proteins in the sequence referred to as the MEF2-M2/NF1 region (45). In cultured myotubes, all different mutations in this region result in a similar drop in the pM-driven CAT activity (45). Here we show that the mutation in the NF1 consensus half-site (mM2) results in an important decrease in promoter activity in fast muscles and especially in one of the two fast muscles studied, the gastrocnemius. Mutation of the adjacent AT-rich sequence (mMEF2) has a similar but milder effect on pM activity in gastrocnemius muscle. These results, together with the observation that the NF1 factors appear to represent the main binding activity in the MEF2-M2/NF1 region (45), suggest that both the mild mMEF2 and strong mM2 phenotypes are mainly due to a disturbed NF1 binding to this region. This hypothesis is supported by the observation that the NF1 factors present in adult skeletal muscle nuclei do not bind to mM2 DNA but still bind to the MEF2-M2/NF1 region when the AT-rich sequence is changed, but with an approximately 30% reduced affinity (45).

Interestingly, the mutations in the MEF2-M2/NF1 region result in dramatic differences in the promoter activity in two fast mouse muscles that are composed of nearly the same proportions of identical fiber types if classified according to the expression of different MHC isoforms. Both vastus lateralis and gastrocnemius are composed mainly of glycolytic fibers of type IIB (20) in which pM is the most active (44). However, the fiber type classification according to MHC isoforms is highly simplified and does not correlate to a specific metabolic status (39). Therefore, vastus lateralis and gastrocnemius muscles may contain metabolically different type IIB fibers which could correspond to the specialized functions of these muscles in the animal. Consequently, the pM activity could be regulated in

distinct ways in these metabolically different fiber subtypes. It has already been proposed that the phenotypes of individual muscles are specified by a unique combination of transcription regulators. In addition, different muscles are innervated by distinct sets of motoneurons which could for their part have an influence on the final muscle identity (13). Furthermore, integration site-dependent expression of some transgenes has provided indirect evidence that individual muscles differ in their gene expression potentials (38). Our results may support this hypothesis in showing that one regulatory element has a different functional importance in seemingly very similar fast muscles.

The tissue-specific and position-independent pM activity results from multiple DNA-protein interactions in a highly restricted promoter region. The molecular mechanism by which gene expression becomes restricted to specific fiber types has remained largely unknown. The only transcription factors shown to be expressed at different levels in distinct fiber types are MyoD and myogenin (23, 54). However, slow and fast fiber types form despite the absence of these proteins (40, 53), and we show here that fiber-type-specific expression can be achieved without high-affinity binding sites for the MyoD family members. Therefore, additional regulatory factors are likely to participate in the establishment of myofiber diversity.

In the present study we show that a very short and compact proximal sequence from bp -164 to +45 directs pM expression specifically to fast skeletal muscles and that multiple promoter elements present in this region seem to contribute remarkably to the promoter activity. Although the more upstream sequences between bp -310 and -165 are dispensable for tissue-specific pM activity, they seem to contribute to the overall level of promoter activity. However, they do not overcome the dramatic effects of the mutations introduced into the most proximal region downstream of bp -164.

Our previous studies including transient transfection assays (45) together with the present work suggest that the M1 and MEF2-M2/NF1 elements present in the most proximal pM region serve as important determinants for promoter specificity. However, additional pM elements, the MEF3 motif and the USF binding E box, are required to highly activate the promoter in the context of the whole organism. Identification of the factors that interact with the crucial pM regulatory elements and their possible cofactors will help to better understand the mechanism by which genes are activated in distinct muscle fibers and in different individual muscles.

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