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Fast-Muscle-Specific Expression of Human Aldolase A Transgenes

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The expression of the human aldolase A gene is controlled by three alternative promoters. In transgenic mice, pN and pH are active in all tissues whereas pM is activated specifically in adult muscles composed mainly of fast, glycolytic fibers. To detect potential regulatory regions involved in the fast-muscle-specific activation of pM, we analyzed DNase I hypersensitivity in a 4.3-kbp fragment from the 5′ end of the human aldolase A gene. Five hypersensitive sites were located near the transcription initiation site of each promoter in those transgenic-mouse tissues in which the corresponding promoter was active. Only one muscle-specific hypersensitive site was detected, mapping near pM. To functionally delimit the elements required for muscle-specific activity of pM, we performed a deletion analysis of the aldolase A 5′ region in transgenic mice. Our results show that a 280-bp fragment containing 235 bp of pM proximal upstream sequences together with the noncoding M exon is sufficient for tissue-specific expression of pM. When a putative MEF-2-binding site residing in this proximal pM region is mutated, pM is still active and no change in its tissue specificity is detected. Furthermore, we observed a modulation of pM activity by elements lying further upstream and downstream from pM. Interestingly, pM was expressed in a tissue-specific way in all transgenic mice in which the 280-bp region was present (32 lines and six founder animals). This observation led us to suggest that the proximal pM region contains elements that are able to override to some extent the effects of the surrounding chromatin.

The developmental basis for different skeletal muscle fiber types is not completely understood, but the discovery of distinct myoblast lineages in early stages of myogenesis indicates that intrinsic properties could determine the early fiber types. In later stages of myogenesis, innervation and hormones influence muscle fiber development and maturation (51, 53) (see references 36, 37, and 56 for recent reviews). In mature vertebrate muscles, four major types of myofibers are detected on the basis of their metabolic properties and expression of different myosin heavy-chain isoforms. Slow-twitch fibers (type I) use primarily oxidative respiration. Fast-twitch type IIA fibers use both oxidative and glycolytic pathways in energy metabolism, whereas fast-twitch type IIB fibers are purely glycolytic. The third fast-type, IIX, fibers are classified as intermediate fast fibers (reviewed in reference 45).

The regulatory factors responsible for the establishment of the different fiber phenotypes are yet unknown. Nevertheless, the identification of a family of skeletal muscle-specific nuclear factors which can activate the myogenic program in nonmuscle cells has led to an increased understanding of muscle development. This MyoD family of factors includes MyoD, myogenin, Myf-5, and MRF4, which bind to a DNA consensus sequence referred to as the E box, found in the regulatory regions of many muscle genes (reviewed in references 14 and 40). These factors are differentially expressed in developing (3, 42, 50, 53) and adult muscles (48) as well as in different types of mature muscle fibers (27), suggesting that they could play a role in muscle fiber diversity. In addition, other nuclear proteins such as M-CAT-binding factor (35), SRF (38), and the MEF-2/RSRF family of factors (13, 19, 47, 62) are thought to contribute to myogenesis. To better understand the potential role of all these factors in the formation of the different fiber phenotypes, it is important to know how the expression of fiber-type-specific genes is controlled.

We have used the human gene coding for aldolase A, a glycolytic enzyme, as a model to study the *cis*-acting elements involved in the control of fast-muscle-specific expression. The regulation of the human aldolase A gene is particularly complex: three alternative promoters are localized within a small 1.6-kbp region and give rise to three types of mRNAs, which differ only in their 5′ noncoding ends (28, 34). We have recently shown (10) that a 4.3-kbp fragment of the human aldolase A gene containing the three promoters and the first two coding exons linked to the 3′ end of the human β-globin gene (EAccI) (Fig. 1 and 2) is sufficient to reproduce correct tissue-specific and developmental regulation in transgenic mice. The two ubiquitously active promoters pN and pH are both transcribed in fetal tissues, and in adult tissues the highest levels of N- and H-type mRNAs are observed in the heart and skeletal muscle tissues. The third promoter, pM, which lies in between the two ubiquitous ones, is highly specific to skeletal muscle. It is turned on at birth and becomes strongly active in mouse muscles after the 15th day, reaching a maximal level of expression in mature adult muscles. This M-type mRNA accumulates in muscles composed mainly of glycolytic fast-twitch fibers. A strong ubiquitous enhancer, shown to be necessary for both pH activity and pN activity in cultured cells, has been localized upstream of pH (Fig. 2) (9). When this pH/enhancer region was deleted in a 1.8-kbp fragment (EAccIΔ7) (Fig. 1 and 2), pN lost its activity in nonmuscle tissues but remained active together with pM in fast skeletal muscles (10). pN and pM also have similar expression patterns during muscle development (38a). This has led us to propose that these two promoters share some regulatory elements for their coactivation in fast muscles.

In the present study, we have further investigated the regulation of this fast-fiber-specific gene expression in trans-

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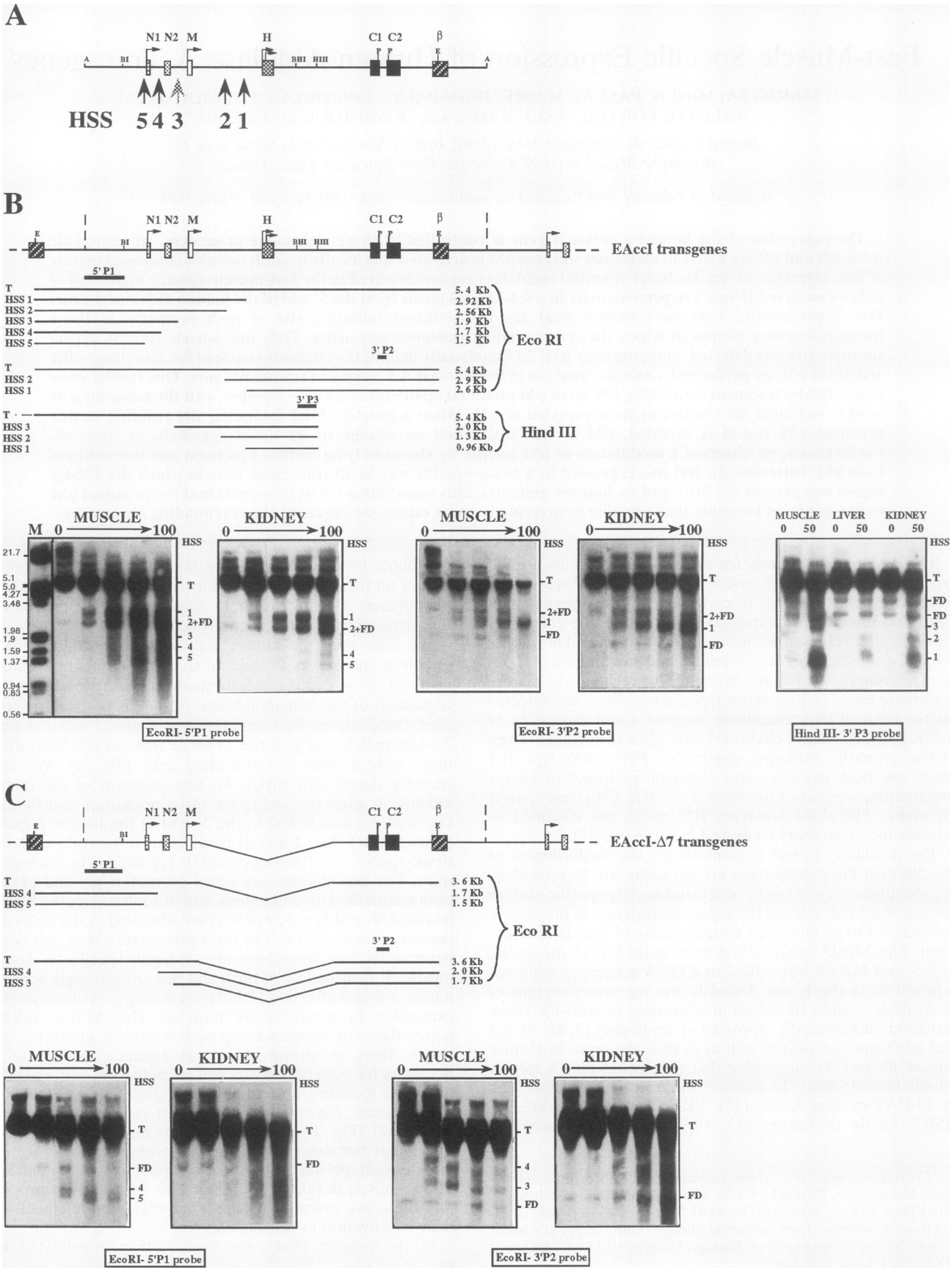
A
N1 N2 M
H
C1 C2
β
HSS 5 4 3 2 1
B
EAccI transgenes
5' P1
T
HSS 1
HSS 2
HSS 3
HSS 4
HSS 5
5.4 Kb
2.92 Kb
2.56 Kb
1.9 Kb
1.7 Kb
1.5 Kb
Eco RI
3' P2
5.4 Kb
2.9 Kb
2.6 Kb
3' P3
5.4 Kb
2.0 Kb
1.3 Kb
0.96 Kb
Hind III
MUSCLE
KIDNEY
LIVER
HSS
EcoRI- 5'P1 probe
EcoRI- 3'P2 probe
Hind III- 3' P3 probe
C
EAccI-Δ7 transgenes
3.6 Kb
1.7 Kb
1.5 Kb
2.0 Kb
1.7 Kb
FD

genic mice. In mice, all mechanisms influencing the formation of a fast-fiber-specific mRNA expression pattern are present and the transgenes are subjected both to intrinsic control systems and to modulation by innervation or hormones. The description of *trans*-acting nuclear factors which mediate this regulation will be important for understanding the mechanisms involved in the establishment and maintenance of muscle fiber diversity. To enable the identification of these factors, we have defined the minimum control region required for fast-muscle-specific expression of pM. This consists of a 280-bp DNA sequence near the transcription start site, characterized by muscle-specific DNase I hypersensitivity. Furthermore, fast-muscle-specific expression of pM was shown to be independent of a MEF-2-binding site located in this region.

For most genes, expression in transgenic mice is influenced by the chromatin surrounding the site of integration. As a result, no expression is detected in several transgenic lines created for one construct, and it is usual to find ectopic activity of promoters (reviewed in references 29 and 43). For a few transgenes, integration-site-independent expression has been observed (1, 5, 20, 22, 26, 39, 44, 60). DNA sequences conferring such position-independent expression to linked genes in transgenic animals are generally characterized by a hypersensitivity to nuclease (1, 20, 22).

In all lines of transgenic mice carrying a 4.3-kbp fragment from the 5′ end of the aldolase A gene, the three promoters were tissue-specifically active, suggesting dominant elements of some type capable of overriding the effects of the surrounding chromatin could be present in our transgene (10). In this study, this unusual behavior of aldolase A transgenes was further investigated with respect to pM expression. The same 280-bp region which is needed for tissue-specific expression seems to be sufficient to ensure expression in all transgenic lines.

MATERIALS AND METHODS

DNase I hypersensitivity assay. Two lines of transgenic mice with a large number of integrated transgenes EAccI-3 and EAccIΔ7-17 (10) were used to detect nuclease hypersensitivity. Nuclei from adult-mouse liver and kidney tissues were prepared as described in reference 31. Nuclei from adult-mouse skeletal muscle tissue were prepared by a method modified from that of reference 31. Muscles from mouse hind limbs were first homogenized in a Waring blender in 10 volumes of 2.4 M sucrose buffer. Triton X-100 was added up to 0.5% (vol/vol) in muscle homogenates, and they were additionally homogenized in an electric Teflon glass homogenizer and filtered through gauze. Twenty-seven milliliters of homogenate was layered on 10-ml cushions of the same buffer containing 2.3 M sucrose. The tubes were centrifuged at 25,000 rpm for 2 h at 0°C in an SW27 rotor. Nuclei from HepG2 hepatoma cells were isolated as described in reference 52.

The pelleted nuclei were resuspended in DNase I buffer [15 mM Tris (pH 7.6), 60 mM KCl, 15 mM NaCl, 0.2 mM EDTA, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 0.15 mM spermine, 0.5 mM spermidine, 0.3 M sucrose, 5% glycerol (vol/vol), 0.5 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride]. The DNA concentration was measured, and nuclei corresponding to 200 μg of DNA were digested for 10 min at 4°C with increasing concentrations of DNase I (Boehringer Mannheim) ranging from 0 to 100 U/ml in a final volume of 500 μl containing 5 mM $MgCl_2$. Ten microliters of 0.5 M EDTA was added to stop the reaction. The nuclei were washed once with DNase I buffer without phenylmethanesulfonyl fluoride or dithiothreitol and lysed in proteinase K buffer (50 mM Tris [pH 7.4], 0.1% sodium dodecyl sulfate, and 2 mM EDTA). The samples were incubated overnight at 37°C with 300 μg of proteinase K per ml, and genomic DNA was purified by standard methods.

To analyze hypersensitive (HS) sites, 10 μg of genomic DNA from DNase I-treated or untreated nuclei from different mouse tissues were digested with *Eco*RI or *Hin*dIII. Twenty micrograms of HepG2 DNA was used for *Eco*RI digestion. The digested DNAs were subjected to electrophoresis in a 0.8% agarose gel and transferred on Hybond N^+ filters (Amersham) as described elsewhere (10). *Eco*RI- and *Hin*dIII-digested λ-phage DNA was loaded into each lane with the sample DNAs as a molecular weight marker. After transfer, filters were successively hybridized with three different genomic probes, 5′P1 (*Acc*I at position 723 to *Bgl*I at 1249), 3′P2 (*Pst*I at 4705 to *Pst*I at 4830), and 3′P3 (*Bam*HI at 3581 to *Hin*dIII at 3865) (Fig. 1), and finally with a λ-probe to visualize the molecular weight marker.

Creation of transgenic mice. The aldolase A–β-globin hybrid transgenes EAccI, EAccIΔ7, EAccIΔ8, and EAccIΔ14 were derived from the pE14 and pE14Δ7 constructs previously described (10) with the restriction enzymes indicated in Fig. 2. pE14 and pE14Δ7 were then digested with *Bal*I at positions 1792 and 6132, and the released fragments were subcloned into the Bluescript II KS vector (Stratagene Cloning Systems), giving rise to pKBalIΔ1 and pKBalIΔ2 plasmids, respectively. Transgenes KBalIΔ1, KBalIΔ2, KAgeIΔ2, and KBamHIΔ2 were derived from these two plasmids (Fig. 2).

A mutation of a potential MEF-2-binding site (CTAAAT ATAG) between positions 1981 and 1990 in the M promoter was created by using a site-directed mutation system (Amersham) with an oligonucleotide (5′-CAGGGACCagatctgctGG AAACCTGAAGCC-3′) which substitutes the MEF-2-binding site with sequences containing a *Bgl*II restriction site for

FIG. 1. Analysis of DNase I HS-site formation in the human aldolase A EAccI-3 and EAccIΔ7-17 transgenes. (A) Schematic representation of the analyzed region indicating the positions of the main transcription start sites as well as the alternative noncoding exons (shaded and open boxes) and the coding exons as (filled boxes). The β-globin third exon is also indicated (β). Enzymes: A, *Acc*I; BI, *Bgl*I; BHI, *Bam*HI; HIII, *Hin*dIII; P, *Pst*I; E, *Eco*RI. The distribution of the five DNase I HS sites detected in this region is indicated (vertical arrows). (B) Analysis of the EAccI-3 transgenic line carrying 40 copies of the transgene. A head-to-tail arrangement of the 5.4-kbp transgene is presented at the top. DNase I-treated samples were digested either with *Eco*RI or with *Hin*dIII, and the resulting fragments were visualized with genomic probe 5′P1, 3′P2, or 3′P3 as indicated for each experiment. The resulting fragments are presented diagramatically, and the signals corresponding to the entire transgene (T) fragment, different HS sites, or flanking DNA (FD) fragments are indicated on the right of each blot. As the results obtained with liver and kidney DNAs were identical, only blots corresponding to kidney DNA are shown for *Eco*RI-digested samples. To take into account slight variations in the migration of the DNase I samples, *Eco*RI- and *Hin*dIII-digested λ-phage DNA was added in each lane as a molecular weight marker and visualized later with a λ-probe. The positions of these markers (lane M) are indicated in kilobase pairs on the left. (C) Analysis of the EAccIΔ7-17 transgenic line carrying 190 copies of the transgene. The EAccIΔ7 construct was derived from EAccI by deleting a 1.8-kbp fragment containing the pH/enhancer region (Fig. 2). A head-to-tail arrangement of the *Eco*RI-digested 3.6-kbp transgene is shown at the top.

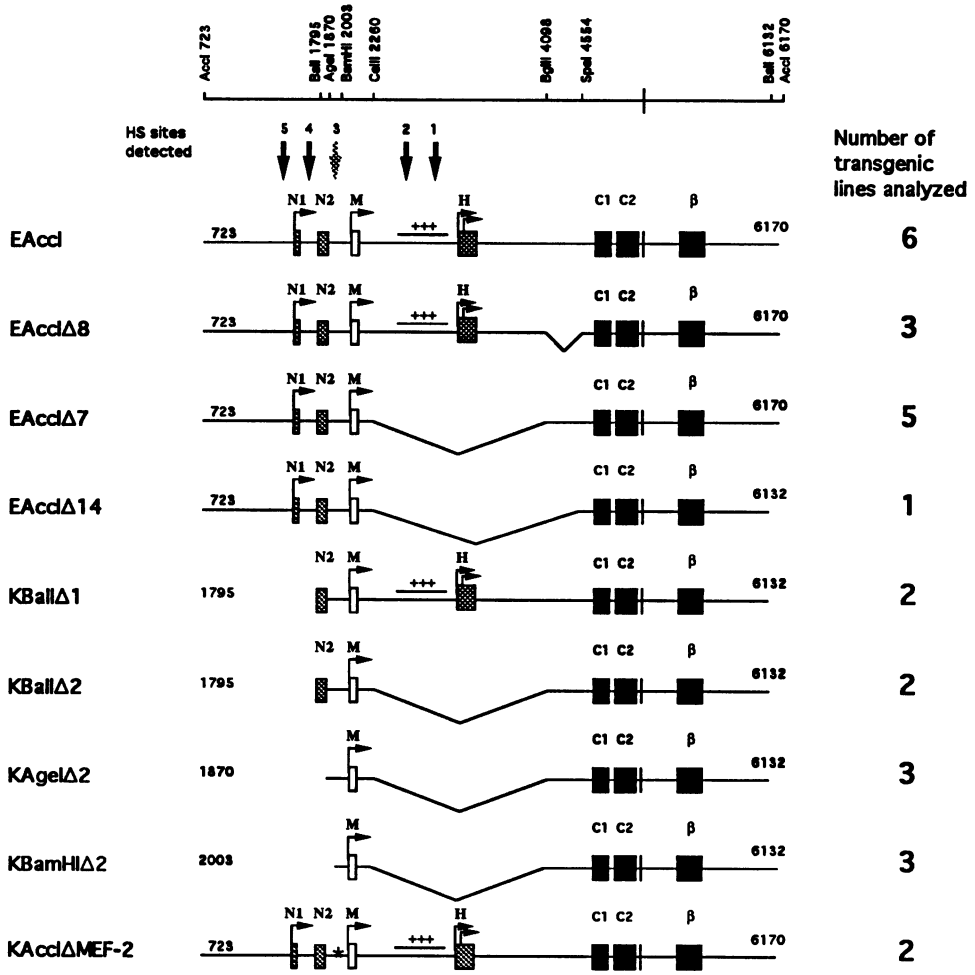

FIG. 2. Aldolase A–β-globin transgenes. The restriction enzymes used to generate the different transgenes are indicated at the top. The numbering is derived from previous publications (10, 34), with an *Eco*RI site upstream of the N1 exon defined as position 1. This practice was chosen for the human aldolase A gene because of the presence of multiple transcription start sites in a small 1.6-kb region, which renders the conventional minus and plus numbering of the promoters confusing. *, mutated MEF-2 element (positions 1981 to 1990); +++, H enhancer; β, β-globin third exon.

convenient detection of mutants. The introduced mutation was verified by sequencing.

The pMCAT construct was created by ligation of the pBLCAT2 plasmid (33) digested with *Xho*I and *Xba*I (which deletes the herpesvirus thymidine kinase sequences) with a PCR-created fragment from pKBalIΔ2. The oligonucleotides used for PCR were the universal primer in the KS− vector and a primer containing the last nucleotides from M exon and a *Sal*I site for cloning (5′-CAGTCGACCTGCGGCGAGGCC GAAGG-3′). To avoid any base changes, the entire PCR fragment covering the M promoter and exon between positions 1795 and 2150 was verified by sequencing.

Production and detection of transgenic mice were performed as previously described (10). The estimation of transgene copy numbers was done by Southern blot analysis of 5 μg of genomic tail DNA from F_1 offspring heterozygous for the transgenes. The signals detected on autoradiographs were scanned with a Shimadzu scanner CS-930 and compared with standard DNA signals corresponding to 1 or 10 copies of the injected fragment used in the creation of transgenic animals.

RNA and CAT analysis. All transgenic mice used in this study were heterozygous F_1 offspring. Total RNA was prepared from several mouse tissues from two or three adult animals of each transgenic line and from control animals as described in reference 7. Northern (RNA) blots and RNase protection assays were performed as previously described (10). The quantification of M- or H-specific mRNAs from vastus lateralis muscles was done by densitometric scanning of Northern blot autoradiographs exposed for different times from several experiments. The signals obtained with the rRNA (R45) probe (10) served as standards between different lanes. At least three different Northern blot analyses were performed with each line. After scanning, all values were normalized to that obtained with transgenic line EAccI-3, taken as 100. For the chloramphenicol acetyltransferase (CAT) assay, mouse tissues were homogenized in 0.5 to 1.0 ml of 0.25 M Tris (pH 7.5)–5 mM EDTA. The homogenates were centrifuged, and the supernatants were heated at 65°C for 8 min and recentrifuged. The concentration of soluble proteins in supernatants was determined by the Bio-Rad protein assay, and CAT activity was assayed according to standard methods using from

0.2 to 50 μg of protein extracts, keeping the enzyme activity in a linear range.

RESULTS

Muscle-specific and ubiquitous DNase I HS-site formation in transgenic animals. DNase I HS sites are found at the 5′ end of actively transcribed genes and are often associated with gene regulatory regions such as promoters, enhancers, and silencers. These sites are thought to reflect gaps in the distribution of nucleosomes and the binding of *trans*-acting proteins (reviewed in references 15 and 21). We were interested in detecting regulatory sequences involved in the control of the human aldolase A gene, particularly those involved in the specific activation of the M promoter in the adult-mouse fast skeletal muscle. Therefore, we determined the locations of the DNase I HS sites in the chromatin of two previously described transgenic lines harboring transgene EAccI or EAccI-Δ7 (Fig. 2), from which pM is specifically expressed in skeletal muscle (10).

Nuclei from transgenic muscle, liver, and kidney tissues were digested with increasing amounts of DNase I, and the resulting *Eco*RI- or *Hin*dIII-generated fragments were detected with 5′P1, 3′P2, and 3′P3 probes (Fig. 1B and C). The molecular weight determination and comparison of the various DNase I-liberated fragments in the two analyzed transgenic lines allowed the mapping of a total of five DNase I HS sites, which are shown in Fig. 1A. As the results obtained with liver and kidney samples were similar, only one representative example is presented.

In the transgenic line EAccI-3, one major HS site (HSS1) mapping near the pH transcription initiation site at the 3′ border of the ubiquitous H enhancer (around position 2900) could easily be detected in both *Eco*RI- and *Hin*dIII-digested DNase-treated DNAs (Fig. 1B). This site was present in all three tissues studied. A second site (HSS2) was localized between the M and H noncoding exons on the 5′ end of the H enhancer, around position 2600. This HS site was best observed in *Hin*dIII-digested liver and kidney DNAs with the 3′P3 probe. Although detectable with *Eco*RI digestion in all three tissues studied, as shown by the marked increase in the intensity of the corresponding fragment, this site was difficult to analyze because a signal of a similar size was already present in samples which had not been treated with nuclease. We supposed that this signal was due to the flanking DNA fragment of integrated transgenes, created by *Eco*RI digestion. When DNAs were digested with *Hin*dIII, different flanking fragments were created and the HSS2 was clearly detected in liver and kidney samples. In this case, HSS2 is only faintly detected in skeletal muscle, probably because of the relative strength of HSS1 in this tissue.

Two additional HS sites were detected in all three tissues. HSS5 was localized near the multiple transcription initiation sites 5′ of the N1 exon around position 1500, and HSS4 was localized between the N1 and N2 exons at position 1700 (Fig. 1B), where transcription is also initiated (18). The four ubiquitous HS sites detected occurred at identical sites within the intact aldolase A gene studied in a human hepatoma cell line (data not shown) in which the endogenous N and H promoters are active (18).

On the other hand, a unique muscle-specific site (HSS3) was faintly detectable in *Eco*RI-digested DNA with the 5′P1 probe. This site was clearly revealed when the DNAs were digested with *Hin*dIII (Fig. 1B) and could be localized in between −200 to −100 bp upstream of the pM transcription start site around position 1950.

The EAccIΔ7 transgene was derived from EAccI by deleting a 1.8-kbp fragment encompassing the pH/enhancer region (Fig. 2). In EAccIΔ7 mice, pN and pM are active only in skeletal muscles. The HS-site pattern generated by the analysis of the EAccIΔ7-17 line confirmed that obtained with the EAccI-3 line. No HS sites were present in liver or kidney samples for which no promoter activity was found. In contrast, in skeletal muscle tissue, the regions proximal to the two promoters pN and pM proved to be hypersensitive. In *Eco*RI-digested DNA, the HS sites closest to the probe used were the easiest to detect (Fig. 1C).

Taken together, our results show that potential regulatory regions in the human aldolase A gene reside near the transcription initiation sites of each promoter. The presence of each site reflects the activity of the corresponding promoter nearby. Importantly, the HSS3 near pM was detected only in skeletal muscle samples. This implied that muscle-specific control elements for pM activity could reside in this region.

Elements proximal to the M promoter are required for its fast-muscle-specific activity in transgenic mice. In order to further define regions responsible for the muscle-specific expression of pM, six new aldolase A–β-globin hybrid constructs were used to generate transgenic mice (Fig. 2). For each construct, one to three independent transgenic lines were analyzed for pM activity by RNase protection analysis of total RNA isolated from various adult-mouse tissues including fast-fiber-enriched (gastrocnemius and vastus lateralis) and slow-fiber-enriched (soleus) skeletal muscles. As for any given construct, all transgenic lines behaved in the same way; only one representative RNase protection result is presented in Fig. 3.

The analysis of the EAccIΔ7 lines has previously shown that pM is still active in fast skeletal muscles when the pH enhancer/promoter region including HSS1 and HSS2 is deleted from *Cel*II to *Bgl*II (10) (Fig. 2). Here, we show that deleting the remaining part of the intron preceding the first coding exon, C1 (Fig. 2, transgenes EAccIΔ8 and EAccIΔ14), does not suppress pM activity in fast muscles (Fig. 3).

When upstream sequences were progressively deleted up to *Age*I, leaving only 235 bp upstream of the pM transcription start site (transgenes KBalIΔ1, KBalIΔ2 and KAgeIΔ2 [Fig. 2]), pM was still active in fast muscles (Fig. 3). By contrast, a further deletion of a 130-bp fragment up to *Bam*HI (transgene KBamHIΔ2) (Fig. 2) led to a loss of detectable pM activity in all tissues studied including skeletal muscles (Fig. 3). Two additional founders obtained with this transgene were also analyzed and showed the same result.

This deletion analysis of transgenic mice indicates that a 130-bp region located upstream of pM (between *Age*I and *Bam*HI), where the previously described muscle-specific DNase I HS site was mapped, is essential for high-level tissue-specific expression of pM. The most-proximal promoter fragment up to 100 bp upstream of the pM transcription start site is not sufficient together with the 3′ sequences present in the construct to activate pM in transgenic mice.

Another feature arose from the above results. When the relative activity of pM in soleus (a slow muscle) was compared with those in the two fast muscles examined (vastus lateralis and gastrocnemius), a marked difference was repeatedly noticed. In transgenic lines EAccI, EAccIΔ8, and KBalIΔ1, in which the 1.8-kbp *Cel*II-*Bgl*II region is present, M-type mRNAs were clearly detectable in soleus samples. In contrast, when this 1.8-kbp region was deleted, as in EAccIΔ7 (10), EAccIΔ14, KBalIΔ2, and KAgeIΔ2, pM activity became barely detectable in this slow muscle (Fig. 3). This shows that the 1.8-kbp region which includes the previously described strong

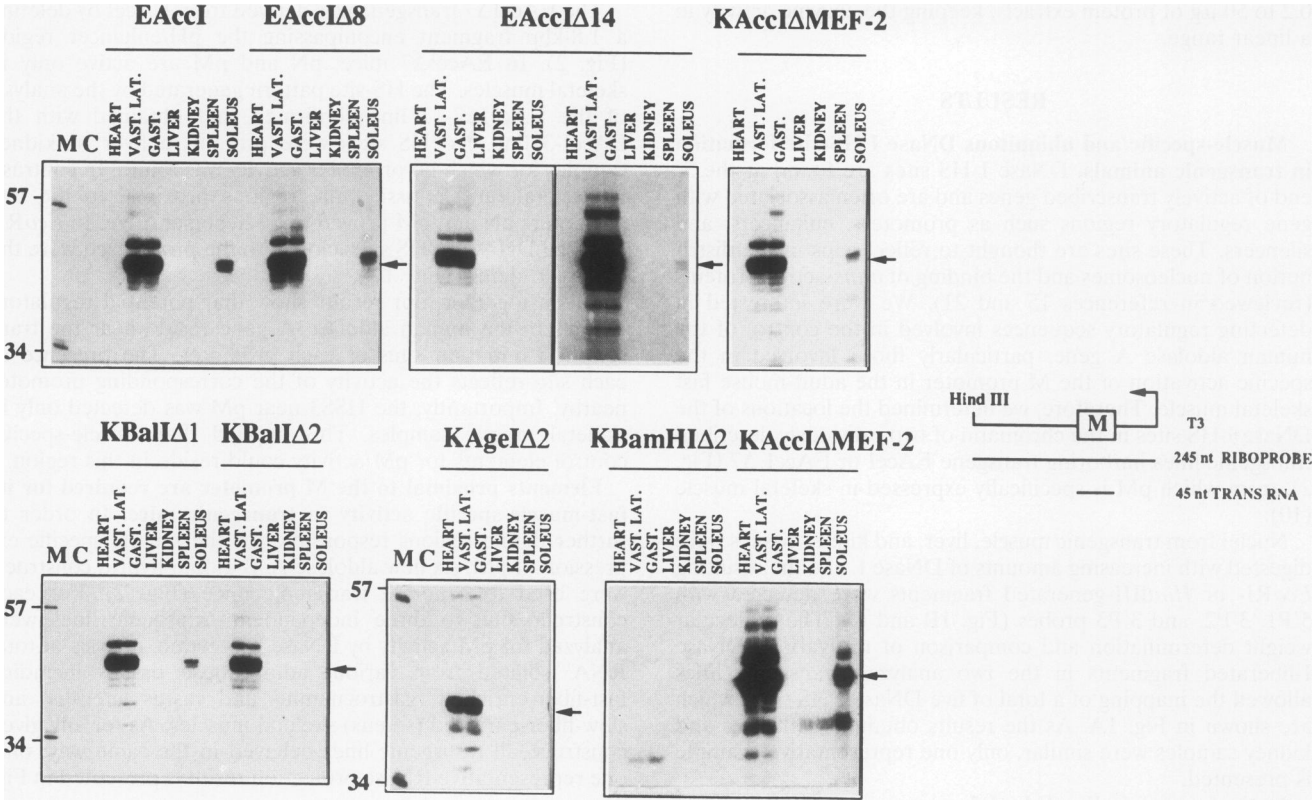

FIG. 3. pM expression patterns of aldolase A–β-globin transgenes. Total RNAs (5 μg) from adult-mouse tissues were analyzed by RNase protection with the RNA probe diagrammed on the right. In the experiment with KBamHIΔ2 and KAccIΔMEF-2, 20 μg of RNA was analyzed. The resulting 45-bp M-exon-specific signal is indicated (arrow). The RNase protection results are shown for lines EAccI-3, EAccIΔ8-9, EAccIΔ14-25, KAccIΔMEF-2-49, KBalΔ1-47, KBalΔ2-4, KAgeIΔ2-4, and KBamHIΔ2-18. To facilitate comparison between expression in fast muscles and expression in slow muscles, exposures with similar signals in fast muscles were chosen. For EAccIΔ14, a longer exposure is also shown to visualize the signal in the soleus muscle. M, molecular size (in base pairs) marker (*Hpa*II-digested Bluescript II KS plasmid); C, control RNA from gastrocnemius (GAST.) muscle tissues of nontransgenic mice; VAST. LAT., vastus lateralis.

ubiquitous H enhancer is able to modulate pM activity in skeletal muscles.

Mutation of a putative MEF-2 site in pM does not impair fast-muscle-specific expression of the promoter. Analyses of *cis*-acting elements required for the activity of muscle-specific promoters in myogenic cell cultures have defined a consensus DNA sequence for the binding of MEF-2/RSRF family of factors (19, 47, 62). Such a potential MEF-2-binding site (CTAAATATAG) is present 140 bp upstream of the pM transcription initiation site in the region between the *Age*I and *Bam*HI sites. This sequence is 100% conserved among mouse, rat, and human genes (55), and it has been shown that the rat sequence is capable of binding MEF-2 family proteins (25).

In order to study the possible role of this motif in fast-muscle-specific expression of pM in the human aldolase A gene, two lines of transgenic mice were created with a KAccIΔMEF-2 construct (Fig. 2), in which the MEF-2 site is substituted by a *Bgl*II restriction site. In both lines analyzed, pM was active (Table 1) and retained its tissue specificity, as illustrated for the KAccIΔMEF-2-49 line in Fig. 3. This indicates that the MEF-2 element is not necessary for fast-muscle-specific expression of the M promoter. Furthermore, the expression pattern of KAccIΔMEF-2 transgenes in different muscles resembles that of EAccI lines, indicating that elements in the 1.8-kbp fragment responsible for detectable pM expression in soleus tissue are capable of interacting with pM in the absence of the MEF-2 site.

Position-independent expression of pM in the muscles of transgenic mice. In our previous studies using EAccI and EAccIΔ7 transgenes, we observed that all nine lines analyzed expressed these transgenes (10). This unusual behavior of aldolase A transgenes is confirmed by the present study of 18 additional lines of transgenic mice carrying aldolase A–β-globin hybrid transgenes. With the exception of KBamHI-Δ2 lines, in all these mice pM was functional, as were the other promoters present in constructs (Table 1) (49a). This kind of position-independent expression is often associated with transcription activity which is related to the number of transgene copies integrated in the genome. We, therefore, compared the levels of M-specific mRNAs in all the transgenic lines with the results of a Northern blot analysis of total RNA isolated from a fast muscle (vastus lateralis). Figure 4 shows a typical set of autoradiographs obtained by hybridizing Northern blots successively with three probes specific to M exon, H exon, and 18S rRNA (R45) (10), allowing for standardization. In order to allow comparison among the various lines, mRNAs from six EAccI lines were used as references on each blot. The analysis of pH-derived mRNAs served as an internal control when pH was present in the transgene. All values obtained after densitometric scanning of autoradiographs were normalized to that obtained with the EAccI-3 line, taken as 100. The mean values of these analyses are shown in Table 1 as relative levels of pM and pH expression. The level of M-type or H-type mRNAs per transgene copy are also given.

TABLE 1. Relationship between transgene copy number and pM expression level in different aldolase A–β-globin transgenic lines

Transgenic line	Copy number	pM[a]	pH[a]	pM/copy	Mean pM/copy	pM/pH
EAccI					4.7 ± 1.4	
48	3	14.5 ± 5	8.5 ± 3.5	4.8		1.7
29	4	18.0 ± 9	16.7 ± 11	4.5		1.1
44	5	26.5 ± 6	17.5 ± 5	5.3		1.5
47	10	43.5 ± 12	42.5 ± 20	4.3		1.0
49	35	237.0 ± 36	140.0 ± 13	6.7		1.7
3	40	100.0	100.0	2.5		1.0
EAccIΔ8					5.4 ± 3[b]	
9	51	325.0 ± 67	301.0 ± 60	6.3		1.1
4	84	665.0 ± 160	883.0 ± 240	7.9		0.75
11	148	305.0 ± 40	356.0 ± 36	2.0		0.85
EAccIΔ7					12.1 ± 5.4[c,d]	
16	2	37.5 ± 8		18.7		
41	10	132.0 ± 28		13.2		
36	100	1,530.0 ± 395		15.3		
43	177	987.0 ± 251		5.6		
17	190	1,474.0 ± 796		7.7		
EAccIΔ14-25	4	68.0 ± 25		17.0	17.0	
KBalΔ1					16.2[c,e]	
38	2	30.0 ± 7	28.0 ± 12	15.0		1.1
47	5	87.0 ± 34	126.0 ± 29	17.0		0.7
KBalIΔ2					8.5[b]	
4	4	61.0 ± 17		15.2		
40	47	90.0 ± 10		1.9		
KAgeIΔ2					15.8 ± 8[c,f]	
4	8	177.0 ± 53		22.0		
5	78	1,470.0 ± 280		18.8		
27	156	1,062.0 ± 188		6.8		
KBamHIΔ2					0	
8	1	0		0		
1	8	0		0		
18	230	0		0		
KAccIΔMEF-2					2.7[b]	
17	1	2.7 ± 1.6	3.5 ± 2.5	2.7		0.8
49	4	11.0 ± 7	43.0 ± 19.5	2.7		0.25

[a] Relative to the value for EAccI-3. The data are means ± standard deviations for three separate Northern blot assays.
[b] Not significantly different from the value for the EAccI lines by Student's *t* test.
[c] Significantly different from the value for the EAccI lines by Student's *t* test.
[d] $P < 0.05$.
[e] $P < 0.02$.
[f] $P < 0.01$.

A correlation between pM expression and transgene copy number was observed with the different lines obtained for a given transgene. The levels of pM expression per gene copy were relatively constant but dropped, often significantly, when a high number of copies were integrated. When present, pH expression dropped in a parallel way (Table 1). This kind of phenomenon has been noticed for other transgenes as well (17, 46).

The fact that pM expression was roughly copy number dependent (at least up to 40 integrated copies) permitted us to compare the expression levels of this promoter among the different transgenes. Significant changes were noticed when the average values per copy for pM expression in the vastus lateralis muscle were compared. When a 1.8-kbp fragment containing pH and the ubiquitous enhancer was deleted, as in lines harboring transgene EAccIΔ7 or EAccIΔ14, a threefold increase in pM activity was detected in comparison with the EAccI or EAccIΔ8 transgene (Table 1). A similar increase in pM activity without any evident change in the pM/pH expression ratio was also detected when upstream sequences were deleted until *Bal*I (in KBalIΔ1). When both the 1.8-kbp fragment and the upstream region were deleted (as in, e.g., the KAgeIΔ2 transgene), no additional effect could be observed. These results suggest that sequences situated further upstream and downstream from pM can modulate the level of activity of this promoter in skeletal muscle when present together.

A 350-bp fragment of the aldolase A gene directs the expression of a CAT reporter gene in fast muscles in transgenic mice. To test whether pM alone was sufficient to target reporter gene expression in fast-type muscles, we created transgene pMCAT, in which 355 bp of pM aldolase A sequences were linked to a CAT reporter gene. This transgene

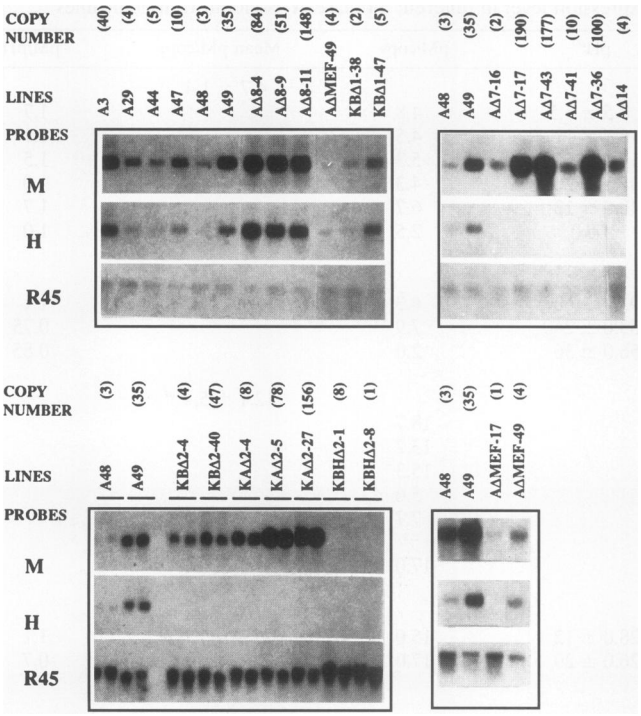

FIG. 4. Analysis of pM expression levels in skeletal muscles of transgenic mice. Northern blot analyses were performed with 5 μg of total RNA from a fast muscle, vastus lateralis, of one or two animals of each transgenic line (20 μg of RNA was analyzed for KBamHIΔ2). The Northern blots were successively hybridized with M- or H-specific probes, the obtained signals were quantified by scanning different exposures of the Northern blots, and the values obtained were standardized with the scanning of the R45 probe signals. Six EAccI-lines (A3, A29, A44, A47, A48, and A49) (upper left) served as references for different blots. To save space, only the last two EAccI lines (A48 and A49) are shown in the other three panels. In the lower left panel, pairs of identical lanes represent RNA samples prepared from two different animals from a given line. The number of transgene copies integrated in each line is indicated. A reevaluation of the transgene copy number in some EAccIΔ7-lines led to some modifications of the numbers we previously reported (10). Abbreviations: A, EAccI; KB, KBalI; KA, KAgeI; KBH, KBamHI.

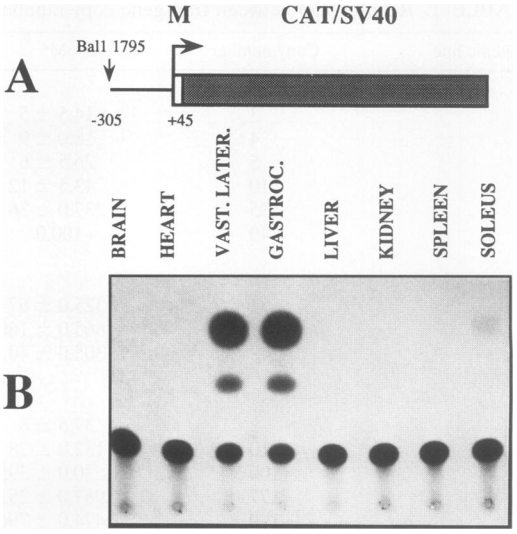

FIG. 5. (A) pMCAT construct used to create 14 independent transgenic founder mice. SV40, simian virus 40; M, M promoter sequences (from *Bal*I at position 1795 to position 2150). (B) Tissue-specific expression of pMCAT transgenes, represented here by line 82B carrying 18 copies of the transgene integrated in the genome. In this experiment, extracts containing 1 μg of protein were assayed for CAT activity. VAST. LATER., vastus lateralis; GASTROC., gastrocnemius.

contains 310 bp of pM proximal sequences, including HSS3, together with the 45-bp M exon (Fig. 5A). Fourteen transgenic founders were obtained, from which eight independent lines were derived. Muscle and nonmuscle tissue extracts from adult transgenic F_1 offspring from five pMCAT lines were assayed for tissue specificity of CAT activity. As shown in Fig. 5B for line 82B and in Table 2, high levels of CAT activity were constantly found in fast muscles vastus lateralis and gastrocnemius while much lower levels of expression were detected in the predominantly slow soleus muscle. No expression in heart, liver, kidney or spleen samples was observed, and in only one case (line 26) ectopic expression was detected in the brain. Three additional transgenic lines (88, 82A, and 106) were studied only in the two muscle types, and here too CAT activity was found to be much higher in fast muscles than in slow ones (Table 2). Fast-muscle-specific expression was also observed in six additional positive founders (data not shown).

In order to study more precisely copy number-related expression of pM, CAT activities in fast and slow muscles of eight transgenic lines were compared. Mean CAT activities of different assays are given in Table 2. In general, the more copies of the transgene integrated, the higher pM expression, with one exception, line 88, in which pM activity per copy was much higher than those in others (Table 2). The results for the pMCAT transgene were more scattered than those for the aldolase A–β-globin transgenes. Nevertheless, once more, it is striking to observe that of a total of 14 lines and founders tested, all expressed pM.

In conclusion, the 355-bp fragment was sufficient alone to drive fast-muscle-specific expression of the linked CAT gene in all 14 independent transgenic mice tested. These results confirm the importance of the pM proximal sequences in tissue-specific expression of the aldolase A muscle-specific promoter. Furthermore, the fact that all pMCAT lines expressed pM suggests that the pM proximal region contains elements capable of ensuring a high level of tissue-specific expression in different integration sites.

DISCUSSION

In fetal and adult human tissues, different amounts of aldolase A enzyme are produced by using a complex combination of regulatory mechanisms to differentially activate three alternative promoters, pN, pM, and pH. In fast muscles, in which glycolysis is most active, a high level of aldolase A is ensured by the activation of pM. In order to better understand the molecular basis of this activation, we have sought to identify elements that regulate this promoter and confine its expression to fast muscles. For this purpose, a study of chromatin accessibility to DNase I was combined with a functional analysis of transgenic mice.

We demonstrate that a small DNA fragment near the pM transcription start site characterized by the presence of a muscle-specific DNase I HS site is sufficient to obtain fast-muscle-specific expression in vivo. Furthermore, we show that

TABLE 2. CAT activities in tissues from pMCAT transgenic lines

pMCAT line[a]	Copy number	CAT activity (cpm/μg of protein/min)								pM expression/copy in vastus lateralis
		Brain	Heart	Vastus lateralis	Gastrocnemius	Liver	Kidney	Spleen	Soleus	
88	2	0[b]	ND[c]	836.3	995.6	ND	ND	ND	1.8	418.2
82A	3	ND	ND	34.6	27.3	ND	ND	ND	1.4	11.5
106	6	ND	ND	90.3	119.2	ND	ND	ND	2.8	15.1
24	6	ND	0	196.0	80.9	0	0	0	3.1	32.7
26	10	55.4	0	815.3	489.5	0	0	0	17.6	81.5
82B	18	0	ND	1,387.8	1,385.8	0	0	0	58.3	77.1
20	20	0	0	1,838.2	1,745.6	0	0	0	138.6	91.9
98	22	0	0	1,604.4	1,929.6	0	0	0	3.0	72.9

[a] Lines 82 A and 82 B are derived from the same founder animal.
[b] No activity above background level detected.
[c] ND, not determined.

pM activity is modulated by upstream and downstream sequences.

The presence of muscle-specific and ubiquitous HS sites in the 5′ region of the aldolase A gene reflects the activity of the corresponding promoters. Regulatory events that take place at the chromatin level were analyzed in order to better understand the different aspects of the complex control of the aldolase A gene. Transgenes spanning the 4.3-kbp 5′ region of the human aldolase A gene permit correct expression of the three promoters (10). Many transgenes mimicking endogenous gene expression have been shown to display a DNase I HS pattern similar to that of the gene in its natural chromatin context (4, 32, 60). For these reasons, we used previously described transgenic mice in which pM is active to characterize the localization and tissue-specific formation of DNase I HS sites within the 4.3-kbp segment encompassing the three promoters.

This study has allowed the description of five DNase I HS sites whose presence and specificity proved to correlate with the function of the adjacent promoter in different tissues. Only one muscle-specific HS site (HSS3) could be detected in the region studied, and it was localized between −200 and −100 bp upstream of the pM transcription start site. Remarkably, the M promoter remains insensitive to DNase I in nonmuscle tissues of the EAccI line, despite the presence of two ubiquitously active and nuclease-sensitive promoters surrounding it. Even if transcription starting at the upstream pN and proceeding through pM leads to a "loose" chromatin structure (58), this is not sufficient to allow access of ubiquitous factors to pM. This indicates that muscle-specific factors are required to disturb the nucleosomal arrangement at this promoter region. This observation suggests also that the restriction of pM activity to fast muscles is regulated by the presence of tissue-specific factors and not, e.g., by silencing factors being active in nonmuscle tissues. Such a silencing mechanism has been shown to occur in the repression of the α-fetoprotein gene in liver (57).

The other four sites, HSS1, -2, -4, and -5, were detected in all tissues studied, reflecting the ubiquitous activity of the other two promoters, HSS4 and HSS5, mapping near the two main transcription start sites identified for pN (18), with HSS1 and HSS2 mapping to the 3′ and 5′ borders, respectively, of the ubiquitous H enhancer located immediately upstream of pH. The deletion of a 1.8-kbp fragment encompassing the H enhancer/promoter region was shown to inactivate pN in nonmuscle tissues (10). Here, we show that the two HS sites observed around pN in the context of the complete promoter region are absent in EAccIΔ7-17 tissues in which pN activity is no longer detectable. We believe that the opening of the chromatin at pN in nonmuscle tissues requires elements residing in this 1.8-kbp fragment, most likely in the H enhancer shown to be important for pN activity in transient transcription assays (9). Thus, the strong ubiquitous H enhancer functions as a classical enhancer in activating both pN and pH promoters (9), and it seems also to be necessary to ensure the alterations of chromatin needed for access of the transcriptional machinery on these promoters in nonmuscle tissues.

In skeletal muscle tissue, the HSS4 and HSS5 sites on pN are capable of forming in the absence of the H enhancer, reflecting the activity of pN in this tissue. So far, we have tested pN function only in the company of pM and its regulatory elements. Thus, we cannot say if pN could be independently activated in muscle tissue. As previously suggested (10), the elements needed to activate pM could have a role in coactivating pM and pN in fast-type muscles.

Proximal pM sequences encompassing HSS3 are necessary for tissue-specific activity of pM. The fact that a unique muscle-specific HS site could be detected in the proximity of pM suggested that this region participates in its specificity. This was confirmed with the functional analysis of the different aldolase A–β-globin and pMCAT transgenes. In contrast to what has been observed for many muscle-specific genes (reviewed in reference 30), no important regulatory elements for muscle-specific pM expression could be found in the intron between the noncoding exon and the first coding exon. We were able to delineate the minimum region needed for tissue-specific control of pM to a 280-bp fragment containing 235 bp of upstream sequences and the noncoding 45-bp M exon. This was deduced from the following data. (i) In all the mice transgenic for the pMCAT construct, a 355-bp fragment from *Bal*I (position 1795) to the end of the M exon (position 2150) was able to target the expression of a CAT reporter gene to fast muscles. (ii) The deletion analysis performed with the aldolase A–β-globin constructs showed that the upstream sequences could be deleted until *Age*I (position 1870) without affecting pM expression. (iii) By contrast, a further 130-bp deletion, until *Bam*HI (position 2003), corresponding to the region in which HSS3 maps, resulted in the loss of all detectable pM activity.

This study confirms the unusual behavior of aldolase A transgenes. pM expression was found to escape at least from some of the position effects generally observed in transgenic-mouse studies because pM was always active independently of the integration site of the transgene in mouse chromatin. In all transgenic mice in which the 280-bp region was present (32 lines and six founder animals), pM was expressed in a tissue-specific way.

Tissue-specific regulatory elements conferring position-in-

dependent and copy-dependent expression to linked transgenes have been described for the human β-globin locus (22) and the human CD2 (20) and adenosine deaminase (1) genes. The β-globin locus control region is characterized by four erythroid-specific DNase I HS sites, and it functions as a particularly strong enhancer overriding the effects of the surrounding chromatin (reviewed in references 11, 16, and 42). A speculative but attractive hypothesis would be that our transgenes contain such elements conferring position-independent expression. However, despite the low variability in expression per transgene copy that we observed for the aldolase A–β-globin transgenes, transgenic pM did not function in a strictly copy-dependent manner when the transgenes carried only a small region of aldolase A sequences, as in pMCAT lines.

The muscle-specific HSS3 found at pM reflects the occupancy of the regulatory region by nuclear proteins. A deletion of this regulatory region between the *Age*I and *Bam*HI sites results in the inactivation of pM, showing that important transcription factors for pM activity bind the 130-bp *Age*I-*Bam*HI fragment. We know that this fragment binds nuclear proteins isolated from both skeletal muscle and liver tissues (49a). The mode of action of these transcription factors regarding the opening of the chromatin, the activation of the basal transcription, and the specificity of this activation is under investigation.

A putative MEF-2-binding site is not important for pM specificity. A motif that bears sequence similarity to a binding site for the MEF-2/RSRF family of transcription factors is present in the 130-bp *Age*I-*Bam*HI region. As this 130-bp fragment is necessary for pM activity, we suspected that the MEF-2 motif could be a determinant of pM activity level and specificity. Furthermore, it has recently been shown that this highly conserved motif has an important role in muscle-specific induction of the rat aldolase A gene when transfected in chicken muscle primary cells (25). In this study we have mutated the human aldolase A MEF-2-binding site in the context of the three promoters and analyzed the expression of the mutant transgene. We found that this mutation seems not to affect significantly pM activity (Table 1) and does not alter its specificity. This motif is thus dispensable for the fast-muscle-specific opening of the chromatin at pM.

The effect of a mutation in a MEF-2-binding site in transgenic mice has been studied also for the myogenin gene (6, 61). While the MEF-2 site is required for correct myogenin expression in embryos (and in cell cultures [13]), it is not necessary for the expression in differentiated muscle fibers, suggesting that activation and maintenance of myogenin transcription rely on different mechanisms. In light of these results it would be interesting to examine the expression of mutated pM during development to see whether the MEF-2-binding site could have a privileged role in pM control at some steps of myogenesis.

Additional interactions take place in the aldolase A 5′ region: pM activity level and/or specificity is modulated by upstream and downstream sequences. A remarkable feature of the aldolase A 5′ region is the coexistence of several promoter/enhancer elements with different specificities in a restricted 1.6-kbp DNA fragment. This proximity results in a variety of interactions, such as the sharing of regulatory elements between promoters (9, 10). Some mechanisms are also supposed to maintain the specificity of these interactions.

For the mouse aldolase A gene, a so-called sequestering mechanism by sequences mapping between positions −1100 and −350 with respect to the pM transcription start site was proposed (54). In a transient transfection analysis, the mouse pM was strongly stimulated by downstream sequences when this restrictive element was deleted, suggesting that it prevents constitutive activation of pM by the downstream ubiquitous H enhancer. In this study, we show that deleting upstream sequences up to *Bal*I (position 1795, corresponding to −305 bp relative to the pM start site) in transgene KBalIΔ1 does not alter human pM specificity. Although only two KBalIΔ1 lines were analyzed, this deletion seemed to increase pM activity per transgene copy in fast muscles from both lines, without significant modification in the pM/pH ratio (Table 1), indicating that we may have deleted some negative regulatory elements for the function of these two promoters. It is interesting that the deleted region includes pN, which is supposed to share regulatory elements with both pM and pH (10).

We previously showed that deleting the sequences spanning the pH/enhancer region in EAccIΔ7 transgenes does not result in a decrease of pM activity in a fast skeletal muscle. Here, a more quantitative analysis of pM activity in the vastus lateralis muscles of five of these lines together with the analysis of an EAccIΔ14 line in which the deletion is slightly extended in the 3′ part of the intron has been performed. In these lines, pM activity per transgene copy seemed to increase in proportion similarly to what was observed when upstream sequences were deleted. No additional effects could be observed when the upstream and downstream sequences were deleted together as in the KAgeIΔ2 transgene. A possible overall explanation of these effects could be that the supposed interactions taking place between the downstream H enhancer and upstream pN have a negative effect on pM activity. The deletion of one or both of them would disrupt this interaction and therefore result in enhanced pM activity.

Interestingly, here, we have observed that the deletion of the downstream pH/enhancer region also has a qualitative effect on pM activity: its deletion rendered pM more specific to fast muscles. In transgenes in which this region was present, M-type mRNAs were easily detected in a slow muscle such as the soleus. In contrast, when this region was deleted, very few M-type mRNAs were detected in this muscle even in mice harboring a high transgene copy number. Thus, some regulatory elements present in this region may activate pM in additional fiber types and, in this way, extend its specificity. However, these elements are likely to act only in a subset of fibers, since the overall level of pM does not decrease but, rather, increases when they are deleted. This effect could be due to the H enhancer, as no other regulatory elements in this deleted region have yet been characterized.

How is the fiber type specificity of pM activity achieved? Muscle maturation occurs during the first weeks of postnatal life in mice. This period is characterized by the appearance of adult fast-type myosin heavy chains IIA, IIX, and IIB (2, 59). During muscle maturation, transgenic human pM becomes highly active in fast-type muscles, as does the mouse endogenous pM (8). The muscle fiber specificity of the human pM is not known in detail. However, the fact that we found human pM expressed at high levels in mouse gastrocnemius and vastus lateralis muscles (composed of 90 to 94% of type IIB fibers and 6 to 10% of type IIX fibers) and at low levels in the soleus (composed of 58% of type I, 38% of type IIA, 6% of type IIX, and 2% of both type IIA and type IIX fibers) (12, 24) suggests preferential expression of this promoter in the glycolytic IIB fibers, with less activity in the other fast fibers, IIX and IIA. Results obtained with the mouse pM (8, 38a) suggest that the mouse pM pattern of expression resembles the one obtained in this study for the human pM with transgenes including the H enhancer/promoter region. Thus, in vivo, pM expression could, in fact, result from the combinatory effect of the proximal

regulatory elements specific to a subset of fast fibers (mainly IIB) with downstream sequences enabling its expression in a broader range of fibers. Such a counterbalancing effect of fiber type IIB-specific elements by other sequences spreading promoter specificity has been suggested to explain the complex (IIB > IIX > IIA) expression pattern of the quail TnI transgenes in different fast fibers (23). This kind of expression pattern has been also detected for the fast-fiber MLC1-CAT transgenes (12), suggesting the presence of some common regulatory mechanisms.

Very little is known about the transcription factors required for fast-fiber-specific gene expression. It has been recently shown that MyoD mRNA is preferentially expressed in adult-rat fast type IIB and IIX glycolytic fibers (27), indicating a potential role for MyoD in fiber-type-specific transcription activation. However, MyoD is unlikely to be the only key fast-fiber-specific factor, since mice lacking a functional MyoD gene develop normal proportions of adult muscle fiber types (49). It is clear that in the human aldolase A gene, the pM 130-bp regulatory region from position 235 to 105 upstream of the M exon is required for high-level muscle-specific promoter activity in transgenic mice, although it does not contain any E box. An E box is present in the remaining 105 bp upstream of the pM transcription start site, but it is not conserved in the rat and mouse genes.

No detailed information about the *cis*-acting elements needed for fiber-type-specific gene expression is yet available. In the present study, we show that the human aldolase A pM proximal sequences behave as an unexpectedly compact and powerful regulatory segment in fast muscles of transgenic mice, thus constituting a nice model for characterizing mechanisms that regulate fast-fiber-type-specific expression.

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