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Distinct Gene Expression Patterns in Skeletal and Cardiac Muscle Are Dependent on Common Regulatory Sequences in the *MLC1/3* Locus

MICHAEL J. MCGREW,[1,2] NATALIA BOGDANOVA,[2] KOJI HASEGAWA,[3] STEPHEN H. HUGHES,[4] RICHARD N. KITSIS,[3] AND NADIA ROSENTHAL[2]*

Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118[1]; Cardiovascular Research Center, Massachusetts General Hospital-East, Charlestown, Massachusetts 02129[2]; Departments of Medicine (Cardiology) and Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461[3]; and ABL-Basic Research Program, Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, Maryland 21702-1201[4]

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The myosin light-chain 1/3 locus (*MLC1/3*) is regulated by two promoters and a downstream enhancer element which produce two protein isoforms in fast skeletal muscle at distinct stages of mouse embryogenesis. We have analyzed the expression of transcripts from the internal *MLC3* promoter and determined that it is also expressed in the atria of the heart. Expression from the *MLC3* promoter in these striated muscle lineages is differentially regulated during development. In transgenic mice, the *MLC3* promoter is responsible for cardiac-specific reporter gene expression while the downstream enhancer augments expression in skeletal muscle. Examination of the methylation status of endogenous and transgenic promoter and enhancer elements indicates that the internal promoter is not regulated in a manner similar to that of the *MLC1* promoter or the downstream enhancer. A GATA protein consensus sequence in the proximal *MLC3* promoter but not the *MLC1* promoter binds with high affinity to GATA-4, a cardiac muscle- and gut-specific transcription factor. Mutation of either the MEF2 or GATA motifs in the *MLC3* promoter attenuates its activity in both heart and skeletal muscles, demonstrating that MLC3 expression in these two diverse muscle types is dependent on common regulatory elements.

Although embryologically distinct, striated myocytes in the skeletal and cardiac muscle lineages share many specialized gene products. Investigations into the molecular mechanisms involved in the expression of these skeletal and cardiac genes have revealed that both distinct and common regulatory regions are responsible for their expression. Distinct regulatory regions of several genes have been shown in vivo to activate transcription either in skeletal or cardiac muscle tissue (30, 39, 53). Conversely, skeletal and cardiac muscle-specific expression of other striated genes has been shown to be modulated in vitro or in vivo by the same *cis* regions and elements (28, 49, 55, 63). Such results support the hypothesis that certain shared regulatory elements are present in the two striated muscle lineages.

Multiple regulatory factors involved in differentiation of skeletal muscle lineage have been recently characterized (reviewed in reference 66). The myogenic determination factors, MyoD, myogenin, myf-5, and mrf-4, have been shown to bind to the regulatory regions of muscle-specific genes through E-box consensus sequences and thereby activate their transcription. Additionally, overexpression of this class of proteins in nonmuscle cells will convert them to a skeletal muscle phenotype. Isolation of a similar class of proteins which will convert cells to the cardiac lineage has not been as successful. However, several factors which are involved in the establishment of the cardiac muscle cell lineage and cardiac-muscle specific gene expression have recently been identified. An example of the former is the homeobox gene *Nkx-2.5*, which is necessary for correct cardiac muscle morphogenesis and ventricular gene expression (43). Transcription factors expressed in cardiac muscle cells include the GATA-4 protein, which contains a zinc finger DNA binding domain (2), and members of the MADS protein family, MEF2, isoforms of which are found in skeletal and cardiac muscles (72). GATA-4 has been shown to activate cardiac muscle-specific transcription of the rat B-type natriuretic peptide (rBNP), cardiac troponin C, and α-myosin heavy chain (α-MHC) regulatory regions (23, 29, 47). MEF2 isoforms are necessary for the transcriptional activation of both cardiac and skeletal genes (21, 48, 51). Moreover, disruption of the single *MEF2* gene in *Drosophila melanogaster* blocks the differentiation of all smooth and striated muscles (40). These results further indicate that both separate and common transcription factors are involved in the differentiation of skeletal and cardiac muscles.

To further delineate the regulatory mechanisms involved in striated muscle gene expression, we have examined the expression of MLC3, the fast alkali light-chain isoform encoded by the internal promoter of the *MLC1/3* locus. This locus consists of two widely separated promoters, *MLC1* and *MLC3*, and a distal downstream enhancer element which generates two transcripts which vary in their two 5′ exons and contain five common 3′ exons (Fig. 1) (12, 54, 56). Transcription from the two promoters occurs at developmentally distinct stages (4, 42). The *MLC1* promoter and enhancer elements are essential for the expression of a reporter construct in skeletal muscle cell lines, although the *MLC3* promoter appears to contain independent transcriptional activity (11, 57, 60). *MLC1* promoter and enhancer elements driving a chloramphenicol acetyltrans-

* Corresponding author. Mailing address: Cardiovascular Research Center, Massachusetts General Hospital-East, 149 13th St., 4th Floor, Charlestown, MA 02129. Phone: (617) 724-9560. Fax: (617) 724-9561.

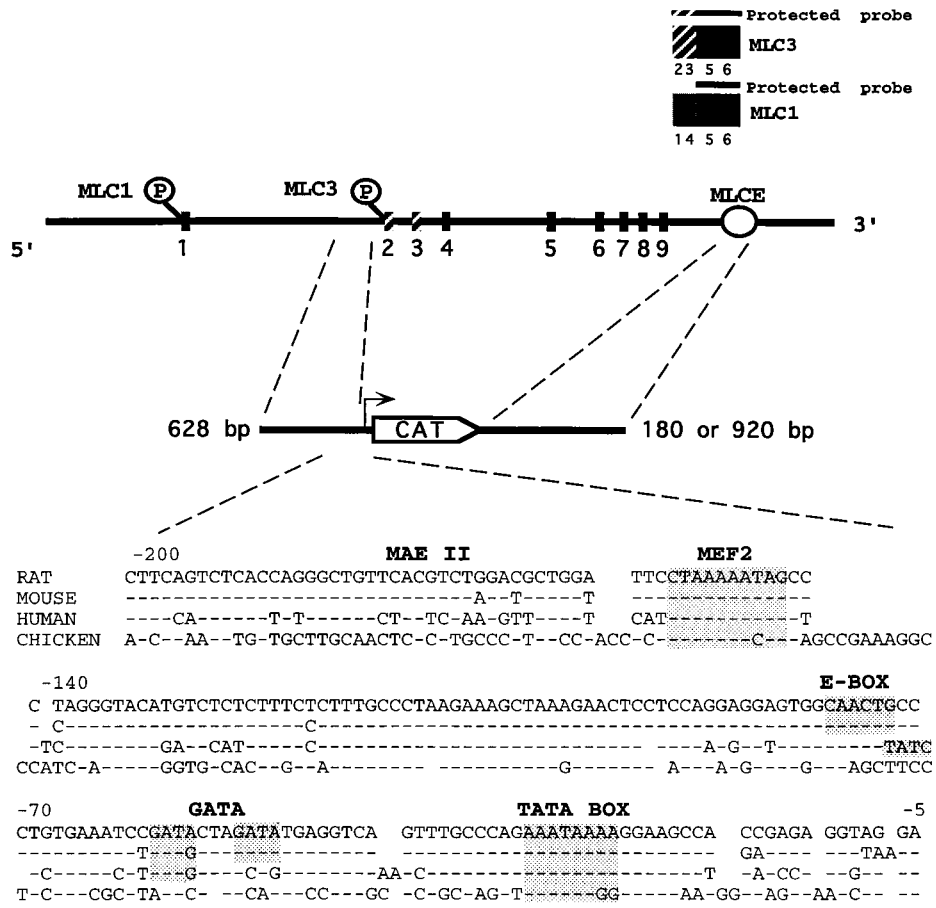

FIG. 1. *MLC1/3* locus and regulatory regions used in transgenic animals. Organization of exons and regulatory regions in the rat *MLC1/3* locus (12, 50). The locations of the two promoters and the downstream enhancer are indicated. (Top) Transcripts and exons detected by riboprobe are indicated. (Middle) Arrangement of regulatory regions used in MLC3CAT transgenic animals. Transgenic constructs contained a 628-bp *MLC3* promoter region driving the transcription of a CAT reporter gene. Some constructs also contained a downstream 920-bp or minimal 180-bp enhancer element (63). (Bottom) Sequence comparison of *MLC3* promoter regions in several vertebrates. Consensus sequences for potential striated muscle regulator elements are stippled (per reference 56). The *Mae*II restriction site assayed for genomic methylation is also indicated.

ferase (CAT) reporter gene in transgenic mice display correct temporal and spatial expression in fast fibers (58). Unexpectedly, a rostrocaudal gradient of expression is present in the intercostal musculature (13). This expression pattern is established early in development, is maintained in culture, and is correlated with a gradient of methylation in the downstream enhancer (14, 24, 25).

In this study we demonstrate that *MLC3* is expressed in cardiac tissue. This transcript is primarily in the atria and precedes skeletal muscle expression during development. We assay promoter and enhancer elements in vivo by generating transgenic mice carrying a minimal *MLC3* promoter region driving a CAT reporter construct with or without the downstream enhancer element. We find that both temporal and spatial transgene expression patterns in the cardiac muscles of these mice are solely dependent on the *MLC3* promoter, which is also able to activate moderate levels of expression in most adult skeletal muscles. The addition of a downstream enhancer is necessary for high levels of expression in skeletal muscle and for expression in a larger number of muscle groups. The examination of the methylation status of promoter and enhancer elements of the endogenous and transgenic regulatory elements reveals differential mechanisms that regulate the regulatory regions of this locus: the *MLC1* promoter and *MLC* enhancer exhibit skeletal muscle-specific demethylation while the *MLC3* promoter is ubiquitously demethylated. In addition, delineation of *MLC3* promoter elements indicates that a double GATA element present exclusively in the *MLC3* promoter binds in a sequence-specific manner to the GATA protein, GATA-4. Furthermore, both the GATA and MEF2 *cis* elements in the *MLC3* promoter are necessary but not individually sufficient to activate expression in cardiac and skeletal muscle. These results demonstrate a new role for GATA elements in striated muscle.

MATERIALS AND METHODS

Vectors and site-directed mutagenesis. Plasmid MLC3^{2H}CAT was generated by deletion of the internal *Hin*dIII site of rat MLC3CAT (12) and the insertion of a *Hin*dIII linker into the downstream *Sma*I restriction site. Rat enhancer fragments were cloned into the *Bam*HI site to generate MLC3^{2H}CAT180 and MLC3^{2H}CAT920. Plasmid p628Luc was prepared by subcloning of a 628-bp *Hin*dIII MLC3 promoter fragment into pGL2-basic (Promega). The enhancer fragment from pMLC1CAT920 (53) was subsequently cloned into the downstream *Bam*HI site (p628Luc920). Promoter deletions were constructed by digestion of the entire *MLC3* promoter with *Apa*I (position −434), *Afl*II (position −257), or *Avr*II (position −147), filling in with DNA polymerase (Klenow fragment) to generate a blunt end, and digestion with *Hin*dIII. The resulting promoter subfragments were recloned into pGL2-basic.

The MEF2 site and the GATA consensus binding sites in the rat *MLC3* promoter were mutated by PCR (31) with the following oligonucleotides: CTG GAT TCC GAC CAC TCG CCC TAG G for the MEF2 mutant and GTG AAA

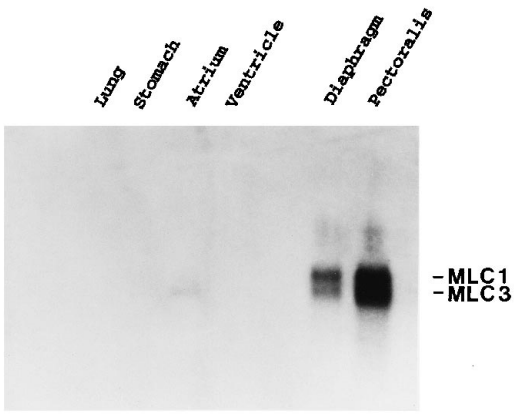

FIG. 2. *MLC3* transcripts are present in mouse atria. Northern blot containing 10 μg of total RNA per lane was hybridized with a mouse cDNA probe to *MLC3*-specific exons and common exons. The *MLC1* transcript is 1,050 bp, and the *MLC3* transcript is 900 bp.

TCC GCC ACT CGC CAT GAG G for the GATA mutant. The MEF2 mutated sequence (mutated bases are indicated by underlining) was previously shown not to bind to the MEF2 protein (1). The GATA site was mutated at bases essential for the high-affinity binding of GATA factors (2, 36, 46, 68).

In vitro transcription-translation and EMSAs. Coupled in vitro transcription-translations were carried out with 1.5 μg of a circular GATA-4 expression plasmid (gift of M. Parmacek) and T7 RNA polymerase in a rabbit reticulocyte kit (Promega) according to the manufacturer's instructions. For electrophoretic mobility shift assays (EMSAs), 2 μl of a 50-μl rabbit reticulocyte reaction mixture either with or without added plasmid was mixed with 5 μl of 4× buffer (40 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.8], 200 mM potassium glutamate, 20.0 mM $MgCl_2$, 4.0 mM EDTA, 20% glycerol) (15), 0.1 μg of sonicated salmon sperm DNA (Sigma), and cold competitor oligonucleotides in a final volume of 19 μl. Reaction mixtures were incubated at room temperature for 10 min, 1.0 μl (0.1 pmol) of the ^{32}P-end-labelled probe was added (described below), and the reaction mixtures were incubated on ice for 30 min. Samples were loaded on a 5% nondenaturing polyacrylamide gel and separated by electrophoresis in 0.5× Tris-borate-EDTA (TBE) at 200 V.

The following complementary 27-mers with overhanging ends were synthesized for EMSAs: wild-type GATA-F, TGTGAAATCCGATACTAGATATGA GGT; wild-type GATA-R, ACTGACCTCATATCTAGTATCGGATTT; mutant GATA-F, TGTGAAATCCGCCACTCGCCATGAGGT; and mutated GATA-R ACTGACCTCATGGCGAGTGGCGGATTT. The GATA motif was mutated to the same bases (indicated by underlining) as those described above for the mutant MLC3 reporter construct. Annealed wild-type GATA oligonucleotides (1.0 pmol) were end labelled with the Klenow fragment (Promega). Unlabelled annealed oligonucleotides were used as cold competitors.

Transgenic mice. MLC3CAT expression cassettes contained in a 2.2-, 2.4-, or 3.1-kb *Hin*dIII fragment were excised from pMLC3^{2H}CAT, pMLC3^{2H}CAT180, or pMLC3^{2H}CAT920, respectively (Fig. 1), and injected into the pronuclei of fertilized mouse eggs as described previously (27). Potential founders were screened for transgene integration by tail blotting with the ^{32}P-labelled CAT DNA probe (58). Founder lines were established by mating transgene-positive animals to C57BL/6 mice. F_1 progeny were checked for presence of the transgene by PCR amplification of tail genomic DNA with primers specific for the CAT gene. The copy number was determined by digestion of 10 μg of genomic DNA with *Eco*RI followed by Southern blot analysis with the CAT probe. The signal intensity for each transgenic line was compared with that of a control transgenic line which contained 40 copies (MLCCAT, line 7) (58). All transgenic lines contained less than 40 copies of their respective transgenes with the exception of line 1 which contained more than 200 copies (data not shown). CAT activity was assayed in adult heterozygotes (5 weeks or older).

Cardiac injections of plasmid DNA. Cardiac injections of plasmid DNA were performed as previously described (34). Briefly, 5 μg of test plasmid and 0.2 μg of Rous sarcoma virus CAT control plasmid were coinjected directly into the apex of the left ventricles of anesthetized adult female Wistar rats. Rats were sacrificed 5 days after injection. The apical two-thirds of the heart was homogenized in homogenization buffer (1.5 μl/mg of tissue). A CAT assay was performed chromatographically with 15 μl of the homogenate (3% of total) (20). Luciferase assays were conducted as described below with the exception that the emitted light was quantitated for 20 s with a Monolight 2010 luminometer (Analytical Luminescence Laboratory) and 15 μl of extract was used.

RNA isolation for Northern blotting and RPAs. Total RNA was isolated from C57BL/6 mice by guanidinium isothiocyanate-cesium chloride centrifugation (3). A riboprobe specific for both *MLC1* and *MLC3* transcripts was generated by cloning a 293-bp PCR product from mouse skeletal muscle cDNA containing exons 2 and 3 (specific for *MLC3*) and exons 5 and 6 (common exons) into the *Sma*I site of pBluescript KS (Stratagene) (Fig. 1). Antisense RNA probes labelled with ^{32}P were generated by in vitro transcription with T3 RNA polymerase of the *Bam*HI-digested mouse construct. RNase protection assays (RPAs) were performed with 10 μg of cesium chloride-purified RNA as described previously (74). For Northern blot analysis, 10 μg of total RNA per lane was electrophoresed on a 1.5% gel and hybridized with a ^{32}P-labelled probe to the 293-bp mouse DNA fragment generated by priming with random oligonucleotides (Amersham).

Quantitation of genomic methylation. Quantitative analysis of genomic methylation was carried out by restriction enzyme analysis coupled with ligation-mediated PCR (LMPCR) (45, 61). For promoter of enhancer analysis, 1 or 2 μg of DNA, respectively, was digested overnight with a 10-fold excess of *Afl*II (New England Biolabs) or *Pvu*II (New England Biolabs) restriction enzyme units per microgram. The DNA was ethanol precipitated and digested overnight with equal amounts of *Mae*II (Boehringer Mannheim) or *Hha*I (New England Biolabs) restriction enzymes, respectively, followed by ethanol precipitation and LMPCR.

PCR was performed with the restriction-digested genomic DNA samples as originally described (18, 50) and as modified by McGrew and Rosenthal (45). For quantitation of methylation, the labelled DNA samples were run on a 6% acrylamide sequencing gel (0.4-mm thickness). The gel was dried and exposed, and the intensities of the bands in the autoradiograph were quantitated with a scanning densitometer (Molecular Dynamics). The primers used for the enhancer analysis have been described previously (24). The primers used for the endogenous rat and transgenic rat *MLC3* promoters were R3P-1, AAA ACA GCA GCG GGA CTG; R3P-2, AAC TGA AGA CAC CTC CAG TGG GTC; and R3P-3, TCC AGT GGG TCC CTG AGT GGA GTC C.

In situ hybridizations. Transgenic mouse embryos were identified by PCR of CAT sequences from genomic DNA isolated from the amnion of each embryo. The morning of the vaginal plug formation was designated 0.5 day postcoitum (dpc). Embryo fixations and in situ hybridizations were performed as previously described (25, 59). The *MLC3* probe was prepared as previously described (42) with an oligonucleotide corresponding to the 5′ untranslated region of the mouse *MLC3* gene (56) cloned into a Bluescript vector, to eliminate cross-hybridization with other members of the *MLC* multigene family. The CAT probe was prepared as previously described (25).

Rat primary cultures and transfections. Primary myocytes were isolated from 1-day-old neonatal rat pup hind limbs as previously described (11). Myoblasts were grown in plating medium (Dulbecco modified Eagle medium [DMEM] plus 20% fetal calf serum, 2% glutamine, 12.5 U of penicillin-streptomycin per ml) for 3 days and transfected by calcium phosphate coprecipitation (22) with 10 μg of the MLC3 test construct and 2 to 3 μg of a pSV2-CAT control construct (Promega). After 24 h, the medium was changed to differentiation medium (DMEM plus 4% horse serum, 2% glutamine, 12.5 U of penicillin-streptomycin per ml). The cultures were harvested for luciferase assays after 2 days in the differentiation medium.

Luciferase and CAT assays. Twenty microliters of rat primary cell extract (100 μl) was assayed for luciferase activity for 10 s in a Berthold LK 9200 luminometer with the Promega luciferase assay system. The remaining extract (30 μl) was heat treated for 10 min at 65°C, and CAT activity was determined by the phase-exchange assay (52).

RESULTS

MLC3 expression in adult skeletal and cardiac muscle tissues. Previous studies have indicated that the *MLC1* and *MLC3* promoters are differentially regulated and that the *MLC3* promoter displays transcriptional activity without the downstream enhancer in primary muscle cell cultures (57, 60). Recently it was shown that a large intronic fragment containing the mouse *MLC3* promoter and a 260-bp downstream enhancer was sufficient to drive CAT transgene expression in skeletal and cardiac muscles (33). We therefore decided to perform a detailed comparison of endogenous *MLC1* and *MLC3* expression in adult tissues. Northern blot analysis of total RNA from adult mouse tissues revealed a transcript in the atria which comigrated with the *MLC3*-specific transcript in the diaphragm (Fig. 2). To eliminate possible artifacts arising from cross-hybridization of the probe with other myosin light chains which have high levels of sequence identity, we performed RPAs of mouse tissues with a mouse cDNA riboprobe which could distinguish *MLC1* and *MLC3* transcripts (see Materials and Methods) (Fig. 1). The *MLC3* transcript was present in the atria and at a lower level in the ventricles

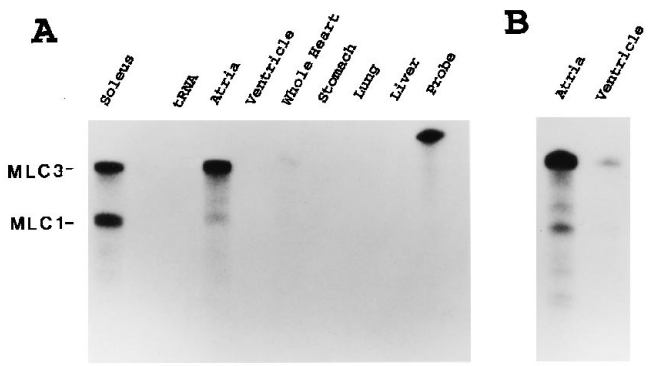

FIG. 3. *MLC3* expression pattern in various cardiac and skeletal muscle tissues. RPAs were performed with 2 μg of mouse total RNA (overnight exposure) (A) or 5 μg (2-day exposure) (B) with a mouse probe specific to *MLC1* and *MLC3* (see Materials and Methods).

(Fig. 3). The amount of transcript was equal in intensity to expression in the soleus, a low expressor of *MLC1* and *MLC3*. A faint lower band specific for common exons 5 and 6 was also visible in heart tissue and possibly represents an *MLC3* transcript which was not yet fully spliced. Additional transcripts were not detected in any other mouse tissues (data not shown). In addition, *MLC3* transcripts were also detected in the atria and ventricles of rat and canine hearts (44a). Therefore, the internal *MLC3* promoter is transcriptionally active in both mammalian skeletal and cardiac muscles.

Temporal expression of *MLC1* and *MLC3* in striated muscle. To examine the spatial and temporal expression patterns of *MLC1* and *MLC3* transcripts during embryogenesis, we assayed tissues of limb, body wall, and cardiac muscles at staged developmental times (Fig. 4). Previous studies have shown that the *MLC1* promoter is first transcribed at 9.5 dpc in the myotome, and the *MLC3* promoter is transcribed at 15.5 dpc in the axial and limb musculature (42). Accordingly, we detected the *MLC1* transcript at 12.5 dpc in trunk and limb muscles. At later stages this transcript increased linearly. In contrast to previous reports, a low level of the *MLC3* transcript was detected at 12.5 dpc. *MLC3* transcripts dramatically increased between 14.5 and 16.5 dpc, concomitant with the formation of secondary muscle fibers and polyinnervation. The increase in *MLC3* transcript accumulation at 15.5 dpc has been shown to be caused by an increase in the rate of transcription of the *MLC3* promoter (10).

The expression of the *MLC3* transcript in cardiac tissue was first assayed by RPA at 12.5 dpc and was found to be expressed in the atria at a level equivalent to *MLC1* expression in skeletal muscle (upper band versus lower band). The endogenous transcript was also detected at 11.5 dpc by in situ hybridization and was restricted to the atria (Fig. 5). The expression levels in cardiac muscle remained constant throughout embryogenesis and persisted in the adult. This comparison reveals new aspects of the differential regulation of the *MLC3* promoter in cardiac and skeletal muscles during development. While expression in the atrium is initiated at early embryonic stages and is constant, skeletal muscle-specific expression begins at very low levels, at stages earlier than previously reported, and undergoes a dramatic upregulation during the fetal stage of mouse development.

***MLC3* regulatory elements drive reporter gene expression in vivo in both skeletal and cardiac muscle lineages.** To define the *cis* regulatory elements responsible for the skeletal and cardiac muscle expression of *MLC3*, we generated transgenic mice containing a 628-bp *MLC3* promoter fragment driving a CAT reporter construct with or without a downstream MLC enhancer fragment (Fig. 1). As shown in Table 1, analysis of CAT activity in several independent lines revealed that the *MLC3* promoter alone was sufficient to drive transgene expression in cardiac muscle tissue. MLC3CAT transgene expression most resembled the endogenous pattern of expression in cardiac muscle in two transgenic lines, 479 and 533, which showed higher levels of expression in the atria than in the ventricles. Further, the level of ventricular expression in all cardiac muscle lines expressing *MLC3* (excluding line 533) was higher than expected in comparison to atrial and skeletal muscle transgene expression. In situ hybridizations on transgenic embryos from line 479 demonstrate that the *MLC3* promoter drove correct temporal expression in the atria of mice at 11.5 dpc although no signal was detected in any embryonic muscle masses (Fig. 5 and data not shown). Surprisingly, the *MLC3* promoter also proved to be sufficient to activate high levels of skeletal muscle transgene expression in some adult muscle groups without the downstream *MLC* enhancer. This transgene expression did not correlate well with endogenous *MLC3* expression. The level of the endogenous *MLC3* gene is expressed highest in muscles that contain a high percentage of fast-type 2B fibers, such as the extensor digitalis longus (EDL), and lowest in the diaphragm and the soleus, which predominately do not express fast-type 2B muscle isoforms (64, 65). The majority of transgenic MLC3CAT lines expressed the CAT gene in most adult skeletal muscles groups assayed, although different muscle groups displayed various levels of CAT activity in each line. These differences can presumably be attributed to position effects.

Additional transgenic lines containing either a 180- or 920-bp MLC downstream enhancer element, MLC3CAT180 and MLC3CAT920, demonstrated an increased range and level of transgene expression in skeletal muscle. This is exemplified by the expression of CAT transcript in the EDL, the highest expressor of endogenous *MLC3* transcript, and by the increase in CAT expression levels in the intercostal muscles relative to the levels in other muscle groups (Table 1, lines 552

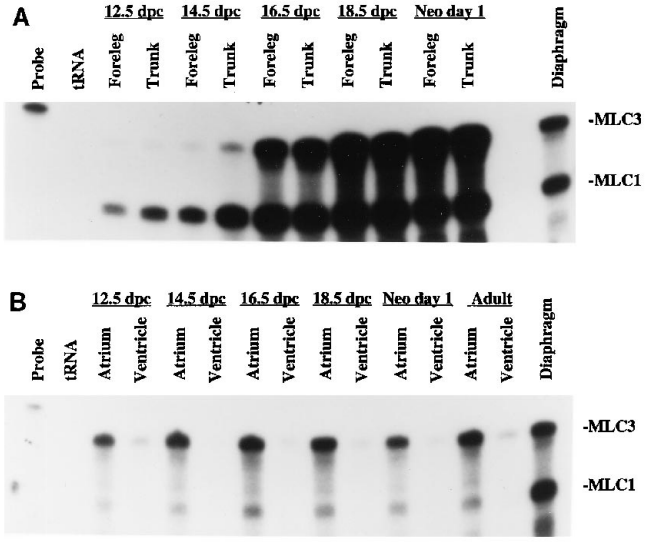

FIG. 4. Development expression of *MLC3* transcripts in the striated musculature. RPAs were performed with 5 μg of mouse total RNA (2 μg from an adult diaphragm), isolated at the indicated times, with a probe specific to *MLC1* and *MLC3*. (A) Skeletal muscle tissue; (B) cardiac muscle tissue. Neo, neonatal.

FIG. 5. Distribution of *MLC3* endogenous and *MLC3CAT* transgene in embryonic hearts at 11.5 dpc. Dark-field micrographs of *MLC3* (a) and CAT (b) transcripts in parallel parasagittal sections from a transgenic animal at 11.5 dpc (transgenic line 479). Endogenous expression is predominately localized over the atria while CAT expression is detectable in the ventricle. (c and d) Bright-field micrographs of panels a and b, respectively. RBC, red blood cells; A, atrium; V, ventricle.

and 193). Unexpectedly, most lines containing the *MLC* enhancer element no longer expressed the CAT transgene in cardiac muscle tissue. This may be due to the close juxtaposition of the promoter and enhancer elements in the concatenated transgenes. In summary, the expression patterns obtained in transgenic mice demonstrate that regulatory elements sufficient to drive transgene expression in embryonic and adult cardiac muscles are present in the proximal *MLC3* promoter. This regulatory region is also able to activate expression in most adult skeletal muscle groups, although the complete skeletal muscle phenotype is not generated in the absence of the downstream distal enhancer.

Previous work has demonstrated that the *MLC1* promoter and 920-bp enhancer driving a CAT reporter construct generated a gradient of expression in the intercostal muscles. Recently, in a transgenic mouse the murine *MLC3* promoter and a 260-bp enhancer fragment driving a *lacZ* transgene were reported to not be expressed in a gradient in the intercostal muscles (33). In contrast to these results, several MLC3CAT transgenic lines containing the promoter alone (lines 479 and 459) or with the downstream enhancer (lines 690, 194, and 256) displayed a significant gradient of expression in the intercostal muscles (4- to 40-fold). This suggests that redundant *cis* elements present in the *MLC3* promoter and *MLC* enhancer regulatory regions are responsible for the gradient of transgene expression.

Differential methylation of regulatory regions in the *MLC1/3* locus. Previous studies of the endogenous *MLC1/3* locus have implicated DNA methylation in the control of gene expression during embryogenesis and in different adult muscle groups. Examination of the *MLC1/3* locus in the chicken showed that the late activation of transcription of the *MLC3* promoter corresponded with the timing of demethylation of promoter CpG dinucleotides (37). The endogenous rat *MLC1* promoter and enhancer also displayed muscle-specific demethylation (45, and this paper). Both elements were highly methylated in cardiac and nonmuscle tissues. In addition, transgenic mice carrying the *MLC1* promoter driving a CAT reporter gene with the downstream *MLC* enhancer exhibited muscle-specific expression which closely correlated with demethylation levels of both promoter and enhancer elements (14, 24). We investigated the methylation status of a CpG dinucleotide located at −175 bp in the rat *MLC3* promoter (Fig. 1), using a quantitative PCR-based technique (45, 61). This method combines the use of methylation-sensitive restriction enzymes with LMPCR to generate two amplified DNA products whose ratio correlates with the level of methylation at the site being assayed. We found that unlike the *MLC1* promoter and *MLC* enhancer which are demethylated in a muscle-specific pattern, the CpG sequence analyzed in the endogenous *MLC3* promoter was demethylated ubiquitously. We extended this analysis to examine the methylation pattern in transgenic mice containing the *MLC3* promoter and the 920-bp downstream *MLC* enhancer element driving a CAT reporter construct. As seen in Fig. 6, the transgenic *MLC3* promoter was ubiquitously demethylated, while the *MLC* enhancer was demethylated solely in muscle tissue. This study demonstrates that methylation pat-

TABLE 1. Expression of MLC3 constructs in transgenic mouse lines

Construct and cell line	% of CAT activity in tissue from[a]:															
	Liver	Lung	Spleen	Atria	Ventricle	Masseter	Dig	Dia	Tricep	Pec	Thigh	Soleus	EDL	RIC	MIC	CIC
MLC3CAT																
459	−	−	−	+++	+++	−	−	−	+++	+++	±	−	−	−	±	+
533	−	−	−	+++	−	−	+++	−	−	−	+	+	−	−	−	−
479	−	−	−	++	+	−	−	±	+	++	+++	−	+	±	±	+
MLC3CAT 180-bp enhancer																
552	−	−	−	++	++	+++	ND	+++	+++	+	+++	++	+++	+++	+++	+++
599	−	−	−	−	−	+	−	−	+++	+++	+++	±	++	+++	+++	+++
690	−	−	−	−	−	−	−	−	++	++	+++	±	++	+	+	++
MLC3CAT 920-bp enhancer																
1	−	−	−	++	++	+++	ND	±	+	−	++	−	+++	+	−	±
193	−	−	−	−	−	−	−	−	+++	+++	+++	±	+++	+	+	++
194	−	−	−	−	−	−	−	−	++	++	++	±	++	++	+++	+++

[a] Relative CAT activity, normalized to the protein concentration, was determined in the indicated tissues for transgenic mice from each transgenic line (highest level in tissue set to 100). A minimum of two animals were assayed for each line, and the expression pattern is shown for a single representative animal. Expression levels varied highly between cell lines containing the same construct (100-fold). Lines 479, 552, and 193 were the highest expressors for their constructs and had comparable copy numbers, and levels of CAT activity (25 μg of total protein assayed for 1 h gave 25% conversion to the acetylated form). Dig, Digastricus; Dia, Diaphragm; Pec, pectoralis; EDL, extensor digitalis longus; RIC, MIC, and CIC, rostral (1 to 3), mid (5 to 6), and caudal (9 to 10) muscles of intercostal muscles 1 to 12, respectively. CAT activity: 0 to 3% (−), 4 to 9% (±), 10 to 20% (+), 21 to 50% (++), 51 to 100% (+++). ND, not determined.

terns in vivo are established independently on the multiple regulatory regions of this locus.

In vivo analysis of cardiac muscle-specific elements in the *MLC3* promoter. To further extend the transgenic analysis of MLC3 regulation, and to delineate the *cis* elements responsible for cardiac muscle-specific expression, we assayed activity of the *MLC3* promoter driving a luciferase reporter gene when directly injected into adult rat ventricles. Direct DNA injection results in specific transfection into myocytes and has been shown to be useful for the analysis of cardiac regulatory regions in vivo (7, 34, 35). Luciferase reporter constructs are commonly used for cardiac injections because of the increased sensitivity of the luciferase assay. Figure 7A shows the expression pattern obtained for MLC3Luc constructs in cardiac tissue. Comparison of MLC3Luc reporter constructs MLC628Luc and MLC628Luc920 indicated no increase in expression with the presence of the downstream enhancer. These data support our results obtained with transgenic lines indicating that cardiac regulatory elements are located in the 628-bp proximal promoter and not in the *MLC* enhancer. Deletions of the promoter alone to −434 and −257 bp did not cause a substantial loss of activity. This demonstrates that three E boxes present in this 371-bp upstream region are not necessary for cardiac activity. A further deletion to −147 bp led to a dramatic loss of activity (20-fold), indicating the presence of a positive cardiac element in this region. An MEF2 consensus site located at −155 bp was deleted in this construct. To determine whether the MEF2 site was functional, we mutated the consensus sequence in the context of the 628-bp *MLC3* promoter fragment. Additionally, a repeated binding motif for another known cardiac transcription factor, GATA-4, located at −60 bp, was also mutated. Mutation of either the MEF2 site or the GATA site caused a dramatic reduction (10-fold) in activity compared with that of the intact promoter. These results demonstrate that the proximal 257 bp of the *MLC3* promoter are sufficient for cardiac muscle-specific activity and that the MEF2 and GATA sites present in the proximal promoter region are both singularly necessary for its cardiac muscle-specific expression.

Analysis of skeletal muscle-specific elements of the *MLC3* promoter. To compare the function of *MLC3* regulatory elements in skeletal versus cardiac muscles, we analyzed the expression profile of MLC3Luc constructs in transient transfections of primary skeletal muscle cultures. Rat primary cultures were chosen because, unlike established muscle cell lines, they contain regulatory factors able to activate the *MLC3* promoter without the presence of the downstream enhancer (57, 60). As seen in Fig. 7B, a construct containing the *MLC3* promoter alone was 25% as active as a construct containing both the promoter and *MLC* enhancer. Deletions of the *MLC3* promoter alone to −257 bp did not decrease its activity, as was also seen for cardiac injections. These data indicate that the three upstream E boxes in the *MLC3* promoter are not necessary for expression in skeletal or cardiac muscle. However, an additional deletion to −147 bp resulted in a loss in activity (twofold). This deletion removes the single MEF2 site in the *MLC3* promoter. Mutating the MEF2 site in the context of the entire 628-bp promoter also resulted in a twofold loss of activity. These results suggest that MEF2 is more important for *MLC3* promoter function in cardiac muscle than in skeletal muscle. In addition, the construct containing a mutation of the GATA site in the context of the full-length promoter also showed a significant loss of activity (10-fold). This result was unprecedented since GATA-binding factors have not previously been shown to be involved in skeletal muscle-specific gene expression. In summary, both MEF2 and GATA sites located in the proximal *MLC3* promoter are required for activity in both striated muscle lineages.

Tandem GATA binding sites in the proximal *MLC3* promoter bind with high affinity to mouse GATA-4 protein. The repeated GATA sequence at −60 bp in the *MLC3* promoter does not strictly conform to the consensus GATA protein-binding site, A/T GATA A/G. However, GATA protein family members have been shown to bind specifically to sequences divergent from the consensus sequence (36, 46). To determine if the *MLC3* promoter element was a specific binding site for GATA proteins, EMSAs were carried out with an oligonucleotide containing the double GATA site as a probe and in vitro-transcribed-translated mouse GATA-4 protein. As seen in Fig. 8, a shifted complex was present in the lane containing GATA-4 protein. Competition with 200- to 400-fold excess of cold wild-type GATA oligonucleotide resulted in the loss of this complex, while competition with cold mutated oligonucleotide did not. Therefore, the GATA element present in the

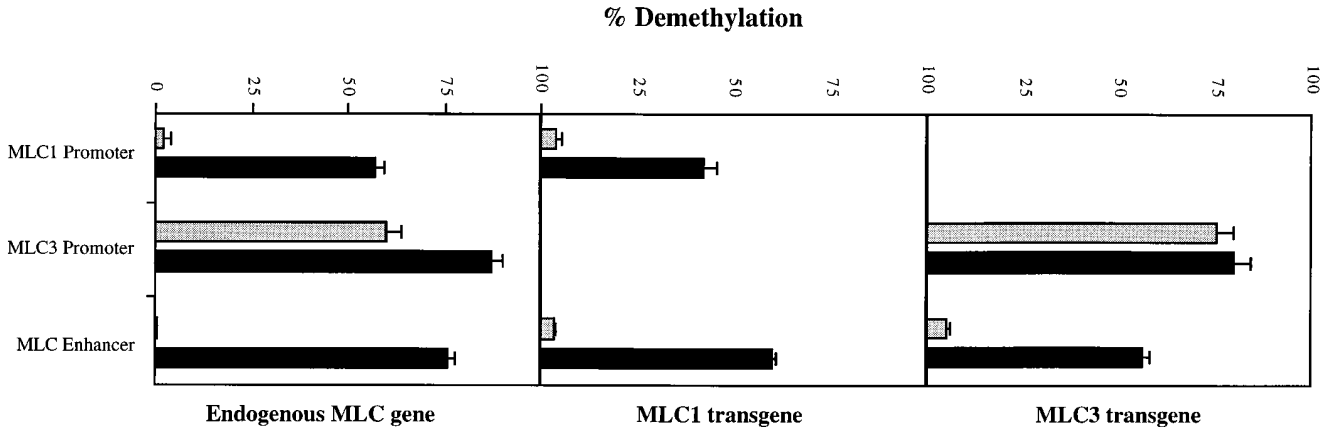

FIG. 6. Methylation of regulatory elements in *MLC* locus. Demethylation levels were determined at CpG sites in the *MLC1* promoter, the *MLC3* promoter, and the downstream *MLC* enhancer of the endogenous rat locus (41) or of transgenic mice carrying the MLC1CAT920 transgene (23) or the MLC3CAT920 transgene (transgenic lines 1, 193, and 228). Genomic DNA was isolated from adult tissues (rat liver and extensor digitalis longus; mouse liver and thigh). The percent demethylation was calculated from photodensitometric scans of labelled LMPCR products analyzed by gel electrophoresis and autoradiography. The values shown are the averages from three to six independent experiments with a minimum of two animals; error bars indicate standard errors. ▨, nonmuscle; ■, muscle.

MLC3 promoter is not only functionally important for transcriptional activity in striated muscles but comprises a sequence-specific binding site for GATA proteins.

DISCUSSION

This report documents the expression of fast skeletal muscle *MLC3* in cardiac muscle tissue and delineates regulatory regions in the *MLC1/3* locus responsible for this expression in the proximal promoter region. *MLC3* expression in cardiac tissue is activated early and is constant throughout development whereas skeletal muscle *MLC3* expression is present in early embryos at very low levels and undergoes a dramatic increase at 14.5 dpc. The present study extends previous analyses of *MLC* regulation to show that reporter gene expression both in transgenic animals and direct cardiac injections reveals that the downstream *MLC* enhancer is not involved in cardiac transcription and may actually silence cardiac expression when placed close to the promoter element. However, high levels of *MLC3* expression in skeletal muscle in vivo appear to rely on the enhancer element. Mutational analysis of the *MLC3* proximal promoter region has identified two sequence motifs, MEF2 and GATA, which are important for expression in both skeletal and cardiac muscles. The GATA element is further shown to bind in a sequence-specific manner to GATA-4 protein. Finally, the *MLC3* promoter is ubiquitously demethylated. This is in contrast to the upstream *MLC1* promoter or the downstream enhancer which exhibits tissue-specific demethylation. These results emphasize the differential regulation of the internal *MLC3* promoter.

Expression of MLC3 in the mouse atrium is unexpected, considering that the alkali myosin light chain, MLC1A, is highly expressed in this cardiac domain. It is conceivable that before the expansion of the alkali myosin light-chain gene family, an *MLC3*-like gene was expressed to a greater extent in cardiac tissue. Barton and Buckingham (5) argued that the antecedent *MLC* would most resemble *MLC3* on comparison of intron and exon structures and the sequence of the locus in different species. This is supported by the similarity of *MLC3* and the single *Drosophila* alkali MLC, *MLC-alk*, which possesses a similar exon/intron structure, contains a separate first exon encoding the initiator methionine codon (62), and is expressed throughout the fly striated musculature (16, 17). Examination of *MLC-alk* upstream promoter regions reveals the presence of an MEF2 and a repeated GATA motif (17), indicating these regulatory elements may be evolutionarily conserved. Future work will determine if other vertebrate organisms retain cardiac expression of the *MLC3* gene or if this potentially vestigial expression pattern is peculiar to mammals.

Transcriptional activation of the *MLC3* promoter in striated muscle tissues appears to depend on MEF2 and GATA sites in the proximal promoter region. By contrast, it appears that three upstream E boxes are not required for promoter activity. It is possible that the MEF2 and GATA sites coordinate the activity of separate upstream regulatory regions which drive transcription in cardiac or skeletal muscle tissues. For example, Iannello et al. (28) observed that the activity of the cardiac troponin T gene in skeletal and cardiac muscles depended on two separate promoter regions which acted through common proximal elements. Notably, the three MLC3CAT transgenes analyzed here are expressed exclusively in a subset of skeletal muscle fibers that express the endogenous gene (IIX or IIA [52a]), indicating that other regulatory regions may contribute to the full spectrum of endogenous *MLC3* expression. For example, it is likely that other intronic regions in the *MLC* locus may be involved in expression from the *MLC3* promoter. A single transgenic line that contains an additional 1.4 kb of *MLC3* promoter sequence and the entire second intron expressed in all skeletal muscle groups suggest the existence of other positive regulatory regions of the *MLC3* promoter in the mouse locus (33). Moreover, since the transgene analyzed in that study also included promoter regions containing the conserved MEF2 and GATA motifs, it is likely that these elements will be key components in driving the striated muscle expression from the *MLC3* promoter. Altogether, it appears that the internal *MLC3* promoter is sufficient for transcriptional activity in most striated muscle tissues, although this expression may be modulated by additional regulatory regions.

Mutational analysis of the MEF2 site in the *MLC3* promoter indicates that this motif is necessary for MLC3 expression in cardiac muscle and to some extent in skeletal muscle. MEF2 sites have been shown to be involved in the cardiac and skeletal muscle expression of several genes (21, 48, 51). In a tissue culture model of hypertrophy, the HF-1 site in the *MLC2* promoter, which contains an MEF2 binding site, has been shown to be important for inducible expression during hyper-

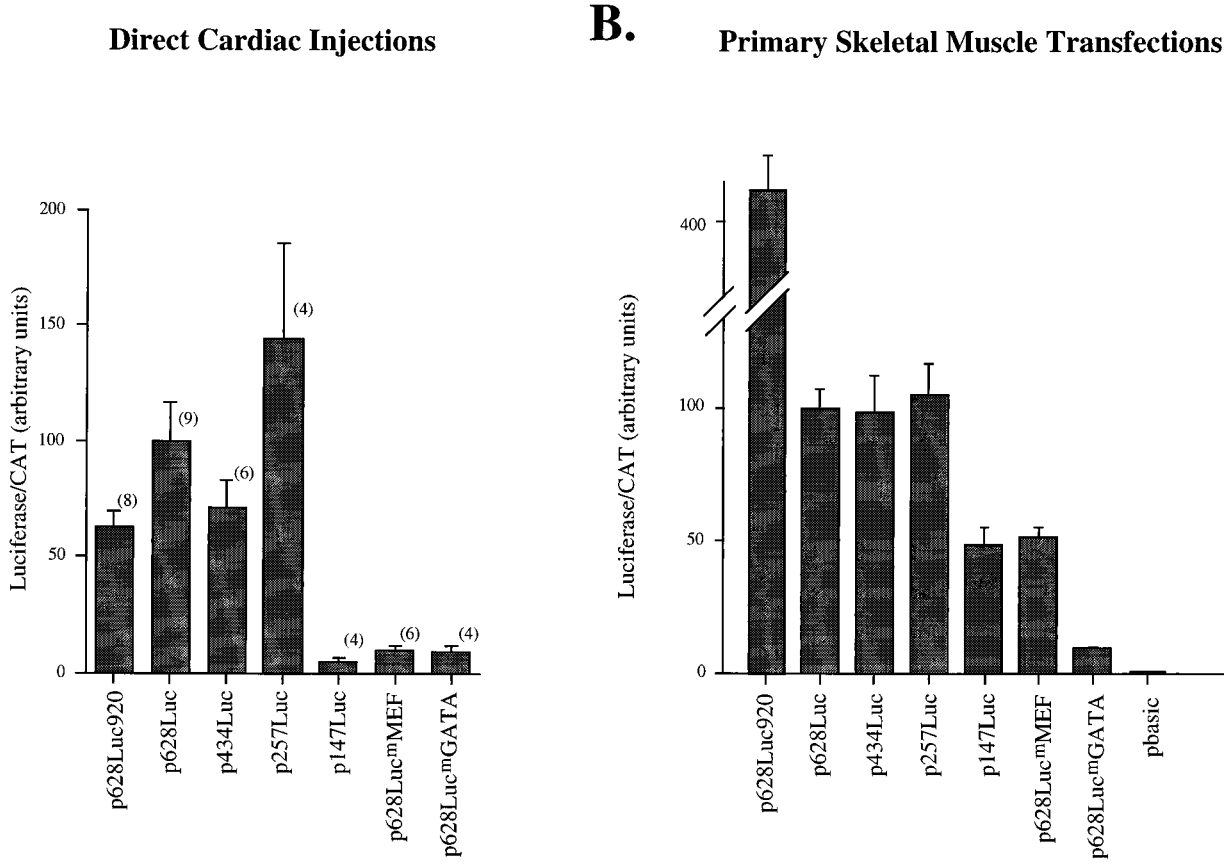

FIG. 7. MLC3 activity in cardiac and skeletal muscles. (A) Luciferase activity in MLCLuc constructs was assayed in injected rat hearts (see Materials and Methods). Injected plasmid DNA consisted of the 628-bp *MLC3* promoter driving a luciferase reporter gene (p628Luc) or 5′ deletions of that construct (p434Luc, p257Luc, and p147Luc). The MEF2 site or GATA site was mutated separately in the construct p628Luc, p628Luc^{m}MEF, and p628Luc^{m}GATA, respectively. The 920-bp rat *MLC* enhancer was cloned downstream of the luciferase gene (p628Luc920). Injections were normalized to CAT activity and shown relative to that of the p628Luc construct (set to 100). The numbers in parentheses indicate the number of animals assayed. (B) Neonatal rat primary skeletal muscle cultures were transfected with the plasmids described for panel A. Transfections were normalized to CAT activity and shown relative to that of the p628Luc construct (set to 100). Each transfection is a minimum of three separate transfections. Error bars indicate standard errors.

trophy (73). Furthermore, the correct spatial and temporal expression of the myogenin promoter in transgenic mice was shown to depend on the presence of an MEF2 site (9, 70). The role of this transcription factor in regulating expression of striated muscle-specific structural proteins in vivo is still unclear. The protein isoforms MEF2A and MEF2C are known to be expressed in both of these tissues and to activate gene expression from multimerized binding sites in cell culture (32). Future experiments will determine if a specific member of this family interacts with the *MLC3* promoter in both skeletal and cardiac muscles.

This report also defines a GATA element that is necessary for *MLC3* skeletal and cardiac muscle-specific expression and binds GATA-4 protein with high affinity. GATA sites are also necessary for expression of the α-MHC and *BNP* promoters in cardiac tissue, and these genes are transactivated by the transcription factor, GATA-4 (23, 29, 48). The *MLC3* gene joins a number of other genes such as *MLC1A, BNP, MLC2,* and α-*MHC* whose domain of expression includes the atria (6, 23, 39, 41). Comparison of the *MLC3* promoter region with the regulatory regions of these genes indicates that they also contain a repeated GATA motif separated by 3 to 7 bp. Since GATA proteins bind their consensus sites with equal affinity (52b), it is likely that the subset of GATA factors abundant in cardiac tissue (38) and in other embryonic tissues (19, 26, 69) are all capable of binding with similar affinity to the GATA motif in the *MLC3* promoter (36, 46).

This report is also the first documentation of the involvement of a GATA motif in skeletal muscle-specific expression. GATA-2 and GATA-3 are expressed in many tissues outside of the erythrocyte lineage (19, 69) and may be involved in muscle gene expression. Although no systematic analysis of GATA factor expression in skeletal muscle has been reported, both GATA-2 and -3 transcripts are present in both skeletal muscle tissues and cell lines (44). It remains to be determined if a novel muscle-specific factor acts through this site or if one of the known GATA transcription factors is responsible for *MLC3* activity.

In contrast to mechanisms activating gene transcription through cell-specific *trans*-acting factors, epigenetic modifications have been shown to play a role in establishing accessibility to and spatial expression of tissue-specific genes (reviewed in reference 8). In particular, skeletal muscle genes undergo a tissue-specific demethylation of regulatory regions (71). The CpGs analyzed in the *MLC1* promoter and the *MLC* enhancer follow this pattern and undergo a tissue-specific demethyla-

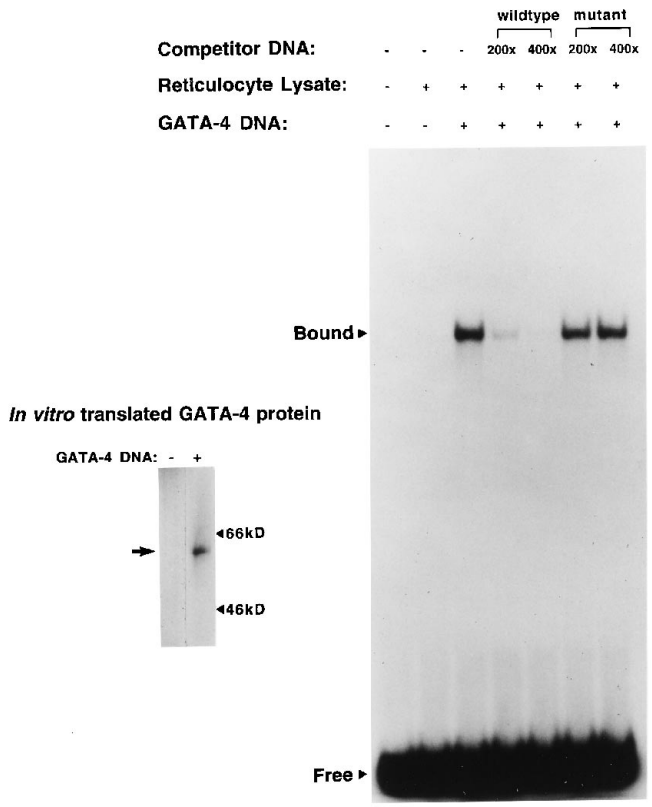

FIG. 8. GATA-4 protein binds specifically to the GATA site present in the proximal *MLC3* promoter. (Left panel) In vitro-transcribed-translated products from a rabbit reticulocyte reaction mixture containing [^{35}S]methionine and a GATA-4 expression plasmid were separated on a sodium dodecyl sulfate–12% polyacrylamide gel. Molecular mass markers (in kilodaltons [kD]) are indicated on the right. (Right panel) EMSA of the radiolabelled GATA site from the *MLC3* promoter with in vitro-transcribed-translated GATA-4 protein products. Binding assays contained rabbit reticulocyte programmed with a GATA-4 expression plasmid as indicated. For competition assays, 2 (200×) or 4 (400×) pmol of cold competitor oligonucleotides was used (see Materials and Methods). The positions of bound and free oligonucleotides are indicated.

tion. However, the CpG analyzed in the proximal *MLC3* promoter is demethylated in all tissues. The lack of tissue-specific demethylation in the *MLC3* promoter may be a component of differential regulation of MLC3 expression in both skeletal and cardiac muscle. In addition, the activation of the upstream *MLC1* promoter precedes the *MLC3* promoter during embryonic development. This results in transcripts from the *MLC1* promoter extending through the downstream *MLC3* promoter region before the latter is transcriptionally active. It remains to be determined if the chromatin structure of the *MLC3* regulatory region assumes an active conformation at these early embryonic stages which may preclude the regulatory control of the *MLC3* promoter by methylation. In conclusion, these results provide evidence for independent differential epigenetic regulation of the two *MLC* fast muscle isoforms, which may underscore their specific roles in muscle function.

In summary, analysis of the *MLC3* promoter in vitro and in vivo has identified common *cis* elements driving skeletal and cardiac muscle-specific expression. Current experiments are focused on defining the transcription factors interacting with these elements in both striated muscle lineages. These findings will help in the further identification of the members of regulatory gene families involved in the expression of genes in subdomains of skeletal and cardiac tissues.

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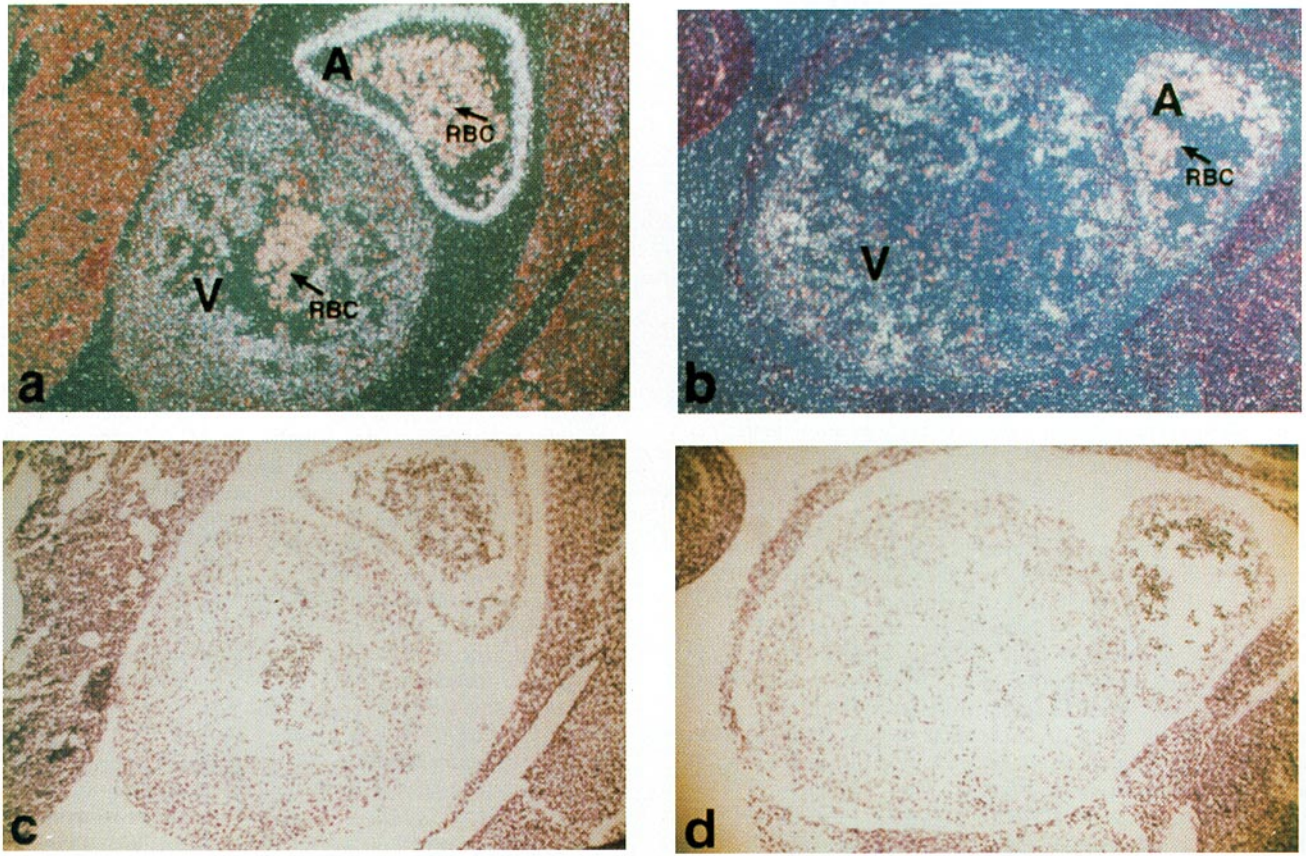
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