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ARTICLE

Mouse Muscle Identity: The Position-Dependent and Fast Fiber-Specific Expression of a Transgene in Limb Muscles Is Methylation-Independent and Cell-Autonomous

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We previously characterised transgenic mice in which fast-muscle-specific regulatory sequences from the human aldolase A pM promoter drive the chloramphenicol acetyltransferase gene expression. Mutation of a NF1/MEF2 binding site (M2 motif) in this promoter does not affect fibre-type specificity of the transgene but modifies its expression in a subset of fast-twitch fibres at the limb level, preferentially affecting distal limb muscles. We investigated the molecular and cellular bases of this peculiar expression pattern that provided an adequate model to characterise the mechanisms responsible for muscle positional information. By direct electrotransfer of mutated M2 construct in adult muscle, we demonstrate that positional differences in mutated M2 transgene expression are not observed when the transgene is not integrated into chromatin. Also, this transgene expression pattern does not seem to be correlated with the extent of CpG methylation in its promoter sequence. Finally, we show that positional values reflected by CAT levels are maintained in primary cultures established from different adult limb muscles, as well as in heterotopically transplanted muscles. Our results suggest that mutation of the M2 site contributes to reveal a molecular memory of fibre fate that would be set up on pM promoter during development and persist into adulthood possibly through a chromatin imprint maintained in satellite cells associated with various limb muscles. ***Developmental Dynamics 228:594–605, 2003.*** **© 2003 Wiley-Liss, Inc.**

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INTRODUCTION

The uniqueness of each individual muscle in terms of body coordinates, morphology, contractile properties, and innervation pattern reflects skeletal muscle diversity. Such an intrinsic heterogeneity in muscle innervation of mouse hindlimb muscles has been recently pointed out by the existence of distinct post-synaptic differentiation processes that influence the formation of neuromuscular junctions during development and their maintenance in the adult (Pun et al., 2002).

Muscle identity has been extensively investigated in *Drosophila,* where particular muscles or muscle groups can be distinguished by expression of particular genes (reviewed in Frasch, 1999). Whether similar mechanisms exist in vertebrates is still unclear. Nonetheless, recent studies have shown that muscle precursor cells, or myoblasts, are heterogeneous and that muscle identity may result from the interplay between intrinsic myoblast heterogeneity and extrinsic influences during development (reviewed in Ordahl et al., 2000). For instance, studies using transgenic models indicate that several muscle-specific

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genes rely on different regulatory sequences for their expression in different muscle territories (reviewed in Firulli and Olson, 1997; Kelly and Buckingham, 2000; Hadchouel et al., 2000; Summerbell et al., 2000). The spatiotemporal specificity of activation of myogenic regulatory genes, such as *Myf5,* or of myosin isoform genes, such as *MLC1F/3F,* would thus be achieved in each subset of muscle precursor cells through the combinatorial use of different regulatory modules and their corresponding transcription factors. The *MLC1F/3F* gene, encoding alkali myosin light chain MLC1F and MLC3F isoforms, has been thoroughly studied in the transgenic mouse model to characterise regulatory modules responsible for positional and fibre-type specific expression (reviewed in Kelly and Buckingham, 2000). In particular, the transgenic MLC1F-CAT 3′ enhancer was shown to display varying levels of activity in skeletal muscles, evidenced by a rostrocaudal gradient of chloramphenicol acetyltransferase (CAT) reporter gene expression in muscles of similar fibre type (Donoghue et al., 1991). This transgenic model provided the first demonstration that muscle cells can bear an intrinsic positional memory that was conserved in isolated muscle cell culture (Donoghue et al., 1992) and could be correlated with the extent of CpG methylation in the MLC1 promoter and enhancer sequences (Grieshammer et al., 1995).

In the same way, we recently described some striking in vivo differences in the requirements for activation of the pM human aldolase A fast-muscle-specific promoter in individual fast limb muscles (Spitz et al., 2002). Within the minimal pM proximal promoter fragment that is sufficient to direct fast glycolytic fiber-type specific expression in a transgenic mouse model (pM_{310}-CAT transgene, here shortened as pM-CAT), three sequences, M1, M2 and MEF3, were shown to be required for such tissue-specific expression (Salminen et al., 1994, 1996). The M2 region consists of overlapping MEF2 and NFI binding sites, and was shown to bind preferentially NFI proteins, which were further found to be differentially expressed in different muscles (Spitz et al., 1997). Surprisingly, mutations in the M2 region led to great differences in the corresponding mM2-CAT transgene expression levels among individual fast muscles (Salminen et al., 1996). This peculiar expression pattern was observed in several independent transgenic lines, thus excluding influence of integration site. Analysis of mM2-CAT transgene expression in several muscles throughout the body, combined with fibre-type identification, revealed that the expression of the transgene is differentially affected according to the proximal vs. distal position of the muscle along the limb axis (Spitz et al., 2002). Whereas transgene expression is mildly affected by M2 mutation in body muscles and in proximal limb muscles, it is drastically decreased in distally located limb muscles. Analysis of expression at the cellular level indicated that this graded expression results from a graded proportion of fast fibres expressing the transgene, rather than from an overall decrease of transgene expression in the whole fast fibre population. Altogether, these observations suggested that fast glycolytic IIB/IIX fibres from different muscles could be endowed with different transcriptional competencies. The mM2 transgene thus provided a new model to investigate cellular and molecular mechanisms involved in the specification of individual muscle characteristics.

To dissect out the molecular mechanisms responsible for mM2 expression pattern along the limb axis, we transiently transfected the mM2-CAT construct in vivo in adult proximal and distal limb muscles by electrotransfer. Our results revealed no difference in transcriptional activation of episomal mM2 promoter between proximal and distal limb muscles, suggesting that epigenetic modifications could be involved in the position-specific expression of the mM2-CAT transgene. Because DNA methylation was correlated with the rostrocaudal gradient of MLC1F/3F-CAT transgene expression, we compared CpG methylation of the mM2 promoter in adult limb muscles showing differential expression. Our results indicated that regional differences in mM2-CAT transgene expression were not correlated with the methylation pattern of the mM2 promoter, thus excluding DNA methylation as an epigenetic regulatory mechanism accounting for differential mM2 expression. We finally asked whether muscle positional memory revealed by mM2 transgene expression resides within muscle cells themselves, or rather lies within humoral and nerve environment. We show here that myotubes obtained ex vivo from individual mM2-CAT transgenic muscles, expressed CAT levels characteristic of their position of origin in the limb, indicating that intermuscular differences are independent of extrinsic influences. These data are reinforced by in vivo muscle transplantation experiments showing that heterotopically transplanted muscle retained the mM2 expression pattern of the muscle it originated from after regeneration.

RESULTS

Transient Transfection of mM2-CAT Yields Comparable Transcriptional Activation Among Hind Limb Muscles

Previous studies have shown that the pM-CAT transgene is highly and evenly expressed in all axial and limb fast-twitch muscles, while mM2-CAT transgene expression is maintained at a high level in proximal limb muscles but is drastically reduced in distal limb muscles (Spitz et al., 2002).

To investigate whether such mM2-CAT differential expression within fast hindlimb muscles could be reproduced when the transgene is now present in an episomal state, we used the electrotransfer method that allows in vivo transient transfection of adult muscles. This approach already proved to be efficient for analysing muscle-specific gene expression of the pM promoter (Bertrand et al., 2003; Grifone et al., submitted). As a control of specific involvement of the mM2 sequence, parallel sets of mice were used to transfect the wild-type pM-CAT construct, the expression of which is not expected to vary from one muscle

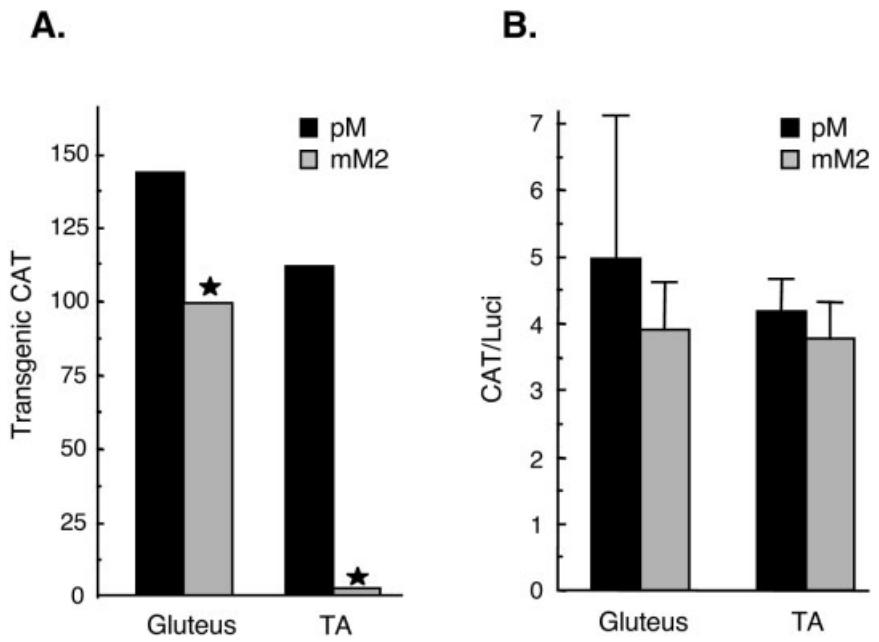

Fig. 1. Comparison between pM- and mM2-driven CAT activity in adult transgenic mice and in transfected adult gluteus and tibialis anterior (TA) hindlimb muscles. **A:** Transgenic CAT activity in adult muscles from pM-CAT$_{98}$ and mM2-CAT$_{57}$ transgenic mice (data from Spitz et al., 2002). **B:** Transfection of pM-CAT or mM2-CAT construct, together with pRSV-Luci as a control for transfection efficiency, was performed by electrotransfer of gluteus and TA as described in Experimental Procedures. CAT activity was normalised to luciferase activity. Values (with SD) are from one experiment and results are representative of the three transfection experiments performed. In the illustrated experiment, gluteus transfected with pM-CAT, 3 animals; gluteus transfected with mM2-CAT, 6 animals; TA transfected with pM-CAT, 4 animals; TA transfected with mM2-CAT, 3 animals. Black bars correspond to pM-CAT and gray bars to mM2-CAT construct transfection results.

to another. Transfected constructs correspond to those used to generate transgenic lines (Salminen et al., 1994, 1995), and transfection efficiency was normalised by co-transfecting the pRSV-luci reporter. The tibialis anterior (TA) was chosen as a distal fast-twitch limb muscle, and the gluteus as a proximal one. As illustrated in Figure 1A (data from Spitz et al., 2002), the pM-CAT and mM2-CAT transgenes are expressed at comparable levels in the gluteus in corresponding transgenic mice. By contrast, in the TA mM2-CAT activity is 100-fold lower than that of pM-CAT.

As shown in Figure 1B, unexpectedly, transfected mM2-CAT did not reproduce the pattern of CAT activity observed in transgenic mouse muscles (compare A with B). CAT levels obtained from mM2-CAT electro-transferred muscles were not statistically different in the gluteus and in the TA. Similar results were obtained when using pCMV-LacZ (Bertrand et al., 2003) instead of pRSV-luci as control plasmid, or by transfecting gastrocnemius instead of TA (data not shown), gastrocnemius being a distal limb muscle in which mM2-CAT transgene expression was also found drastically reduced in the transgenic mouse model. Thus the episomal mM2-CAT transfected gene was not differentially expressed in proximal compared with distal hind limb muscles.

Methylation Profile of the mM2 Promoter Is not Correlated With CAT Activity in Different Adult Limb Muscles

The above results suggested that chromosomal integration of transgenic mM2 regulatory elements was required for the positional variation in mM2-CAT expression in adult limb muscles. Because acquired epigenetic modifications of the mM2-CAT transgene could be involved in this muscle position-dependent expression, we investigated whether differential CpG methylation of the mM2 transgenic promoter in adult limb muscles could be the underlying mechanism. The methylation status of mM2 and pM transgenic promoters in proximal and distal hind limb muscles was explored by using sequencing of cloned PCR-amplified fragments derived from bisulfite-treated genomic DNA. The methylation status of single molecules rather than the average methylation level of a whole population can be thus analysed, that is of particular importance when studying multiple-copy transgenes. The pM promoter region comprises 15 evenly distributed CpG dinucleotides, whereas the mM2 promoter contains 17 sites, two additional sites being brought in by the M2 mutation (Fig. 2A). We analysed the gluteus (proximal) and gastrocnemius (distal) muscles because they display a marked difference (420-fold) when CAT levels are assayed from individual dissected muscles of mM2-CAT transgenic mice (Fig. 4A).

Methylation patterns were found heterogeneous among the 10 to 15 sequenced molecules, each representing a single promoter molecule in a single muscle (Fig. 2B–E). Nevertheless, no significant difference appeared in the overall frequency of cytosine methylation between molecules representing a given muscle, nor between muscles from a given transgene, nor between identical muscles from the two transgenes. Whatever the transgene or the muscle, 40 to 57% of molecules were totally unmethylated, 20 to 33% of molecules contained one methylated CpG residue at variable positions and occasionally one molecule was found methylated at more than five residues. pM-CAT$_{98}$ and mM2-CAT$_{57}$ transgenic lines carry 22 and 25 transgene copies, respectively, and such transgene repetition is frequently found to cause significant transgene silencing and concomitant methylation (Garrick et al., 1998). However, we previously noticed (Salminen et al., 1994) that in the eight different pM-CAT lines that we got, pM is always active, with lines harbouring the highest transgene copy number (as pM-CAT$_{98}$) being also those that express the

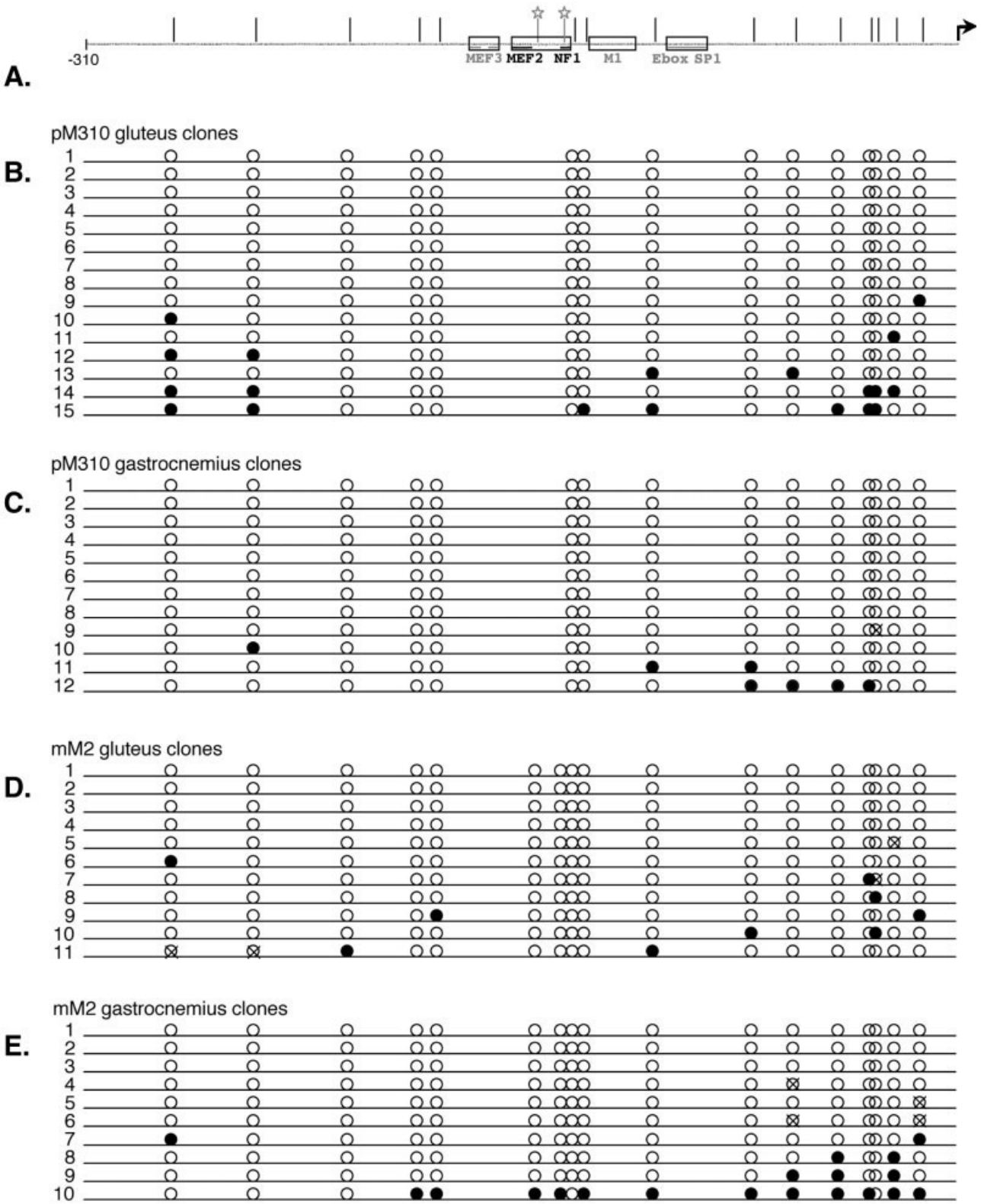

Fig. 2. Methylation analysis of pM and mM2 transgenic promoters. **A:** Schematic drawing of the 310-bp promoter region analysed, with CpG dinucleotides marked as vertical bars, putative protein binding sites (underlined) and nuclear protein binding sites (boxes) (data from Salminen et al., 1995) and the transcription start site (arrow). Asterisks label the two additional CpG dinucleotides brought in by M2 mutation. **B–E:** Analysis of pM and mM2 methylation status was determined by sequencing of individual transgene copies subcloned from bisulfite-treated, PCR-amplified DNA. Gluteus muscle of pM-CAT_{98} transgenic mouse (B), gastrocnemius muscle of pM-CAT_{98} transgenic mouse (C), gluteus muscle of mM2-CAT_{57} transgenic mouse (D), and gastrocnemius muscle of mM2-CAT_{57} transgenic mouse (E) were analysed. Each row represents a single cloned transgene. ○, nonmethylated CpG; ●, methylated CpG; X, not determined.

transgene at the highest level. This was also true for mM2 CAT lines (Salminen et al., 1996) in proximal limb muscles, suggesting that for both transgenes, most integrated copies are active. These observations fit well with the present result showing that for pMCAT in both muscles, as for mM2 CAT in gluteus, we found mostly hypomethylated promoter molecules corresponding to active copies. If CpG methylation was to play a role in differential mM2 transgene expression, we would have expected to find mostly hypermethylated promoter molecules in gastrocnemius of mM2 CAT mice. Surprisingly, in this sample we similarly revealed mostly hypomethylated promoter molecules. Most importantly, no specific methylation pattern was observed in the vicinity of MEF3, MEF2, and NF1 regulatory elements. In particular, the presence of two additional CpG sites brought in by the M2 mutation was not correlated with a modification of either local or global methylation pattern. Altogether, these results excluded DNA methylation as a major factor linked to differential mM2 transgene expression in limb muscles.

Positional Expression of mM2 Transgene Is Maintained in Culture

To test the interaction of environmental cues and cell-autonomous properties as potential determinants of mM2 transgene expression, we compared CAT levels in primary cultures obtained from fast-twitch limb muscles differing in their proximal (gluteus) vs. distal (gastrocnemius) position. To be able to compare mM2 activity among the different muscle primary cultures, and to monitor their degree of differentiation, we used as an internal control a transgene in which placental alkaline phosphatase gene is controlled by the wild-type pM promoter (pM-AP). pM-AP transgene expression in mice mimicked that of pM-CAT in all respects, including recapitulation of endogenous pM postnatal activation and fibre-type specificity (J.P. Concordet and G. Tavernier, unpublished results). Primary cultures were thus obtained from gastrocnemius

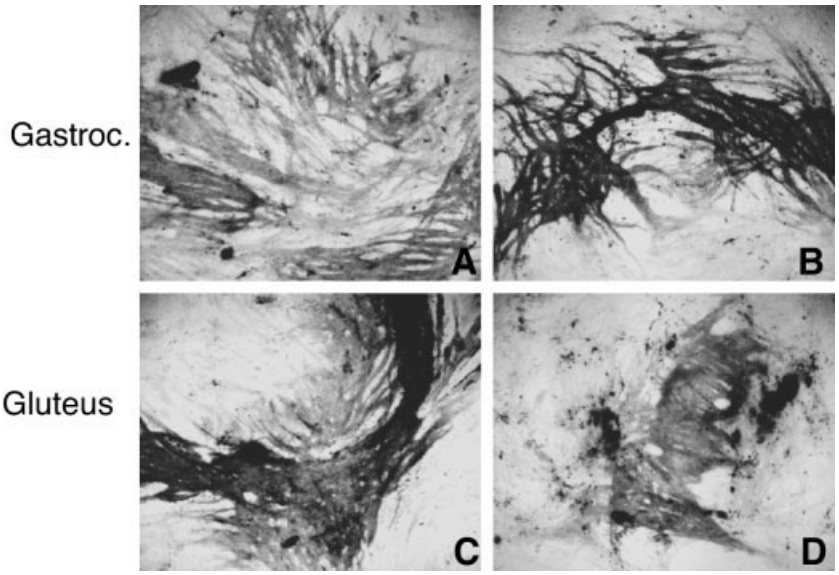

Fig. 3. Myogenic primary cultures derived from proximal and distal hindlimb muscles. Muscles were dissected from double-transgenic adult mice (either pM-AP/pM-CAT_{98} or pM-AP/mM2-CAT_{57}) and primary cultures were performed as described in the Experimental Procedures section. Micrographs of four representative culture dishes 13 days after plating are shown: **(A)** gastrocnemius muscle of pM-AP/pM-CAT_{98} transgenic mice, **(B)** gastrocnemius muscle of pM-AP/mM2-CAT_{57} transgenic mice, **(C)** gluteus muscle of pM-AP/pM-CAT_{98} transgenic mice, **(D)** gluteus muscle of pM-AP/ mM2-CAT_{57} transgenic mice. Dishes were stained for AP activity as described in Experimental Procedures (purple staining).

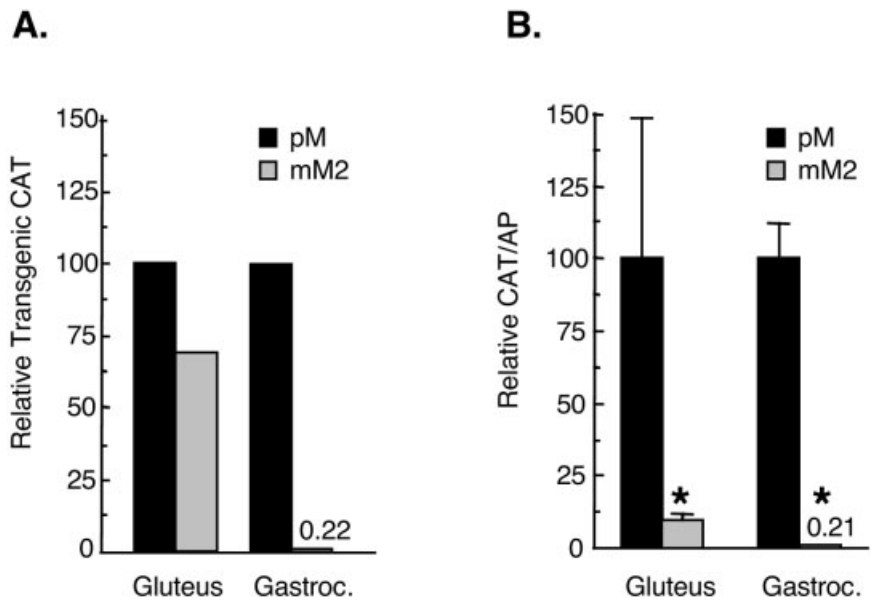

Fig. 4. Comparison of transgenic CAT activity in adult transgenic mice and CAT activity in transgenic primary muscle cultures. **A:** Transgenic CAT activity in adult transgenic mice (data from Spitz et al., 2002). **B:** pM-CAT and mM2-CAT activity in transgenic primary muscle cultures. For each dish, total CAT activity was normalised to total AP activity, which monitored the extent of pM promoter activation level. Background CAT and AP activities measured from nontransgenic primary muscle cultures were subtracted from CAT and AP values obtained from transgenic primary muscle cultures. Results were normalized to the mean value found in the pM-CAT proximal muscle, i.e., the pM-CAT gluteus. Relative CAT/AP ratios (with SEM) are from one experiment representative of three independent experiments. Black bars correspond to pM-CAT_{98} transgene activity and gray bars to mM2-CAT_{57} transgene activity. Asterisks represent two statistically different values according to ANOVA test ($P < 0.01$).

and gluteus of double transgenic animals (pM-AP/pM-CAT_{98} or pM-AP/mM2-CAT_{57}). When myogenic differentiation was achieved after several days in differentiation medium, AP staining of some culture dishes was used to control the differentiation state needed for pM activity (Fig. 3) before collecting cells. CAT activity reflected the level of expression of either the pM-CAT or the mM2-CAT transgene in newly formed myotubes. This activity was normalised from one dish to another by calculating CAT/AP activity in each dish, assuming that pM-AP expressing myotubes were the same that those expressing either pM-CAT or mM2-CAT.

Results from one representative experiment are shown in Figure 4B (for comparison, transgenic mice data from Spitz et al., 2002, are given in Fig. 4A). Most interestingly, in cultures issued from gastrocnemius, a drastic 460 $\pm$ 56 -fold difference between relative pM-CAT and mM2-CAT transgenes expression could be observed. This difference is similar to what was observed in adult gastrocnemius that expressed the pM-CAT transgene 440-fold more than the mM2-CAT transgene (Fig. 4A). However, while pM-CAT expression level in transgenic adult gluteus muscle was 1.5-fold higher than that of mM2-CAT, cultured myotubes issued from gluteus displayed a more accentuated difference since they expressed the pM-CAT transgene 10.7 $\pm$ 5.2 -fold higher than the mM2-CAT transgene (Fig. 4B). Consequently, although mM2 transgene was found far more expressed in cultures obtained from gluteus than those obtained from gastrocnemius, (43-fold), overall this difference was smaller than that found in vivo (300-fold). However, these results demonstrated that myotubes derived from satellite cells obtained from adult transgenic muscles expressed the pM and mM2 transgenes at a level reflecting the positional origin of the muscle from which they are derived. This indicates that satellite cells that are associated to various limb muscles bear cell-autonomous positional information.

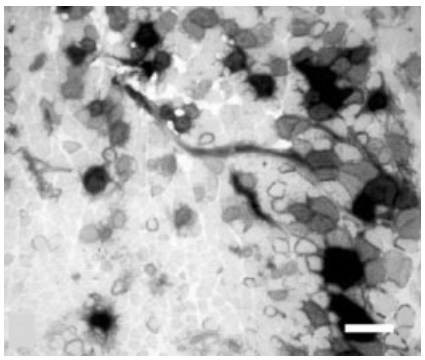

Fig. 5. Muscle fibres regenerated from transplanted muscle. Muscles were dissected from double transgenic adult mice (either pM-AP/mM2-CAT_{57} or pM-AP/pM-CAT_{98}) and transplanted in wild-type mice as described in the Materials and Methods section. Micrograph of a frozen transverse section of a pM-AP/pM-CAT_{98} gastrocnemius external wing transplanted in homotopic location, collected 3 weeks after transplantation, stained for AP activity and with eosin. Purple staining denotes pM-expressing muscle fibres regenerating from transplanted muscle. Scale bar = 100 μm.

Positional Identity Is Maintained in Heterotopically Transplanted Muscle

To further ascertain the cell-autonomous properties of satellite cells to express the mM2 transgene according to their initial position, we performed muscle transplantation experiments in which low CAT-expressing gastrocnemius from mM2-CAT_{57} transgenic mice was relocated in the gluteus muscle bed. In the conditions that we used, in which tendon attachments were not preserved and muscle fibres are sharply cut, regenerating muscle originated exclusively from satellite cells of the graft and of the remaining host muscle (Mauro, 1961; Snow, 1978). Host mice were wild-type B6/CBA adult mice, whereas donor mice carried either the mM2-CAT_{57} or pM-CAT_{98}, and pM-AP transgenes as described in primary culture experiments. As illustrated in Figure 5A, AP staining of frozen sections of regenerating muscles was performed to assess the location and the amount of myofibres formed from satellite cells originating from grafted muscles. After 3 weeks, as judged by AP staining, a noticeable amount of pM-expressing fibres could be observed in the regenerating muscle. CAT/AP ratio measured on grafted muscle cell extracts thus reflected a normalised level of expression of the pM-CAT or mM2-CAT transgenes in myofibres regenerated from grafted muscle.

As shown in the right panel of Figure 6, CAT/AP ratio calculated from two independent experiments was low whether mM2-CAT gastrocnemius was relocated in the homotopic gastrocnemius position or in the heterotopic gluteus position. By contrast, CAT/AP ratio was high whether mM2-CAT gluteus was relocated in the homotopic gluteus or in the heterotopic gastrocnemius position. The average ratio between normalised CAT activity issued from regenerating gluteus cells and that issued from regenerating gastrocnemius cells was 50. This result indicates that the ability of gluteus to express the mM2 transgene at a much higher level than that observed in gastrocnemius was maintained in fibres formed from gluteus grafted satellite cells.

As a control, transplantation of pM-AP/pM-CAT gastrocnemius and gluteus muscles was performed in either position and gave much more comparable results regarding maintenance of CAT expression level whatever the relocation (left panel of Fig. 6). By contrast with transplantation of mM2-CAT muscles, the average ratio between normalised CAT activity in regenerating gluteus and gastrocnemius was 2.3, echoing the comparable levels of CAT activity observed in adult transgenic muscles.

These results indicate that satellite cells contain a cell autonomous information enabling them to express the mM2 transgene according to their initial position in the limb, and that this information is not influenced by neural or humoral cues in adult muscle.

DISCUSSION

We previously showed that a transgene in which the aldolase A pM promoter was mutated on its M2 site is differentially expressed in various limb muscles (Spitz et al., 2002). Because this transgene acts as a marker of unsuspected positional in-

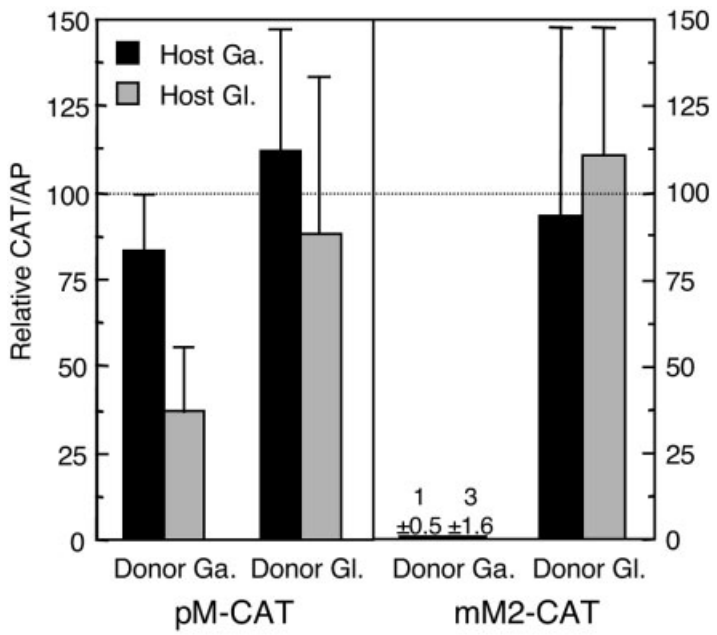

Fig. 6. Muscle transplantation experiments testing mM2-CAT transgene expression levels in homotopically and heterotopically transplanted gastrocnemius and gluteus muscles. Results shown are from two independent experiments. Data are expressed as CAT/AP activity (± SEM or SD) normalised to the mean value found for the proximal donor muscle, i.e., the gluteus (dotted line). Left panel shows transplantation results of pM-AP/pM-CAT_{98} gastrocnemius (Donor Ga.) or pM-AP/pM-CAT_{98} gluteus (Donor Gl.) in the gastrocnemius muscle bed (black bar) or the gluteus muscle bed (gray bar), whereas right panel shows transplantation results of pM-AP/mM2-CAT_{57} gastrocnemius or gluteus muscles. Each result is the mean of 2 to 3 values.

formation within mouse limb muscles, we used it as a tool to investigate the underlying mechanisms. We show here that position-related intermuscular differences in mM2-CAT activity do not exist in the context of in vivo transient transfection of mM2-CAT construct in different adult hindlimb muscles, but are maintained in cultures established from different adult mM2-CAT_{57} transgenic hindlimb muscles, and in heterotopically transplanted satellite cells. We conclude that the corresponding satellite cells bear intrinsic positional information that is cell-autonomous. Our results suggest that an epigenetic mechanism different from DNA methylation could account for such positional memory in adult muscles.

Transgenic Models to Study Muscle Positional Information

The mM2-CAT transgene is, with the MLC1/3F-CAT transgene, one of the few models revealing and thus allowing the study of positional information in muscles. Molecular specification of individual muscles in mice has been investigated through the integration site-dependent expression of the acetylcholine receptor ϵ-subunit promoter driving the *lacz* gene (AChRϵ-lacz), for which expression in particular muscle differ among lines (Sanes et al., 1991). This case exemplifies the influence of nearby genomic regulatory elements that selectively enhance transcription in some muscles. By contrast, the expression pattern of the MLC1/3F-CAT and mM2-CAT transgenes is not dependent on the integration site, providing strong evidence that the relevant regulatory elements leading to positional information are contained within the transgenes themselves.

The MLC1 and mM2 transgenes bear several common features: differential expression is independent of transgene integration site and is established during development. In vitro, the position-dependent expression is maintained in muscle primary cultures obtained from different axial levels of MLC1 transgenic mice and from proximal vs. distal limb levels of mM2 transgenic mice, showing the cell-autonomous nature of positional information retained by the transgene. We show here that not only muscle primary cultures, but also heterotopically transplanted muscle retain mM2 differential expression pattern. Although position-dependent pattern of expression is not found in the endogenous gene (for the *MLC1/3F* gene) or in the nonmutated transgene (for the pM transgene), it indicates that the regulatory sequences of muscle genes contain a variety of control elements conveying tissue-, fibre type-, temporal and spatial control to their expression. Accordingly, the position-dependent expression of the mM2 transgene could result from multiple, independent sites in the promoter, including one that is required for expression in distal limb muscles. Recent studies using transgenic models on cis-regulatory sequences of muscle-specific genes such as *Myf5, MRF4,* and *MyoD* demonstrate that the correct spatio-temporal activation of these genes during myogenesis is driven by alternative use of *cis*-acting regulatory elements (Hadchouel et al., 2000; Summerbell et al., 2000; Chen et al., 2001). In this view, the overall pM pattern of expression may result from the integrated effects of several particular sites, each of which is maximally active in subsets of muscles.

Different Muscle Transcriptional Competencies?

We previously noticed that intermuscular differences in mM2-CAT transgene expression in vivo were much larger than intermuscular differences in fibre type composition, because most of limb muscles are of fast-twitch type and contain high proportions of type IIB fibres. We showed that differential expression of mM2 results from a variation in the number of IIB fibres in which the mutated transgene is expressed (Spitz et al., 2002). We suggested that, according to the muscle, different subpopulations of fast glycolytic IIB fibres exist and that these fibres could be endowed with different transcrip-

tional competencies acquired before post-natal specialization. Such different requirements in transcription factors according to the muscle were revealed by the muscle-specific alteration of MLC1CAT920 transgene activity in variable mice backgrounds for different Hoxc-8 dosages. In this system, maximal levels of CAT activity for specific limb muscles, particularly the triceps and EDL, were not reached in the absence of functional HOXC8 protein (Houghton and Rosenthal, 1999).

Here we tested whether two fast adult hindlimb muscles from the proximal and the distal levels of the limb possess such distinct transcriptional competencies to express mM2-CAT in transient transfection experiments. This assay failed to reveal an overall quantitative difference of the episomal mM2-CAT transcription between the two muscles, and thus to reproduce mM2-CAT transgene expression pattern. This result represents another feature shared by MLC1 and mM2 transgenic promoters since transfection of different primary cell cultures obtained from various axially positioned muscles with MLC1-CAT, also failed to reproduce transgene expression pattern (Grieshammer et al., 1995).

Such finding could be interpreted as the absence of any differences in the transcriptional competency between proximal and distal limb muscles. However, the existence of a qualitative difference in transcription factors that would not be perceived through these experiments cannot be totally ruled out. In particular, if the M2 site-dependent mechanism working in distal limb muscles operates at the chromatin level, this potential qualitative difference would be apprehended only in the chromatin context of an integrated transgene, but not in the case of an episomal cassette. Of interest, analysis of the DNA-protein interactions occurring in vivo at the pM promoter revealed that the formation of a muscle-specific DNAse I hypersensitive site reflected binding of proteins close to the M2 site (Salminen et al., 1995). Moreover, despite the optimised electrotransfer conditions that were chosen (Bertrand et al., 2003), de-repression of a transcriptional factor normally lacking in distal muscles remains also possible.

However, in other respects, the fact that the muscle-specific expression of mM2-CAT requires chromosomal integration of transgenic mM2 regulatory elements and is cell-autonomous, as revealed by primary cultures and muscle transplantation experiments, strongly argues in favour of a mechanism implicating an epigenetic memory. In this view, the existence of an M2 site-dependent and of an M2 site-independent mechanism for pM activation, do not necessarily suggest that qualitative differences in transcription factors still exist between proximal and distal adult muscles. Owing to muscle-specific ontogenic pathways, different transcriptional strategies may have been used during development, resulting in the settling of a silencing epigenetic imprint on the mM2 transgene only in distal limb muscles. If so, one possible role of M2 site-dependent mechanism in distal muscle could be to overcome such positional effect driven by another pM regulatory element.

Epigenetic Mechanisms Involved in mM2 Differential Expression

MLC1 and mM2 transgene behaviours also differ in important ways: first it was thoroughly demonstrated that rostro-caudal gradient of MLC1-CAT expression in axial muscles was faithfully maintained due to differential CpG methylation of an E box adjoining Hox binding sites (Grieshammer et al., 1995; Ceccarelli et al., 1999). In the case of mM2-CAT transgene, CpG methylation does not appear to be involved in differential expression in different fast-twitch limb muscles. Despite the observed difference in CAT expression level between the gastrocnemius and gluteus muscles from a mM2-CAT_{57} mouse, we were unable to detect any difference in the extent of mM2 promoter methylation. While we have not analysed the methylation of every cytosine within the transgene (namely CAT coding sequence) and, therefore, cannot definitively rule out differential methylation at undetected specific sites, these results argue strongly against methylation as the cause of position-dependent transgene activity in this system.

While MLC-CAT gradient of expression is only observed in axial muscles in relation with somitic level and corresponding segmental innervation, no such correlation is encountered in limb muscles, so that positional mM2 transgene imprint leading to limb muscle specific expression may rely on different mechanisms.

Although there are many examples in which the inactivation of a gene has been found to correlate with hypermethylation of its regulatory elements, there are some cases in which a silenced transgene locus is found hypomethylated (Garrick et al., 1996). Of interest, in the native aldolase A gene, pM promoter is located downstream one of the two other ubiquitously active promoters and is likely subject to transcriptional read-through, even in nonmuscle tissues where pM is not active (Moch et al., 1996). Indeed, by in vivo footprinting analysis of this promoter region, we previously observed that pM promoter was accessible to trans-acting factors in liver tissue (Salminen et al., 1996), suggesting that it is not methylated in this tissue. Due to this particular situation, mechanisms different from methylation may have been selected for ensuring a tight control of pM, and could explain why methylation seems not to be involved in mM2 transgene silencing in distal limb muscles. Thus in the case of mM2 transgenic promoter, we may speculate that other epigenetic mechanisms known for modulating gene expression such as histone acetylation or possibly histone methylation, are at work (reviewed in Li, 2002). Histone acetylation/deacetylation has recently emerged as a central mechanism in the control of muscle development (McKinsey et al., 2001). Of note, it is interesting to point out that the M2 site consists of overlapping binding site for MEF2 and NF1 proteins, with NF1 factors constituting the main binding activity (Salminen et al., 1995). While NF1

proteins are able to interact with Ski oncoprotein, that has myogenic properties and is itself a component of an histone deacetylase complex (Tarapore et al., 1997; Nomura et al., 1999), MEF2 factors were shown to control the activation and repression of muscle-specific genes by associating with histone acetylases and histone deacetylases, respectively (McKinsey et al., 2001).

Position-Dependent mM2 Expression and Limb Muscle Ontogeny

Our results strongly suggest that a methylation-independent epigenetic mechanism allows to maintain mM2 expression pattern in vitro, in primary cultures obtained from adult satellite cells, as well as in vivo, in muscle transplants produced by adult muscle grafting. Grafting experiments result in muscle transplants satellite cells activation, and a regeneration process that partly recapitulates the myogenic genetic program operating in the embryo. However, our results show that such a process fails to erase this epigenetic imprint when operating in the adult context. Our previous work showed that differential expression of mM2 was early established, before the onset of muscle metabolic and functional maturation (Spitz et al., 2002). Altogether, these data strongly suggest that position-specific expression of mM2 is established by mechanisms taking place during limb muscle ontogeny. Such mechanisms could be related to the pre-existence of different muscle precursor cells generating different populations of fast IIB fibres, the proportion of which varies according to the proximal vs. distal position of the muscle in the limb, and/or to the various environmental signals these precursor cells receive along their migration route.

The concept of muscle identity has been extensively developed by using mutants in *Drosophila*, allowing to draw a model where different combinations of transcription factors are required at the myoblast stage to drive the formation of each and every muscle (reviewed in Frasch, 1999). Although this aspect of muscle biology is far much less characterised in vertebrates, a growing number of data indicates that a similar system may be at work. *Mox2*, *Lbx1*, *Gab1*, and most recently *Six1* knockout mice reveal the existence of an unexpected diversity in the molecular pathways controlling myogenesis in different limb muscle regions (Mankoo et al., 1999; Brohmann et al., 2000; Gross et al., 2000; Sachs et al., 2000; Laclef et al., 2003). In addition, clonal analysis of muscle precursors in mouse, using the laacz system, has not only demonstrated the regionalisation of the medial and lateral myotomes in the mouse embryo, but that individual anatomic muscles seem to arise from a limited and defined group of myoblasts (Eloy-Trinquet and Nicolas, 2002, personal communication).

These genetic studies are contrasting with the commonly admitted view that myogenic cells present a relatively high plasticity level that enables them to adapt the nature of the mesenchyme they colonise: classic experiments of avian somite transplantation from the wing to the leg level show that the muscles that form from grafted somites adopt the identity of the host limb bud (Jacob and Christ, 1980). These data are supported by recent work using replication-defective retroviral vectors to analyse whether myogenic precursors in the somite are committed to form particular anatomical muscles and fibre types in the chick limb (Kardon et al., 2002).

In this context, our results are in accordance with a model in which during muscle ontogenesis, myoblasts acquire a predetermined fate. We speculate that the molecular differences that would characterise these heterogeneous populations of myogenic cells, and later differentiated limb muscle cells, result from still unidentified epigenetic regulations, probably combined to intrinsic qualitative differences in trans-acting factors. The laying-down of limb muscle positional information during development is probably not exclusively predetermined in muscle progenitors, and most likely results from a fine balance between predetermination and the influence of extrinsic factors encountered in the developing limb mesenchyme. This poorly understood aspect of muscle biology could underlie the striking differential susceptibility of specific muscles observed in most myopathies. Experiments are underway to investigate this key question during mouse limb muscle development.

EXPERIMENTAL PROCEDURES

Transgenic Mice

The generation and characterisation of $pM_{310}CAT_{98}$ (shortened here as pM-CAT_{98}) and mM2-CAT_{57} transgenic lines were described elsewhere (Salminen et al., 1994, 1996; Spitz et al., 1998, 2002). The pM-CAT_{98} and mM2-CAT_{57} transgenic lines contain approximately 22 and 25 copies of the CAT reporter gene, respectively. Transgenic pM-AP mice expressing the human placental alkaline phosphatase gene driven by the muscle-specific proximal promoter of the human aldolase A gene were obtained in the laboratory (J-P Concordet and G Tavernier, unpublished results). Double-transgenic mice carrying either pM-CAT_{98} and pM-AP, or mM2-CAT_{57} and pM-AP were generated by interbreeding and used for primary culture and muscle transplantation experiments. Transgenic mice were identified by Southern blot as previously described (Salminen et al., 1996). All experiments using animals were conducted in accordance with the European guideline for the care and use of laboratory animals.

Transient Transfection in Adult Mouse Limb Muscles

Experiments were performed on 6- to 12-week-old female C57Bl/6 mice (Charles River). For each experiment, 12 mice were used. The pRSV-Luci plasmid used for normalisation of transfection efficiency was obtained by subcloning the 524-bp fragment of RSV containing the LTR (Gorman et al., 1982) upstream of a firefly luciferase reporter vector (Promega). Some experiments (not shown) were also performed with a different pCMV-LacZ control plasmid (described in Bertrand et al., 2003). Transient transfection of pM-CAT or mM2-CAT constructs was per-

formed by injecting the plasmids and electroporating muscles as described in Bertrand et al. (2003) with following minor modifications. Mice were anesthetised with a mixture of ketamine and xylazine (80 mg/kg and 4 mg/kg, respectively). Four to 13 μg of pM-CAT or mM2-CAT and 0.5 to 2 μg RSV-Luci plasmid in 50 or 75 μl sodium chloride isotonic solution were injected in the tibialis anterior (TA) or the gluteus muscle, respectively, by using a 29-G needle. Different amounts of control (pRSV-Luci or pCMV-LacZ) and pM-CAT or mM2-CAT plasmids were tried to test whether promoters used for control plasmids would titrate basal transcription factors and thus alter CAT expression levels. We indeed observed much more consistent results when using small amounts of transfection control and pM-CAT or mM2-CAT plasmids. Six square-wave 130 V/cm pulses lasting 60 msec each with a 100-msec interval were applied by using a BTX electro cell manipulator (ECM). For TA electroporation, leg hair was shaven, DNA was injected and electrical field was applied by using calliper rule electrodes placed on each side of the leg. For gluteus electroporation, hair was shaven and skin was opened to expose the gluteus muscle, DNA was injected and electrical field was applied directly onto the muscle using forceps-shaped electrodes. Skin was sutured close.

To avoid individual variations and to allow accurate comparison of expression level, the TA and gluteus muscles of the same mouse were electroporated. To avoid some transient transcriptional perturbations that could be observed in electroporated muscles, mice were killed 15 days after injection-electroporation by cervical dislocation. When respecting this delay after delivery of electrical pulses, no alteration of pM-CAT transcription could be observed (Bertrand et al., 2003). Electroporated muscles were processed for cell extract preparation, and CAT activity was assayed according to Sambrook et al. (1989). Luciferase activity was assayed using the Luciferase Reporter Gene Assay (Roche).

CpG Methylation Analysis

Genomic DNA was prepared as described in Sambrook et al. (1989) and further purified by using the DNA Cleanup System (Promega). Bisulfite treatment was performed as described by Frommer et al. (1992), as modified by Clark et al. (1994) and Feil et al. (1994). For amplification of the bisulfite-modified sense and antisense strands of both promoters, we designed primer sets S1/S2 and A1/A2 (Genset) respectively, as follows: S1, 5′-ATTTTAACTTCCTTAACTCCTAAAAATCT-3′; S2, 5′-TGGTTAAAGATTTATTTTTTTTGATAATT-3′; A1, 5′-TAACCAAAAATTTATTTCTCTTAACAACC-3′; A2, 5′-ATTTTAGTTTTTTTAGTTTTTGAAAATTT-3′.

PCR amplifications were performed in a 100-μl reaction mixture containing 5 μg of bisufilte-treated genomic DNA in the presence of 0.6 U of Taq polymerase (Life Technologies) and 2.5 mM $MgCl_2$ for S1/S2, or 1.5 mM $MgCl_2$ for A1/A2, using a Perkin Elmer thermocycler. The amplification procedure consisted of 3 min of denaturation at 94°C followed by 40 cycles at 94°C (15 sec), 47°C (45 sec), and 72°C (30 sec), ending with 10 min of extension at 72°C. Cloning of PCR products was performed by using the TOPO-TA Cloning Kit (Clontech) according to the manufacturer's instructions. To ensure statistical significance owing to the number of transgene copies carried by each transgenic line, 10 to 15 clones (each corresponding to one molecule of bisulfite-treated strand of promoter) were sequenced. Results were obtained by analysing three independent series of DNA preparation and bisulfite treatment. Results obtained with sense and antisense strands were pooled in the figure.

Primary Culture From Adult Mouse Limb Muscles

Mice used for primary culture experiments were 3-week- to 4-month-old females, either nontransgenic (for background determination) or double transgenic (pM-AP/pM-CAT_{98} or pM-AP/mM2-CAT_{57}). Primary culture of satellite cells obtained from adult mouse limb muscles was performed according to Rosenblatt et al. (1995). Each culture was initiated from living muscle fibre explants obtained from two gastrocnemius and four gluteus from two mice, and directly plated onto five Matrigel-coated 100-mm Petri dishes with no passaging during the whole time of culturing (11 to 18 days). Cells were grown for 8–15 days in proliferation medium until they reached confluence, and for 3–6 days in differentiation medium (10% horse serum, 2% fetal calf serum, 0.5% chick embryo extract), then collected for CAT and alkaline phosphatase (AP) assay.

During the differentiation process, some dishes were stained in situ for AP activity to monitor the expression of pM-AP transgene in differentiating myotubes. AP staining on primary culture dishes was performed as follows: cells were rinsed in phosphate buffer saline (PBS), fixed in 4% paraformaldehyde in PBS for 10 min at 4°C, then rinsed three times with PBS. Endogenous AP activity was inactivated by incubating the dishes for 45 min at 65°C. AP staining was performed in 100 mM Tris-HCl pH 9.5, 150 mM NaCl containing 0.33 mg/ml nitroblue tetrazolium chloride and 0.17 mg/ml 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt for 1 hr at room temperature in the dark. Reaction was stopped by rinsing with PBS.

CAT assays were performed by using thin-layer chromatography according to Sambrook et al. (1989). Briefly, cells were scraped in PBS and pelleted, then submitted to three freeze-thaw cycles in 0.25 M Tris-HCl pH 7.6. After a 13,000 rpm centrifugation for 15 min, the resulting supernatant was used for CAT assays after a 10-min heat denaturation at 65°C, while the pellet was disrupted and homogenised in 20 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Triton X-100 using a Polytron, and left on ice for 15 min. After a 13,000 rpm centrifugation for 15 min, the resulting supernatant was used for AP assays after a 30-min heat denaturation at 65°C. AP activity was assayed by using the Alkaline Phoshatase kit from Sigma Diagnostics, Inc. (St. Louis, MO) and absorbance at 405 nm was read by using a Shimadzu spectrophotometer.

Muscle Grafts

Muscle grafts were performed on a total of 12 B6/CBA female mice aged 8 weeks. The six donor mice were double transgenic pM-AP/mM2-CAT_{57} or pM-AP/pM-CAT_{98} mice aged 2–4 months. Host mice were anesthetised with a mixture of ketamine and xylazine (80 mg/kg and 4 mg/kg, respectively). A large piece of left external wing of gastrocnemius (this part of the muscle being the richest in type IIB fibres) and of gluteus medius were roughly ablated from host mice. Similarly sized piece of gastrocnemius and gluteus muscles were isolated from donor double transgenic mice (without preserving tendons) and immediately transplanted in the ablated wild-type mouse in homotopic or heterotopic position. Skin was sutured by using Ethicon silk 6-0 sutures and regeneration of grafts was allowed to settle for 3 weeks with a daily intramuscular injection of FK506 (Fujisawa Pharmaceutical Co.) (2.5 mg/kg) to avoid graft rejection, before muscles were processed for histochemical and biochemical analyses. Mice were killed by cervical dislocation, left gastrocnemius and gluteus muscles were dissected out and immediately snap-frozen in isopentane cooled in liquid nitrogen for AP staining on frozen 12-μm sections. AP staining was performed as described for primary muscle culture AP staining. A few sections were stained with hematoxylin-eosin using usual histology procedures.

The remaining muscle was processed for cell extract preparation: liquid nitrogen-frozen muscle was crushed in a fine powder that was resuspended with 600 μl Tris-HCl 0.25 M and submitted to the procedure of CAT and AP activity detection described for primary culture extracts.

Statistics

Statistical comparisons were performed by using one-way analysis of variance followed by Fisher's test with a 99% confidence level ($P < 0.01$) (StatView software).

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