A Muscle-specific Promoter Directs Pitx3 Gene Expression in Skeletal Muscle Cells*

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The Pitx homeobox transcription factor genes have been implicated in different developmental processes, including determination of hind limb identity for Pitx1, left-right asymmetry for Pitx2, and eye development and survival of midbrain dopaminergic neurons for Pitx3. Pitx1 and Pitx2 have partly redundant activities in craniofacial development, including in pituitary organogenesis, as indicated by their names. These genes also exhibit redundant activities in the control of hind limb bud growth. Recent studies have shown expression of the three Pitx genes in muscle, with Pitx3 being the most widely expressed in all skeletal muscles. We now report the identification of a muscle-specific promoter within the Pitx3 gene that is situated between the first exon for eye and brain expression and exon 2 that contains the initiator ATG codon. Sequences proximal to this muscle-specific exon 1 are essential and sufficient to confer muscle-specific expression in transgenic mice, they are responsive to myogenic basic helix-loop-helix regulatory factors, and they recruit these factors in vivo. In agreement with exclusive use of the muscle-specific promoter in aphakia mice that are deleted of the brain promoter, the trimethyl-lysine 4 histone H3 promoter signature shifts to this promoter in embryonic day 13 ak limb bud muscle cells. Myogenic basic helix-loop-helix regulatory factor activation of Pitx3 transcription may be part of a positive feedback loop contributing to establishment of the myogenic program.

The Pitx genes play critical roles in early development of several tissues and organs. The first of these homeobox transcription factors, Pitx1 (Ptx1), was discovered for its role in cell-specific transcription of the pituitary POMC gene (1); on the POMC gene, it acts as an obligate partner of the highly cell-restricted T-box transcription factor, Tpit (2, 3). Beyond their role in pituitary organogenesis and transcription as implied by their names (4, 5), the Pitx genes play several roles in early development. Indeed, Pitx1 is first expressed in the lateral plate mesoderm of the posterior half of the embryo, starting as soon as posterior lateral plate mesoderm is formed at gastrulation (6). Throughout later development, Pitx1 remains highly expressed in developing hind limb bud mesenchyme and was shown to determine hind limb identity in mice (7, 8), chicks (9, 10), and fish (11). In addition, Pitx1 controls the growth ability of hind limb mesenchyme together with Pitx2 (12). Pitx2 is also expressed in the lateral plate mesoderm but preferentially on the left side, where its left-specific expression is controlled by Sonic hedgehog (Shh) and/or nodal (13). The left-specific Pitx2c isoform uses a different promoter than for its bilateral expression in other tissues (14). This alternate promoter is responsible for production of Pitx2a and Pitx2b that arise through differential splicing of exon 2 (4). Left-specific expression is critical for left-right asymmetry and development of the lungs, heart, and stomach (14, 15). In the heart, the left-specific isoform of Pitx2, Pitx2c, has activities that are specific or redundant with those of the bilaterally expressed isoforms Pitx2a and Pitx2b (16).

Finally, the third gene of the family, Pitx3, is expressed in the eye (17) and midbrain dopaminergic neurons (18). In agreement with this expression, a natural mouse mutant of the Pitx3 gene promoter, the aphakia (ak) mouse, has lens developmental defect (17), and in humans, Pitx3 mutations have been associated with eye dysmorphogenesis (anterior segment mesenchymal dysgenesis) and with congenital cataracts (19). The ak mouse was also found to exhibit a severe deficit of dopaminergic neurons in the substantia nigra and ventral tegmental area (20–22) and to have an akinetic phenotype similar to the loss of motor movement presented by patients with Parkinson disease (22). Similar to Parkinson patients, this mouse model exhibits a locomotor response to L-Dopa injection (23).

Early reports on expression profiles of Pitx genes had noted expression of Pitx1 in a small subset of muscles (6), of Pitx2 in early myoblasts (24), and of Pitx3 in extraocular muscles, tongue and head muscles (19). Muscle expression of these genes was not studied systematically until recently.
as we conducted a detailed analysis of their expression patterns in early precursors and in developing muscle (25). It thus appears that Pitx3 is expressed in all skeletal muscles. Since Pitx3 is the only member of the family to be expressed in all muscles, we have undertaken to define the mechanism of its expression and its role in muscle development. Since a natural mouse mutant of the Pitx3 gene already exists, the ak mouse, we assessed muscle expression of Pitx3 in ak mice and found it to be normal. The present report delineates a novel muscle-specific promoter within intron 1 of the Pitx3 gene that is not affected by the ak mutation. Further, we defined the muscle-specific Pitx3 promoter in transgenic mice experiments and showed that it is adequate to target gene expression in early skeletal muscle, thus mimicking expression of the endogenous gene. In normal muscle, both brain and muscle initiation sites appear to be used, but only muscle-specific exon 1m is transcribed in ak mice, in agreement with a shift of the trimethyl-lysine 4 histone H3 (H3K4me3) promoter signature in ak muscle cells. Finally, we show that Pitx3 expression is stimulated by myogenic bHLH regulatory factors (MRFs) in a positive feedback regulatory mechanism that serves to establish the myogenic program of differentiation.

EXPERIMENTAL PROCEDURES

Whole Mount in Situ Hybridization—The protocol used (available on the World Wide Web) is from the laboratory of Dr. Janet Rossant. A rat Pitx3 cDNA EcoRI-BamHI fragment encompassing the entire translated sequence was used as probe.

lacZ Staining and Immunohistochemistry—For β-galactosidase staining, 12.5-day postcoitum mouse embryos were fixed for 25 min in 4% paraformaldehyde at 4 °C; rinsed in cold phosphate-buffered saline, 0.01% sodium deoxycholate, 0.02% Nonidet P-40; and then incubated for 24 h at 30 °C (to minimize endogenous activity) in staining solution (0.4 mg/ml X-gal, 4 mm potassium ferricyanate, 4 mm potassium ferrocyanate, 4 mm MgCl2). Immunohistochemistry was performed as described (26) with the following antibodies: anti-Pitx3 polyclonal antibody (described in Ref. 22) and anti-MyoD monoclonal antibody (purchased from Pharmingen).

RNA Analyses—Total RNA was prepared from adult mouse muscle, spleen, or e14.5 embryos with TRIzol (Invitrogen) according to the manufacturer’s directions. Purification of mRNA was performed using the Qiagen (Valencia, CA) Oligotex Maxi Kit following the supplier’s protocol.

For RT-PCR, reverse transcription was performed with 500 ng of mRNA using a reverse oligonucleotide primer in the Pitx3 3′-untranslated region (TCACAGCCTCTCCGGACAGG), and the resultant cDNA was purified on Qiaquick columns (Qiagen), diluted 100-fold, and amplified with the following primers: reverse primer Rev/cDNA (GGAGTGCCTGCGTCCGGAT), with oligonucleotide 1 (CTCTGGGAGCTCATAGCTTG), 2 (GTGTGTGGTGCGATCATAGTGT), 3 (GTGTTTTGGCCTTTGGCT), 4 (GGGGGCGATGTGAGAGAGA), 5 (TGTCAGAGAGACAGAGAGAG), 6 (AGGGGATGCTGCTCCAAAA), 7 (GGAGTGAAGAGCTGAGGTT), 8 (CCCCCAGACAGTTCTGAA), or lb (ACAAGCCACCCGGAGGCTC). Control reactions on 400 ng of genomic DNA were done with primers 4–8 with reverse primer Rev/gDNA (CAAGCTATGAGCTCCCGAGAG). PCR products were separated on a 1.6% agarose gel. For mRNA quantitation, reverse transcription aliquots prepared as above from ak and WT e14.5 limb bud mRNA were analyzed by quantitative real time PCR (MX-3005; Stratagene, La Jolla, CA). The primers used were oligonucleotides lb and 4 with Rev/cDNA for exon 11 and exon 1m, respectively. Abundance is calculated by comparison with β-actin mRNA amplified with the following primers: TGTAGTGGGAATGGTCAGAA and TCCATGTCGTTCCGATTTGAA.

Chromatin Immunoprecipitation (ChIP)—Chromatin immunoprecipitations were performed as described (27) with modifications. Intact limb buds from e13 wild-type or ak embryos were collected in PBS and formaldehyde (final concentration 1%). After 5 min of agitation, homogenization was performed with a Dounce potter A for 10 min in cross-linking solution. Glycine (final concentration 125 mM) was added to quench excess formaldehyde. Pellets collected by centrifugation (1000 × g, 3 min) were washed twice with PBS and resuspended in Triton buffer (0.25% Triton X-100, 10 mM Tris, pH 8, 10 mM EDTA, 0.5 mM EGTA, 1 μg/ml apronin, 1 μg/ml leupeptin, 0.1 mM phenylmethylsulfonflyl fluoride, and 1 μg/ml pepstatin) and homogenized with Dounce potter B. Nuclei pellets were then resuspended in NaCl buffer (200 mM NaCl, 10 mM Tris, pH 8, 1 mM EDTA, 0.5 mM EGTA, 1 μg/ml apronin, 1 μg/ml leupeptin, 0.1 mM phenylmethylsulfonflyl fluoride, and 1 μg/ml pepstatin). After centrifugation, cells were resuspended in sonication buffer (0.5% SDS, 0.5% Triton X-100, 0.05% NaDOC, 10 mM Tris, pH 8, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1 μg/ml apronin, 1 μg/ml leupeptin, 0.1 mM phenylmethylsulfonflyl fluoride, 1 μg/ml pepstatin) and sonicated to obtain DNA fragments of about 0.5–2 kb. 500 μg of sonicated chromatin was subjected to immunoprecipitation at 4 °C with 5 μg of antibodies against MyoD (Pharmingen), myogenin (Pharmingen), trimethyl-lysine 4 histone 3 (Abcam), RNA polymerase II (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or matched rabbit nonimmune IgG (Sigma) as negative control. Immunoprecipitates were collected with protein A/G-agarose beads (Upstate Biotechnology, Inc., Lake Placid, NY) saturated with tRNA. Beads were washed six times with the following buffer (50 mM Tris·HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1 μg/ml apronin, 1 μg/ml leupeptin, 0.1 mM phenylmethylsulfonflyl fluoride, 1 μg/ml pepstatin). Chromatin was eluted from beads with elution buffer (1% SDS, 50 mM Tris, pH 8, 10 mM EDTA) and purified with a Qiaquick purification kit (Qiagen). Quantitative real time PCR (MX-3005; Stratagene, La Jolla, CA) was performed with the SYBR Green kit (Qiagen). For ChIP analyses of the Pitx3 locus, the following primers (pictured in Fig. 7A) were used: A, ACAAGCCACCCGGAGGCTC; B, ACAAGCTATGAGCTCCCGAGAG.
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AGAA with ATCAGAAGCGTTTGGCACCAC; B, AGTACACATGGCTTTTACCTCCAG with CAACGCTTCTATCCTGCTAAACC; C, AGTCAGTTGGACAGATGTGCTTG with ATAGCAGGAGCGCTCCTACCTCA; D, TGGACAGAGAGGACT with TTTAGACAGAGAAGACACACCTGGAAC; E, TGTTCCAGACCTTGGACGTGTCAGAAGGAGACT with TAGCTGTACACCAAGGCTTCA. Enrichments were calculated relative to PCR analyses (primer TCGGAGTGGAATTACCTATCGTGCTTTGGAC; E, TGATTCAGACTTGCGGTTGAC with TAGCTGTACACCAAGGCTTCA) of a control sequence within the POMC gene promoter that is not expressed in muscles.

Transgene Constructs—Mouse Pitx3 gene sequences were cloned from a genomic 129/sv DASH2 phage library (1). A genomic 4-kb Pitx3 promoter fragment was inserted upstream of LacZ (from α-GSU-LacZ) (28) to create plasmid JA1639. The addition of the 3′ two-thirds of Pitx3 intron 1 yielded JA1642. JA1673 was obtained by deleting the 2.2-kb putative alternative promoter region in JA1642. For TG1704 and TG1707 transgenes, the Pitx3 4-kb promoter in TG1639 was replaced by an intrinsic fragment corresponding to the conserved promoter region (corresponding to −1775/+12 of the muscle promoter) with or without a less conserved 1.5-kb upstream region, respectively. Insertion of the 3′ 5′ part of intron 1 into TG1704 yielded TG1706. All constructs were checked by restriction and sequencing; maps and sequences are available upon request.

Transgenic Mice—All DNA fragments for microinjection were produced by SacII-Sall digestion and purified on agarose gel. Transgenic mice were produced by previously described (29). For transient transgenics, foster mice were sacrificed at 12.5 days postcoitus, taking the day of injection as 0.5 days postcoitus.

Cell Culture and Transfection—L6 and C2C12 cells were cultured in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum and transfected in Dulbecco's modified Eagle's medium without serum for 4 h with Lipofectamine according to the manufacturer's directions. Usually, for 35-mm wells, 1 μg of β-galactosidase reporter was used per well. For differentiation, cells were grown to confluence, and then medium was changed to Dulbecco's modified Eagle's medium containing 2% horse serum, and cells were maintained in culture for 24 or 48 h. In experiments to assess responsiveness to MyoD, expression vectors for both MyoD and E47 (same amount for each) were co-transfected with reporter, and reporter activity was measured 24 h posttransfection in proliferation conditions. β-Galactosidase activity was assayed in lysates with a luminometer using the Galacto-Light system (TROPIX) as described by the manufacturer or by cytochemistry using X-gal as a substrate in formaldehyde-fixed cells to correlate activity with differentiated morphology.

Software—Sequence alignments were performed using ClustalW (available on the World Wide Web), and graphic genomic alignments were performed with LAGAN and VISTA (both available on the World Wide Web). The promoter prediction software is also available online.

RESULTS

Pitx3 Is Expressed in Developing Muscles of Aphakia Mice—In order to establish expression of Pitx3 in developing muscles, we used whole mount in situ hybridization with mouse embryos at day 12.5 (e12.5) of development. This analysis revealed expression of Pitx3 in myotomes and developing muscle masses of the limbs (Fig. 1, A and B). Muscle masses of both fore limb and hind limb buds express Pitx3, and expression is also observed in forming abdominal muscle masses. It thus appears that Pitx3 is very widely expressed in most developing skeletal muscles. We provided a detailed investigation of this expression profile elsewhere (25).

Since the ak mouse was shown to be a promoter mutant of the Pitx3 locus (30, 31), we wanted to verify a putative defect of Pitx3 expression in muscle. Surprisingly, we found that muscle expression of Pitx3 is intact in ak embryos and that in this tissue, it appears indistinguishable from wild-type littermates (Fig. 1, C and D). In order to verify that Pitx3 protein indeed has normal expression in ak muscles and further that it is expressed in adult muscle, we performed Western blot analysis of nuclear proteins from mouse hind limb muscle of ak (lane 1) and wild-type (lane 2) mice revealed with Pitx3-specific antibody. A nonspecific band is present in muscle extracts as well as in Pitx1-positive but Pitx3-negative Att-20 cells (lane 3). The same Pitx3 antibody was used for immunohistochemical analysis of the Pitx3 expression profile (25).
that a different exon 1 and promoter might be active in muscles and that this putative muscle exon 1 could be spliced onto a common exon 2. The quest for an alternate initiation site for muscle Pitx3 expression was facilitated by the search of genome databases for expressed sequence tags containing Pitx3 sequences. Indeed, a BLAST search on the UCSC genome server identified a rat expressed sequence tag (BF543032) matching with exons 2 and 3 of Pitx3 sequences showing percentage identity to the various exons. The top of the diagram shows different fragment end points (in bp) used in the present work relative to muscle transcription start site (TSS) set at +1. VISTA analysis was used to generate genomic analyses. Scale bar, 1 kb.

In order to pinpoint a putative transcription start site for exon 1m, the BDGP neural network promoter prediction software (32) was used to predict putative initiation site(s) as indicated in Fig. 3A; this prediction was made with a high score (0.83, where the maximum is 1). This putative start site lies within a region of high sequence conservation between mouse, rat, and human sequences (Fig. 3A). In order to validate this prediction, a series of RT-PCRs were carried out using e14.5 embryo mRNA and different primers. A common 3' primer located in exon 2 was used in all cases, and different 5' primers (Fig. 3A) located downstream of putative initiation (primer 5, 4, 3, 2, or 1) or just upstream of this predicted start site (primer 6, 7, or 8) were used. No RT-PCR products could be amplified using adult spleen RNA as control (data not shown), and a PCR product of the expected size was obtained with primer 5 located between putative nucleotides −6 and +18 (Fig. 3B). In contrast, no PCR product could be obtained with primer 6, which lies at positions −45 to −26 bp relative to the putative start site (Fig. 3B). In order to verify that primer 6 was active in PCR, control reactions were conducted using genomic DNA substrate; a fragment of the expected size was obtained (Fig. 3C). DNA sequencing of the RT-PCR products confirmed that this fragment contains the expected exon 1m, exon 2, and exon 3 sequences, as predicted for the proposed muscle mRNA shown in Fig. 2A. Similar results were obtained with other reverse primers. It is noteworthy that the initiator ATG codon is found in exon 2 of the Pitx3 gene; it is therefore predicted that the Pitx3 protein should be the same in muscle as in eye and midbrain. These data suggest that muscle expression of Pitx3

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A Muscle Cell-specific Pitx3 Gene Promoter—In order to test whether the putative Pitx3 promoters are active in vivo and show the expected specificity, we tested the two predicted promoter sequences of the Pitx3 locus in transgenic mice. The promoter fragments were fused to β-galactosidase (lacZ) coding sequences, and founder e12.5 transgenic embryos were assessed for transgene expression using LacZ activity as reporter. A 4-kb promoter fragment for exon 11b, that is expressed in eye lens and midbrain (mb) was found to be largely inactive in transgenic embryos (Fig. 4, construct F). This transgene did not exhibit any expression in lens or midbrain and exhibited only weak expression in the myotome region of only one embryo. In contrast, four of seven embryos had faint ectopic expression at unrelated sites. Stable mouse transgenic lines had been established with the same transgene previously, and they showed similar weak and inconsistent patterns of expression.
Various putative promoter fragments for exon 1m were also assessed in transgenic mice, and the most consistent and strongest expression was observed with the shortest transgenic promoter fragment (construct A). This 1.6-kb promoter fragment contains two regions of high sequence conservation: a promoter-proximal region of about 350 bp and another upstream region of about 300 bp at +1450 bp (Fig. 2B). This transgene directed very strong expression in myotomes, limb, and facial muscles with very infrequent ectopic expression (Fig. 4, construct A); LacZ activity was never detected in midbrain or eye lens. The addition of more upstream sequences (constructs B and C) did not further enhance muscle expression of transgenes; in fact, expression was weaker (Fig. 4) with particular loss of expression at the level of craniofacial muscles, of the epaxial domain of the myotome, and of the most caudal somites (beyond the hind limb level). In fact, transgene expression in face muscles was only consistently observed for construct A; nine of 11 founders had facial expression, compared with three of nine for construct B and four of 17 for construct C. A short region of unconserved upstream sequences (absent in construct C) could not be tested in transgenic mice, because the presence of these sequences rendered plasmids very unstable. Since construct F conferred expression at low frequency (one of seven) in putative myogenic cells, we wanted to test whether these sequences may act in concert with the muscle-specific promoter and whether the muscle-specific sequences could act as enhancers. A transgene containing both promoters (construct D) exhibits similar muscle specificity as construct B or C; it is also less active than construct A. In agreement with the hypothesis that the highly conserved sequences around exon 1m (Fig. 2B) that are active in construct A are important for muscle-specific activity, it was observed that deletion of those sequences completely

**FIGURE 3.** The Pitx3 muscle-specific promoter. **A,** mouse, rat, and human sequences in the vicinity of the putative muscle-specific transcription initiation site are compared. A predicted initiation site is shown as +1m. The position of PCR primers used to validate the 5' end of the muscle mRNA is also indicated. **B,** RT-PCR analysis of Pitx3 mRNA using RNA from e15 embryos. The same reverse primer (located in exon 2 as depicted in A) was used for all PCRs. Lanes 1–8 used the corresponding forward primers depicted in A, and lane 11, b used a forward primer in exon 11, b. Similar results were obtained for primers 1–8 using WT and ak samples, whereas the exon 11, b product was only detected in WT RNA. **C,** validation of primers 4–8 using genomic DNA template. **D,** quantitation by quantitative RT-PCR of the two Pitx3 mRNA isoforms utilizing either exon 11, b or exon 1m in e14.5 limb buds. n.d., not detectable.
abolished transgenic promoter activity (Fig. 4, construct E). Collectively, these data clearly identify a muscle-specific promoter/enhancer upstream of exon 1m of the Pitx3 gene and indicate that this promoter is necessary and sufficient for muscle-specific expression of Pitx3. The maintenance of Pitx3 expression in ak muscles is completely consistent with the existence of separate regulatory sequences for muscle-specific expression of the gene.

The Pitx3 Muscle-specific Promoter Is Active in Myoblasts and in Differentiating Muscle Cells—The early pattern of muscle expression for transgene A (Fig. 5, A and B) is extremely similar to the pattern of Pitx3 mRNA expression (Fig. 1). It includes forming skeletal muscle masses of limbs, as revealed by analysis of tissue sections from X-gal-stained embryos (Fig. 5, C and D). In order to ascertain the muscle specificity of this LacZ activity, we performed co-labeling by immunohistochemistry with Pitx3 or MyoD. This demonstrated co-expression of MyoD with LacZ in developing FL muscle masses (Fig. 5, E and F) and in the myotome (Fig. 5, G and H). A similar distribution was observed for Pitx3 and LacZ (Fig. 5, I and J). Only a subset of MyoD-positive cells stain for LacZ in both myotome and limb muscles; in more caudal myotomes (Fig. 5I), LacZ-positive cells are also less abundant than Pitx3-positive cells (Fig. 5F), suggesting a delay between endogenous Pitx3 and transgene A LacZ activity. These data clearly indicate that the muscle-specific exon 1m promoter of the Pitx3 gene is only active in muscle cells.

Myogenic bHLH Regulatory Factors Activate the Pitx3 Muscle Promoter—In order to further define the Pitx3 muscle promoter, we used transfection of promoter constructs into muscle cell lines. Initially, we tested the activity of the −1597 bp Pitx3 promoter (construct A) fused to LacZ in proliferating C2C12 cells, and we observed very low β-galactosidase activity (Fig. 6A). However, significant activity was observed with the same reporter in C2C12 cells following 24 h in differentiation conditions (33). In agreement with this promoter/reporter activation, endogenous Pitx3 mRNA levels were found to increase in similar differentiation relative to proliferation conditions (Fig. 6B). We then assessed the effect of MyoD on the −1597 bp Pitx3 promoter using L6 cells, another muscle-derived cell line; similar results were obtained in C2C12 cells (data not shown). This experiment clearly indicated that MyoD can activate the Pitx3 muscle promoter (Fig. 6C).

We then localized MRF-responsive sequences using deletions of the −1597 bp Pitx3 promoter plasmid. Thus, deletion to −590 bp (Del B) completely prevented responsiveness to MyoD (Fig. 6C). In contrast, deletion to −1388 bp (Del A) did not affect the responsiveness to MyoD, suggesting that active sequences are present between −1388 and −590 bp (Fig. 6C). Interestingly, numerous muscle-specific E-boxes that are the targets of MRFs are present in this interval, and the recruitment of MyoD to the Pitx3 locus was recently shown using a ChIP-on-chip strategy (34) in agreement with the present data showing activation of the Pitx3 promoter.
The differentiation of C2C12 cells is accompanied by up-regulation of MyoD activity, and in agreement with the deletion analyses (Fig. 6C), we found that the different deletion mutants of the Pitx3 promoter exhibit similar activity when assessed upon differentiation of C2C12 cells, with the −590 bp promoter (Del B) showing no activity (Fig. 6D). Collectively, these data are in agreement with the expression of Pitx3 in differentiating muscle cells (25) and with a scheme in which Pitx3 expression is subject to positive feedback up-regulation by MyoD during muscle cell differentiation.

Chromatin Signature of Muscle-specific Promoter—The action of MyoD on the Pitx3 muscle regulatory sequences appeared to depend principally on sequences between −1388 and −590; this 702-bp region contains multiple putative MRF binding sites, and we developed a PCR strategy to investigate MRF recruitment to this region by ChIP (Fig. 7A). We found significant recruitment of both MyoD and myogenin to this region (Fig. 7B, primers C) compared with the exon 1l,b regulatory region (primers A) or with exon 4 of the gene (primers E).

It appears that both promoters 1l,b and 1m are active in muscle (Fig. 3D), although only promoter 1m was active in transgenics (Fig. 4). In order to validate this at the chromatin level, we used ChIP against H3K4me3 that has been reported to be a signature for active promoters (35, 36). This analysis (Fig. 7C) revealed a higher level of H3K4me3 at the exon 1l,b promoter (primers B) than at exon 1m (primers D), but the level of H3K4me3 at exon 1m was significantly increased in ak mice and was absent from the exon 11b region when probed with primers B (Fig. 7A) that are beyond the deletion end point in the ak genome. Although not different in WT and ak limb buds, the levels of H3K4me3 at exon 4 are unusually high for transcribed...
sequences; it is possible that this region may contain another promoter, possibly for antisense transcripts (37). In agreement with the levels of H3K4me3 and with relative promoter usage in muscle (Fig. 3D), more RNA polymerase II was found to be present at exon 1l,b than exon 1m in muscle (Fig. 7D), but this ratio was reversed in ak muscles. Collectively, these data indicate that both promoters 1l,b and 1m are used in muscle cells but that the muscle regulatory sequences are primarily situated upstream of exon 1m.

**DISCUSSION**

The present report defines a skeletal muscle-specific transcription unit of the Pitx3 gene. This gene was previously known to be expressed in the eye lens and in a subset of dopaminergic neurons of the midbrain through the activity of a common promoter and exon 1. The present report defines a new promoter located between the eye/midbrain promoter and the ATG-containing exon 2. The muscle-specific Pitx3 promoter appears to be entirely contained within a 1.6-kb DNA fragment that mimics in transgenic mice the expression of endogenous Pitx3 in muscles. The muscle-specific Pitx3 promoter can be activated by the myogenic bHLH regulatory factor, MyoD. The action of MRFs on the Pitx3 promoter is consistent with the expression pattern of Pitx3 during muscle development.

**A Muscle-specific Promoter and Exon of the Pitx3 Locus**—The skeletal muscle-specific Pitx3 transcription unit defined in the present work includes a new exon 1 (exon 1m) and important regulatory sequences located within 1659 bp upstream (Figs. 4–7). Transgenic mice analyses of this muscle-specific promoter suggest that it is entirely sufficient for muscle expression (Fig. 4). The activity of the exon 1m promoter in transgenic mice is entirely consistent with the expression in developing myotomes of a GFP knock-in allele of the Pitx3 gene (38). The muscle-specific activity of exon 1m regulatory sequences in transgenic mice contrasts with its partial usage in normal muscle. Indeed, all criteria (mRNA abundance, RNA Pol II recruitment, and H3K4me3 enrichment) indicate that exon 1l,b is used preferentially even under the influence of muscle regulatory sequences. It is only upon deletion of exon 1l,b in ak mice that this initiation is no longer used in favor of exon 1m (Fig. 3D) and that H3K4me3 enrichment at exon 1l,b is lost (Fig. 7C). Exon 1l,b appears to have strong transcription initiation capacity; it is used in the brain and eye, but regulatory sequences directing this activity have not yet been identified and are not within the upstream 4 kb (Fig. 4, transgene F). The muscle regulatory sequences (between $\sim 1388$ and $\sim 590$ bp) that are 6 kb downstream from exon 1l,b appear to preferentially use this initiation relative to exon 1m initiation that is closer. The mechanism for promoter selection in such case remains poorly understood.

**Role of Pitx Transcription Factors in the Program of Muscle Gene Expression**—The onset of Pitx3 expression with differentiation of Pitx2-positive progenitors (25) is consistent with the activity of the Pitx3 exon 1m shown in transgenic mice (Figs. 4 and 5). In addition, the maintenance of Pitx2 expression in Pitx3$^{+/+}$ muscles suggests that at least one Pitx factor must be present for maintenance of the myogenic programs (25) and that Pitx2 may contribute in part to activation of Pitx3 expression. The earliest Pitx gene expressed during muscle cell differentiation is Pitx2 with expression in proliferating muscle pro-
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