Myosin Light Chain 3F Regulatory Sequences Confer Regionalized Cardiac and Skeletal Muscle Expression in Transgenic Mice

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Abstract. The myosin light chain 1F/3F locus contains two independent promoters, MLC1F and MLC3F, which are differentially activated during skeletal muscle development. Transcription at this locus is regulated by a 3′ skeletal muscle enhancer element, which directs correct temporal and tissue-specific expression from the MLC1F promoter in transgenic mice. To investigate the role of this enhancer in regulation of the MLC3F promoter in vivo, we have analyzed reporter gene expression in transgenic mice containing *lacZ* under transcriptional control of the mouse MLC3F promoter and 3′ enhancer element. Our results show that these regulatory elements direct strong expression of *lacZ* in skeletal muscle; the transgene, however, is activated 4–5 d before the endogenous MLC3F promoter, at the time of initiation of MLC1F transcription. In adult mice, transgene activity is downregulated in muscles that have reduced contributions of type IIB fibers (soleus and diaphragm). The rostrocaudal positional gradient of transgene expression documented for MLC1F transgenic mice (Donoghue, M., J. P. Merlie, N. Rosenthal, and J. R. Sanes. 1991. *Proc. Natl. Acad. Sci. USA.* 88:5847–5851) is not seen in MLC3F transgenic mice. Although MLC3F was previously thought to be restricted to skeletal striated muscle, the MLC3F-*lacZ* transgene is expressed in cardiac muscle from 7.5 d of development in a spatially restricted manner in the atria and left ventricular compartments, suggesting that transcriptional differences exist between cardiomyocytes in left and right compartments of the heart. We show here that transgene-directed expression of the MLC3F promoter reflects low level expression of endogenous MLC3F transcripts in the mouse heart.

DURING striated muscle development, a dynamic and complex pattern of structural gene expression generates the diversity of muscle types found in the adult vertebrate. Regulation of the majority of sarcomeric genes is under transcriptional control mediated by several families of regulatory proteins, including those of the MyoD and MEF2 transcription factors (Weintraub, 1993; Yu et al., 1992). Through interactions with *cis*-acting regulatory elements, these factors ensure the expression of specific genes whose products are required in particular subsets of cardiac and skeletal musculature. A large number of muscle-specific promoter and enhancer elements have been identified as important in tissue culture (see Rosenthal, 1989); dissection, however, of the complex spatial and temporal control of muscle-specific gene expression in vivo, which is not recapitulated in vitro, requires direct analysis of putative regulatory regions in transgenic mice. Transgenic studies have demonstrated the complexity of *cis*-acting elements controlling the expression of particular muscle-specific genes in different striated muscle types, for example, the separation of cardiac and skeletal muscle regulatory elements in mice containing upstream sequences from the desmin (Li et al., 1993) and MLC2V (Lee et al., 1992) genes. Similarly, transgenic studies have shown that adult skeletal muscle fiber-type diversity is mediated by distinct *cis*-acting elements that are required for slow (Banerjee-Basu and Buonanno, 1993) or fast (Donoghue et al., 1991*b*; Hallauer et al., 1993) fiber-type expression.

The diversity of muscle subtypes is illustrated by the alkali myosin light chain (MLC)[1] family composed of three loci encoding four isoforms expressed in a developmentally regulated pattern in different striated muscle types (Barton and Buckingham, 1985). Alkali MLCs comprise two subunits of hexameric muscle myosin, and they are implicated in the velocity of muscle shortening (Lowey et al., 1993). The MLC 1F/3F locus encodes the two alkali MLC isoforms found in fast skeletal muscle: MLC1F and 3F differ at the amino terminal because of the use of two promoters and a differential splicing event such that exons 1 and 4 are MLC1F specific, and exons 2 and 3 are MLC3F specific (Fig. 1 *a* in this manu-

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1. *Abbreviations used in this paper*: β-gal, β-galactosidase; CAT, chloramphenicol acetyltransferase; MHC, mysoin heavy chain; MLC, myosin light chain; p.c., postcoitum; RT-PCR, reverse transcription PCR.

script; Nabeshima et al., 1984; Periasamy et al., 1984; Robert et al., 1984). Evidence from cell culture experiments has shown that the regulation of these two promoter elements (which are separated by a 10-kb intron) is largely independent (Strehler et al., 1985; Garfinkel and Davidson, 1987; Daubas et al., 1988; Brennan et al., 1990; Seidel and Arnold, 1989; Rosenthal et al., 1990; Pajak et al., 1991). During myogenesis in vivo, the MLC1F isoform is expressed before the MLC3F isoform both in birds and mammals (Hoh, 1979; Gauthier et al., 1982), such that MLC1F and 3F transcripts are first detected at 9.5 and 13.5 d postcoitum (p.c.) respectively in the mouse (Lyons et al., 1990*a*); this differential regulation has been demonstrated to occur at the level of transcription (Cox and Buckingham, 1992). The late onset of MLC3F expression in vivo may be correlated with a dependence on innervation (Merrifield and Konigsberg, 1987; Barton et al., 1989). Furthermore, while MLC1F and 3F isoforms are expressed in fast muscle fibers (type II), the fast fiber-type distribution of the two isoforms is distinct, such that MLC3F is preferentially associated with glycolytic type IIB fibers (Wada and Pette, 1993; Bottinelli et al., 1994). The MLC1F/3F locus, therefore, provides a model for the dissection of regulatory elements controlling differential gene expression during myogenesis.

An enhancer sequence located 3′ to the MLC1F/3F locus confers strong muscle-specific expression on the MLC1F promoter in muscle cells in culture and in transgenic mice, where a chloramphenicol acetyltransferase (CAT) reporter gene is activated up to 1,000-fold over background levels exclusively in skeletal muscle (Donoghue et al., 1988; Rosenthal et al., 1989). MLC1F-CAT transgenic mice containing the 3′ enhancer (1F-CAT-E) initiate transgene expression at the time of myotome formation, when the endogenous MLC1F gene is first expressed (Grieshammer et al., 1992), and CAT expression in adult muscle is specific to fast fibers (Donoghue et al., 1991*b*). Functional binding site motifs for transcription factors of the MyoD and MEF2 families, in addition to a homeobox protein target site, are conserved in both rat and human 3′ enhancer sequences (Wentworth et al., 1991; Rosenthal et al., 1990). Unexpectedly, 1F-CAT-E transgenic mice show a striking rostrocaudal positional gradient of CAT expression, such that caudal muscles (e.g., extensor digitorum longus) express the transgene at levels 100-fold higher than rostral muscles (e.g., masseter, Donoghue et al., 1991*a*). This graded expression is cell autonomous, stably inherited, and correlates with transgene methylation in particular muscles along the anterioposterior axis (Donoghue et al., 1992*a*, *b*). The sequences responsible for the gradient appear to lie within the MLC regulatory elements included in the transgene, although there is no gradient of endogenous MLC1F expression. It remains unclear (*a*) how the differential regulation of the MLC1F and 3F promoters is controlled in the presence of a single enhancer element, and (*b*) whether the *cis*-acting sequences responsible for the caudal-rostral gradient lie in the MLC1F promoter or enhancer element. The experiments described in this paper were designed to address these questions.

We report an analysis of expression from the late activated MLC3F promoter in vivo using transgenic mice containing the mouse 3F promoter upstream of a *lacZ* gene (with a nuclear localization signal), and the mouse 3′ enhancer downstream of the reporter gene (construct 3F-*nlacZ*-E). Our results demonstrate that skeletal muscle-specific expression of *nlacZ* is precocious with respect to the endogenous MLC3F transcript; transgene expression, however, is downregulated postnatally in skeletal muscles with reduced numbers of type IIB fibers in the adult (soleus and diaphragm). Unlike 1F-CAT-E transgenic mice, no positional gradient of rostrocaudal expression is observed at the level of *nlacZ* transcript or product. We report the unexpected finding that *nlacZ* is expressed in a restricted subset of cardiac myocytes from 7.5 d of development, and we demonstrate that there is low level transcriptional activation of the endogenous MLC3F promoter in the heart. This last observation reveals a further level of complexity in transcriptional regulation at the MLC1F/3F locus, and it leads to a reassessment of the overlap in expression patterns between skeletal and cardiac striated muscle–specific genes.

Materials and Methods

Transgene Construction

Plasmid pH4 contains the MLC3F promoter and 3F-specific exons plus MLC1F-specific exon 4 on a 1.9kb HindIII fragment derived from λ phage C isolated from the mouse MLC1F/3F locus (Robert et al., 1984; see Fig. 1 *a* in this manuscript). The HindIII insert of pH4 was subcloned into pBS (Stratagene, La Jolla, CA) and modified by extension of MLC1F/3F sequences at the 5′ and 3′ ends and the introduction of a BglII site, designed for the dual purposes of transgenics and as a homologous recombination replacement vector. (*a*) The BglII site was introduced at the eighth MLC3F codon in exon 2 by site-directed mutagenesis in pBS (Stratagene) to generate pLCM6 (using oligonucleotide 1, see list at end of Materials and Methods). pLCM6 was extended by 620 bp at the 3′ end by the addition of a PCR product generated from BALB/c mouse DNA using oligonucleotide primers 2 and 3; amplification conditions were 94°C 1 min, 57°C 1 min, and 72°C 3 min for 25 cycles (Taq DNA polymerase; Perkin-Elmer Cetus, Norwalk, CT). The purified PCR product was digested with BamHI, subcloned into pBS, verified by DNA sequencing at 5′ and 3′ termini, and subsequently isolated on a BamHI/BspMI fragment for ligation with a 260-bp pLCM6 XhoI/BspMI fragment into pLCM6 digested with XhoI/BamHI to generate pLCM7. (*b*) To extend promoter sequences to −2.0 kb, a 1.8-kb PCR amplified fragment (using primers 4 and 5, conditions as above) was ligated onto the 5′ end of pLCM7. This product contained the expected restriction sites and correct terminal DNA sequence, and it was subcloned into pBluescript (Stratagene) on a PstI/EcoRI fragment, and was subsequently excised as a PstI/ScaI partial fragment for ligation with the 2.2-kb ScaI/BamHI insert of pLCM7 into PstI/BamHI prepared pBS to generate pLCM8. (*c*) *nlacZ*-SV-40 poly(A) (including a nuclear localization signal, Kalderon et al., 1984; details to be published elsewhere) was introduced into the unique BglII site of pLCM8 as a 3.1-kb NcoI-BamHI fragment that was annealed to a double-stranded oligonucleotide (annealed oligos 6 and 7) designed to recreate the NcoI site containing the *nlacZ* ATG in frame with MLC3F sequences and to leave a BglII overhang, generating p3F-*nlacZ*. The resulting 3F-*nlacZ* junction was verified by DNA sequencing. (*d*) A 260-bp mouse MLC1F/3F 3′ enhancer fragment containing the core 173-bp sequence from the rat enhancer (Donoghue et al., 1988; Wentworth et al., 1991), and corresponding to nucleotides 361–620 of the rat enhancer (Donoghue et al., 1988), was amplified as a PCR product using primers 8 and 9, cloned into pBluescript as a BamHI fragment, and verified by DNA sequencing. The enhancer was subcloned on a BamHI fragment into the unique 3′ BamHI site of p3F-*nlacZ*, in the orientation in which the enhancer is found in the MLC1F/3F locus, to generate p3F-*nlacZ*-E.

Muscle Cell Transfections

C2/7 skeletal muscle cells were grown under standard culture conditions in DME with 20% fetal calf serum for proliferation, or 2% fetal calf serum to induce differentiation. Cells were transfected with 10 μg of reporter construct and 1 μg of RSV-luciferase vector per 6-cm dish, using the calcium-phosphate method (see Biben et al., 1994). Cell extracts and luciferase assays were performed as in Biben et al. (1994). β-galactosidase assays were

performed as described in Sambrook et al. (1989); values were normalized with respect to luciferase activities to account for variability in transfection efficiency.

Generation of Transgenic Mice

The insert of p3F-*nlacZ*-E was excised as a 7-kb SphI/KpnI fragment, and was purified by gel electrophoresis and passage through an Elutip column (Schleicher & Schuell, Dassel, Germany). Transgenic mice were generated by microinjection of purified 3F-*nlacZ*-E insert into fertilized (C57BL/6J × SJL) F_2 eggs at a concentration of 700 copies per picoliter using standard techniques (Hogan et al., 1986). Injected eggs were reimplanted into pseudopregnant (C57BL/6J × CBA) F_1 foster mothers.

Identification of Transgenic Mice

DNA was prepared from mouse tails (Laird et al., 1991), and was analyzed by Southern blot or PCR. For Southern blot analysis 15 μg of DNA was digested with restriction endonucleases in the presence of 100 μg/ml BSA, subjected to electrophoresis, and transferred onto Hybond N^+ membranes (Amersham Corp., Arlington Heights, IL) for hybridization in 0.5 M $NaPO_4$ (pH 7.6), 7% SDS plus 100 μg/ml salmon sperm DNA at 65°C overnight; filters were washed in 0.1× SSC, 0.01% SDS at 65°C. Hybridization probes were either from the *lacZ* gene (1kb NcoI/ClaI fragment) or, to determine copy number, 5′ MLC3F fragments (either 1-kb XbaI/BglII or 450-bp EcoRI/BglII pLCM6 fragments). For PCR analysis, one primer in the proximal MLC3F promoter (10) and one within *lacZ* (11) were used, generating a 1,060-bp product. Amplification conditions were 95°C 1 min, 62°C 1 min, and 72°C 3 min, for 25 cycles. Since the 3F-*nlacZ*-E transgene is strongly expressed in adult skeletal muscle, transgenic mice were also identified by in toto X-gal staining (see below) of skinned sections of mouse tails.

Analysis of Transgene Expression

Heterozygous and homozygous transgenic males were crossed with nontransgenic females ([C57BL/6J × SJL] F_1 or CD1). Embryos were dated taking 0.5 d p.c. as the day of the vaginal plug, and they were dissected in 1× PBS, fixed in 4% paraformaldehyde (for 30 min to overnight depending on the size of the embryos), rinsed in 1× PBS, and colored in X-gal solution (X-gal, U. S. Biochemical Corp., Cleveland, OH; Sanes et al., 1986) at 32°C for periods of 30 min to overnight. Transgenic embryos and individual adult muscles were analyzed by whole-mount microscopy or cryostat sections; for the latter, samples were perfused with 15% sucrose, followed by 7% gelatin/15% sucrose for 2 h to overnight and frozen in liquid nitrogen before cryostat sectioning at −20°C. Sections were stained for β-gal activity, counterstained for 5 min in 1% eosin, dehydrated, and mounted in Cytoseal mounting medium (Stephens Scientific, Riverdale, NJ). Antibodies were applied to cryostat sections as described by Tajbakhsh et al. (1994). Slow myosin heavy chain antibody (D5) was supplied by S. Schiaffino (University of Padua, Italy).

In Situ Hybridization

RNA–RNA hybridization was performed as described by Sassoon and Rosenthal (1993); two ^{35}S-labeled nucleotides (UTP and CTP) were incorporated into the riboprobes. Exposure times were from 5–14 d. The MLC3F probe was as described by Lyons et al. (1990*a*). A probe within the first intron of the mouse MLC1F/3F locus was synthesized from a pBluescript clone containing a 7-kb BamHI/HindIII subfragment of the intron, derived from λ phage C (Robert et al., 1984). This plasmid was cut with XbaI and a 500-nt riboprobe complementary to a region ~2 kb upstream of the MLC3F transcriptional start site synthesized using T3 RNA polymerase, and it was hydrolyzed for 20 min before hybridization. A *lacZ* antisense probe was generated by T3 RNA polymerase from ClaI-digested *nlacZ*-SV40 poly(A) in pSK (Stratagene). The RNA product was 2 kb long, and it was hydrolyzed for 45 min before hybridization.

Reverse Transcription Polymerase Chain Reaction

Total RNA from adult tissue was extracted with guanidium thiocyanate followed by centrifugation on a caesium chloride cushion (Sambrook et al., 1989). 1 μg of total RNA was mixed with 50 ng $p(dN)_6$ random primers (Pharmacia, Uppsala, Sweden). The mixture was heated for 10 min at 70°C, chilled on ice, and adjusted to 1x first-strand buffer (Gibco BRL, Gaithersburg, MD), 10 mM DTT, and 0.5 mM dNTPs. RNasin (1 μl [10 U]: Amersham) and reverse transcriptase (1 μl [200 U] SuperScript $RNaseH^-$; Gibco BRL) were added (final volume = 20 μl). Tubes were incubated for 1 h at 42°C, 5 min at 95°C, spun, and chilled on ice. Four different polymerase chain reactions were performed per cDNA synthesis. 5 μl of cDNA solution were amplified in 1× Taq buffer (Amersham) with 50 pmol of each primer, 100 μM dNTPs, and 2 U Taq polymerase (Amersham). Different numbers (15, 20, and 25) of cycles of amplification were carried out to estimate the level of the RNA of interest. Cycle times were 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by 60°C for 2 min and 72°C for 5 min. Oligonucleotide pairs used were: 12 and 13 for MLC3F (163-bp product), 14 and 15 for MLC1F (190-bp product), 16 and 17 for α-cardiac actin (115-bp product), 18 and 19 for MRF4 (mouse gene, 270-bp product), and 20 and 21 for S16 ribosomal protein (102-bp product). Samples were separated on 5% TBE nondenaturing polyacrylamide gels that were subsequently electroblotted onto Hybond N^+ (Amersham) for 1 h at 15 V using a trans-blot apparatus (Bio Rad Laboratories, Hercules, CA). The filter was washed for 10 min in 0.4 M NaOH, rinsed in 2× SSPE and heated for 1 h at 80°C. Hybridization was carried out at 65°C as above, followed by washes in 0.1× SSC, 0.1% SDS at 55°C. Hybridization probes were MLC1F/3F exon 1 for MLC1F products, MLC1F/3F common exon 5 for MLC3F products, and a PCR-amplified fragment from the mouse MRF4 locus (gift of M. Primig, Pasteur Institute, Paris, France) for MRF4 products.

Oligonucleotides

1 5′GTCACTTACCGGAGATCTGGTCAGC3′
2 5′CCATGGATCCACCTGCATAACTGAAGCCCTTGG3′
3 5′AAGCTTGGATCCACATCGTGTGATGACTTCACC3′
4 5′ACGAATTCTGCAGGTACCTTTCCTCTCTGGATTAGCTATAG3′
5 5′ACCCCGGGAATTCAGTACTTTTATGGTCTTTATCTCTG3′
6 5′CCCTCGAGAGATCTT3′
7 5′CATGAAGATCTCTCGAGGG3′
8 5′CCGCGGATCCATCGATAACTTCAGCACACTG3′
9 5′GGATGGATCCGGTACCGTGACCAAGGTAACTTG3′
10 5′CAGGAGGAGTGGCAACTGCCCTGTGAAATC3′
11 5′GGGGGATGTGCTGCAAGGCG3′
12 5′GCTGTTTCGCAGCCTAGAACTC3′
13 5′TCTGCATTGGTGGGATTGGTGC3′
14 5′CAGGTTCTTCTGGAGGAGATCC3′
15 5′CTCCTGTTGCTCCTTAGAGAACTCG3′
16 5′AGTGCCTGCCACCAGCGCCAGCCCA3′
17 5′ACACCAAAGCGGTGGTCTCCTCGTC3′
18 5′GTTTCGGATCATTCCAGGGGCCTC3′
19 5′ATCTCTCGCCTTTCATAAAAGCTGGCA3′
20 5′AGGAGCGATTTGCTGGTGTGGA3′
21 5′GCTACCAGGCCTTTGAGATGGA3′

Results

To examine the in vivo activity of the MLC3F promoter and 3′ enhancer, we generated transgenic mice containing a *nlacZ* reporter gene under the control of a −2kb MLC3F promoter and 3′ enhancer sequence (3F-*nlacZ*-E, Fig. 1). The construct contains 2 kb 5′ and 3′ of the MLC3F transcriptional start site, thus encompassing both MLC3F-specific exons (Fig. 1 *b*). An *Escherichia coli lacZ* gene containing a nuclear localization signal was cloned in frame within the second MLC3F-specific exon such that a fusion protein including the first eight MLC3F amino acids is produced from a transcript containing the entire MLC3F 5′ UTR. The correct MLC3F splicing pattern is maintained (as determined by reverse transcription PCR [RT-PCR], data not shown). *nlacZ* was selected as a reporter gene for sensitivity of both whole-mount and histological assays, while the inclusion of a nuclear localization signal distinguishes specific from nonspecific signals. The 3′ enhancer element is a 260-bp mouse sequence, containing the conserved E-box and MEF2 motifs demonstrated to be necessary for function of the 173-bp minimal rat enhancer fragment (Wentworth et al., 1991); the mouse and rat enhancer DNA sequences are

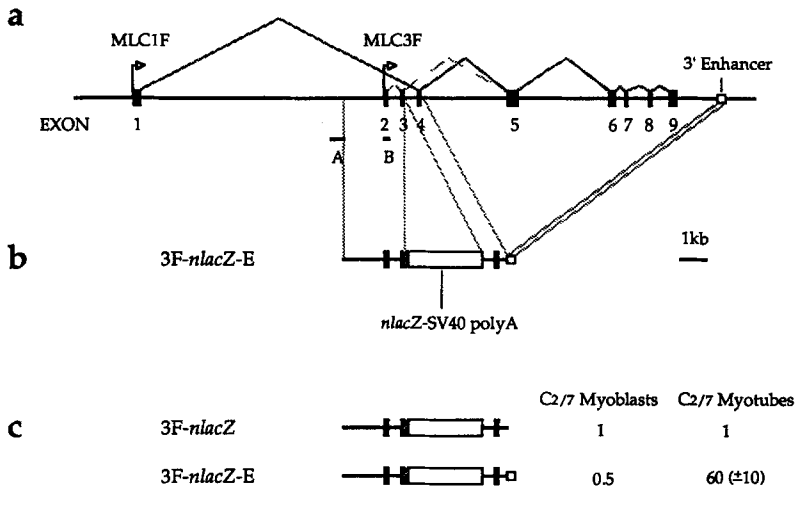

Figure 1. Structure of the mouse MLC1F/3F locus and 3F-*nlacZ*-E transgene. (*a*) Diagrammatic representation of the mouse MLC1F/3F locus showing the distinct 1F and 3F promoters that generate transcripts with differential 5′ splicing patterns (Periasamy et al., 1984; Nabeshima et al., 1984; Robert et al., 1984). The mouse 3′ enhancer element is positioned 1.8 kb downstream of the polyadenylation site. The location of the MLC1F intronic (*A*) and MLC3F 5′ UTR (*B*) in situ hybridization probes is indicated. The 3F-*nlacZ*-E transgene comprises 2 kb of MLC3F sequence on either side of the site of initiation of MLC3F transcription (*b*). The *nlacZ* reporter gene is placed in frame in the second MLC3F-specific exon (exon 3), and a 260-bp 3′ enhancer sequence is included at the 3′ end of the transgene (see Materials and Methods). MLC3F constructs with and without the 3′ enhancer were assayed for *nlacZ* expression by transient transfection into C2/7 cells (*c*). Cells were harvested at myoblast or myotube stages; in myotubes, the relative activity of the construct with the enhancer is 60-fold higher than that containing no enhancer (average of five experiments).

92% identical over this core region (data not shown). In C2/7 mouse muscle cells, this 260-bp enhancer sequence confers an ~60-fold increase of *nlacZ* expression from the −2 kb MLC3F promoter, specifically in differentiated myotubes (Fig. 1 *c*), and is, therefore, an important regulatory element for high level transcription from the MLC3F promoter in tissue culture. Two independent lines of transgenic mice containing the 3F-*nlacZ*-E construct were obtained, each containing 5–10 copies of the transgene. Both lines display strong β-galactosidase (β-gal) activity in skeletal muscle such that in adult mice, *nlacZ* expression is apparent in the majority of skeletal muscles. Adjacent nonmuscle tissues (including blood vessels, connective tissue, and nerve) are negative for transgene expression (Figs. 2 and 3).

MLC3F-nlacZ-E Expression in Developing Skeletal Muscle

In the developing mouse embryo, the first skeletal muscle cells appear in the myotomal compartment of the somites, which differentiates in a rostral to caudal gradient from ~8.5 d p.c. (see Buckingham, 1992). 3F-*nlacZ*-E transgenic mice express *nlacZ* in the myotome from 9 d p.c., positive cells first appearing in rostral somites at about the 18-somite stage, initially scattered throughout the myotome. The last 7–11 formed somites do not express the transgene. Fig. 2 *a* shows a 24-somite mouse embryo (9.5 d p.c.) with transgene expression in the myotome of the 13 most rostral somites (see also Fig. 2 *d*). Note that at this stage, the more rostral somites express the transgene at a higher level than more caudal somites, consistent with a rostrocaudal gradient of somite maturation. Endogenous MLC3F transcripts are not detected at this stage, and they first appear at 13.5 d; MLC1F transcripts, in contrast, are detectable from 9.5 d by in situ hybridization (Lyons et al., 1990*a*). The 3F-*nlacZ*-E transgene is, therefore, activated 4 d earlier than the endogenous MLC3F promoter in the myotome.

Transgene expression in the developing limb musculature of 3F-*nlacZ*-E mice is also early with respect to endogenous MLC3F transcripts; at 10.5 d p.c., β-gal–positive cells are observed in the forelimb bud, and separation into distinct premuscle masses is seen by day 11.5 p.c., when three dorsal and two ventral groups of positive cells are observed (Fig. 2 *b*). In the hindlimb, significant numbers of β-gal–positive cells accumulate from 11.5 d (Fig. 2 *b*), and distinct premuscle masses are detectable from 12.5 d p.c. β-gal–positive cells are detected in developing muscle masses surrounding the eye from 11.5 d p.c. In addition to expression in skeletal muscle, β-gal–positive cells are also observed in the heart throughout development, and transiently in the brain and developing ear (from 9 to ~15 d p.c.). Expression at these sites is observed in both 3F-*nlacZ*-E lines. Unexpectedly, endogenous MLC3F transcripts are expressed in the heart (see later); very low levels of endogenous transcript were detected by RT-PCR in otic vesicle (after 35 cycles, data not shown), but not in brain RNA, at 9.5 d p.c. Transgene expression in the developing ear becomes restricted to the dorsal extremity of the endolymphatic duct at 12.5 d. At later stages of development, the transgene is clearly expressed in the majority of, if not all, skeletal muscle masses throughout the embryo (Fig. 2 *c*).

Postnatal Downregulation of Transgene Expression in Diaphragm and Soleus Muscles

After birth and during adult life, the 3F-*nlacZ*-E transgene is expressed at high levels in the majority of skeletal muscles. Expression of the transgene, however, is downregulated in two muscles during postnatal development, such that in adult mice, extremely low levels of *nlacZ* expression are observed in the soleus and diaphragm. These differences reflect the particular fiber-type distribution of these muscles; both muscles are adapted for fatigue resistance in adult mice, and they lack a significant number of myosin heavy chain (MHC) type IIB fast fibers.

Crural (lower leg) muscles of 3F-*nlacZ*-E transgenic mice express *nlacZ* equivalently at fetal stages (Fig. 3 *a*); however, in adult mice, significantly reduced transgene expression is observed in the deep crural muscles, especially in the soleus (Fig. 3 *b*). This decrease in β-gal activity reflects decreased transgene transcription in these muscles as determined by in situ hybridization using an antisense *lacZ* riboprobe (Fig. 3 *e*). This pattern of expression appears to reflect the abundance of fibers containing type I (Fig. 3, *c* and *d*) and type

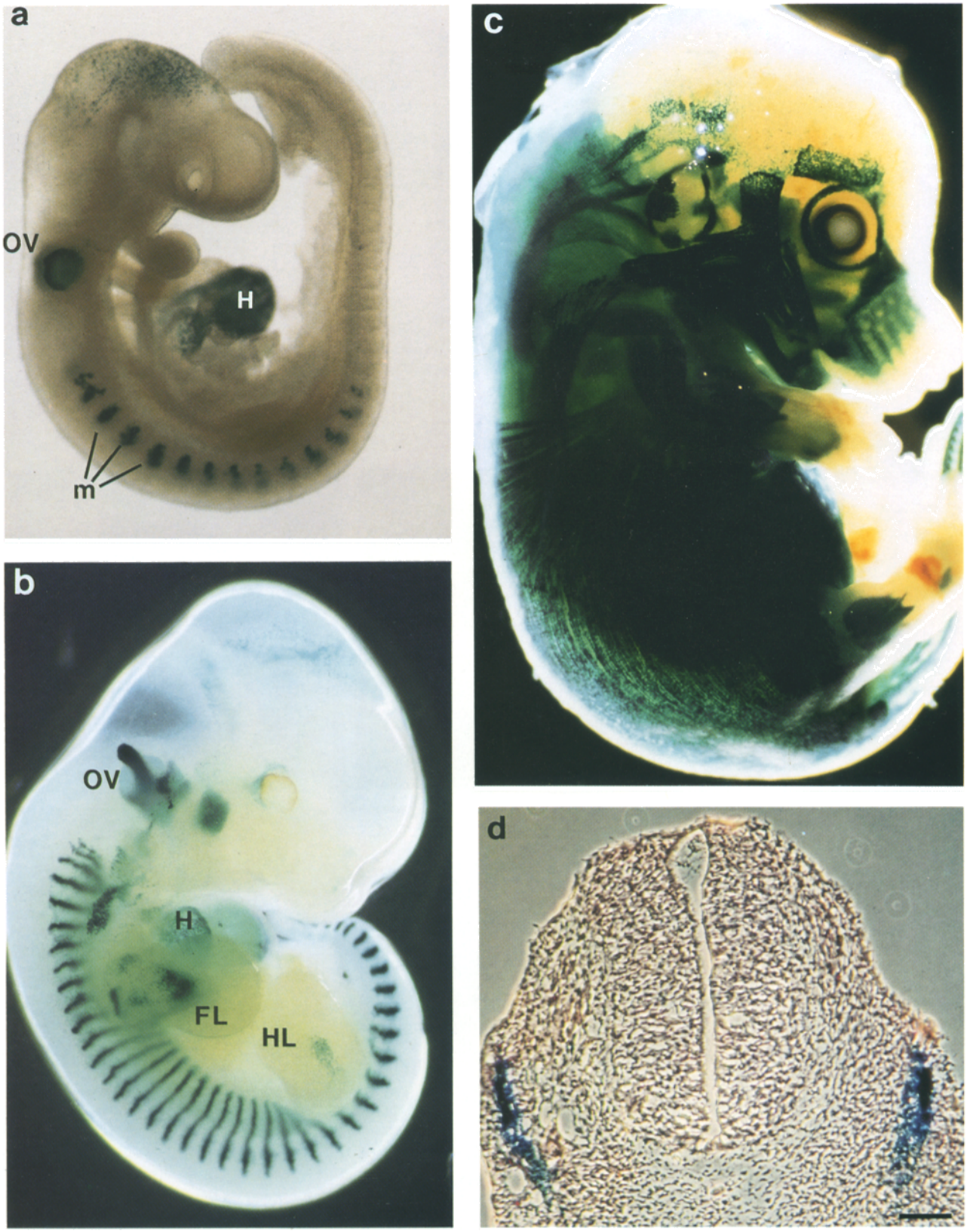

Figure 2. Transgenic embryos at different stages of development illustrating expression of the 3F-*nlacZ*-E transgene in embryonic and fetal skeletal muscles. (*a–c*) Whole-mount embryos stained with X-gal; (*a*) A 24 somite 9.5-d p.c. transgenic embryo (line *2*) where the transgene is active in the 13 most anterior somites. In addition to expression in the myotome (*M*), the *nlacZ* reporter gene is active at this stage in the heart (*H*), the otic vesicle (*OV*), and the brain. (*b*) An 11.5-d p.c. transgenic embryo (line *1*), showing expression in developing myotomal muscles and the premuscle masses of the limb buds; *FL*, forelimb, *HL*, hindlimb. (*c*) A 14.5-d p.c. embryo (line *2*) showing widespread expression in skeletal muscles. A transverse section through a 10.5-d transgenic embryo shows β-galactosidase activity in the myotomal compartment of the somite (*d*). Bar, 100 μm.

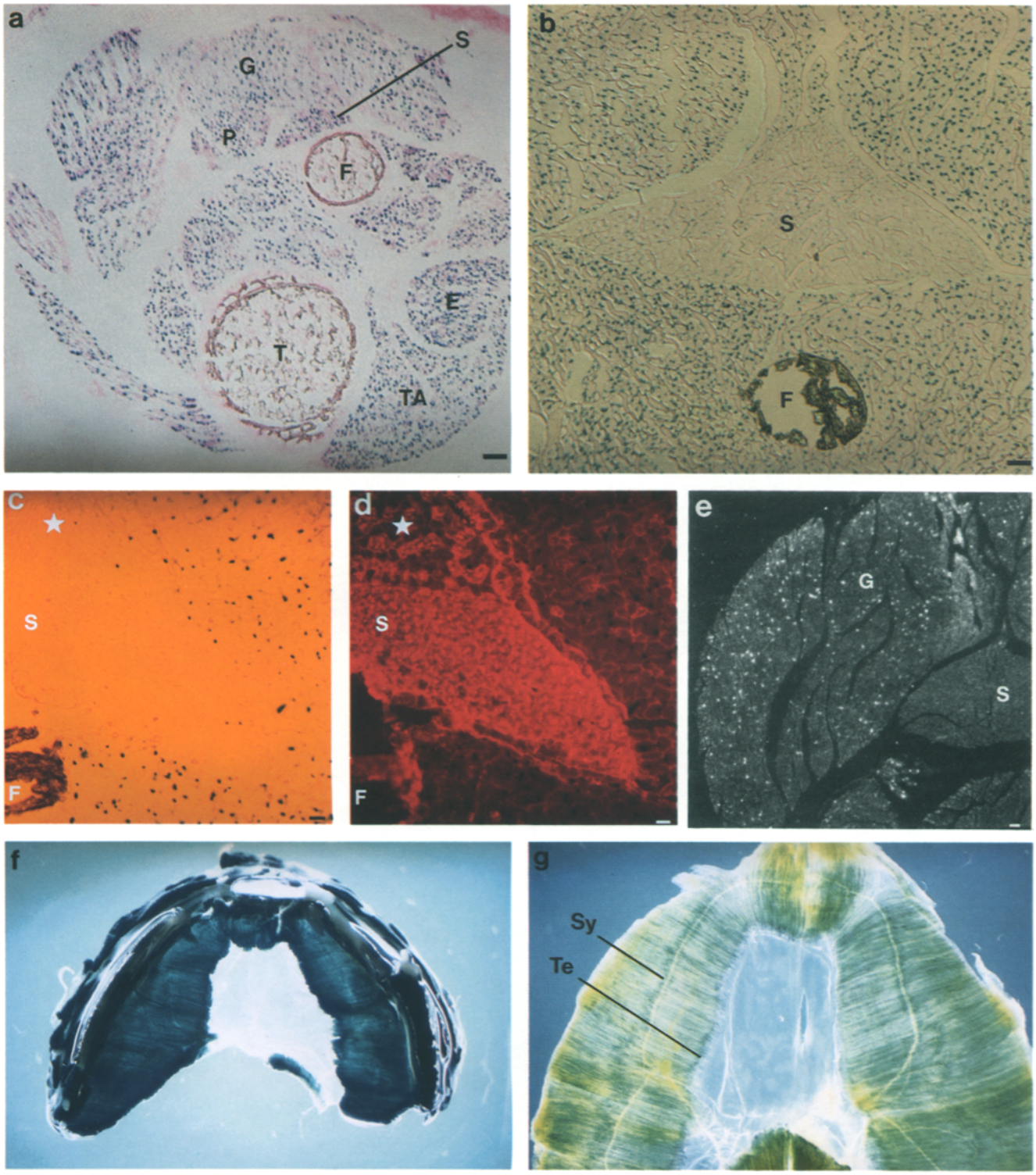

Figure 3. 3F-*nlacZ*-E expression in crural and diaphragm muscles. (*a*) At 17.5 d p.c., β-galactosidase activity is equivalent throughout the crural muscles of a 3F-*nlacZ*-E transgenic embryo, as seen in a cryostat section stained with X-gal. *nlacZ* activity is downregulated in the soleus muscle of an adult (4 mo old) transgenic mouse (*b*). Reduced time of X-gal coloration reveals that transgene expression is also low in the region of the lateral gastrocnemius adjacent to the plantaris muscle (*c*, *asterisk*); both this region and the soleus muscle are rich in type I immunopositive fibers, as seen in an adjacent section reacted with MHC I antibody (*d*). The section in *c* was photographed using an orange filter. The low expression in adult soleus muscle is at the level of transgene transcription; in situ hybridization with an antisense *lacZ* riboprobe shows reduced activity in soleus compared to gastrocnemius muscle (*e*). The punctate signal reflects the perinuclear subcellular localization of the *nlacZ* transcript, and it is comparable to in situ hybridization localization of the CAT transcript in 1F-CAT-E transgenic mice (Sassoon and Rosenthal, 1993). In the diaphragm of a 2-d postnatal mouse, transgene expression remains high (*f*); after 3 wk, transgene expression levels in the diaphragm decrease (*g*), remaining highest in nuclei at the center of the fibers (*Sy*, potential synaptic nuclei) and at the myotendinous junction (*Te*). *G*, gastrocnemius; *S*, soleus; *E*, extensor digitorum longus; *TA*, tibialis anterior; *F*, fibula; *T*, tibia. Bars, 100 μm.

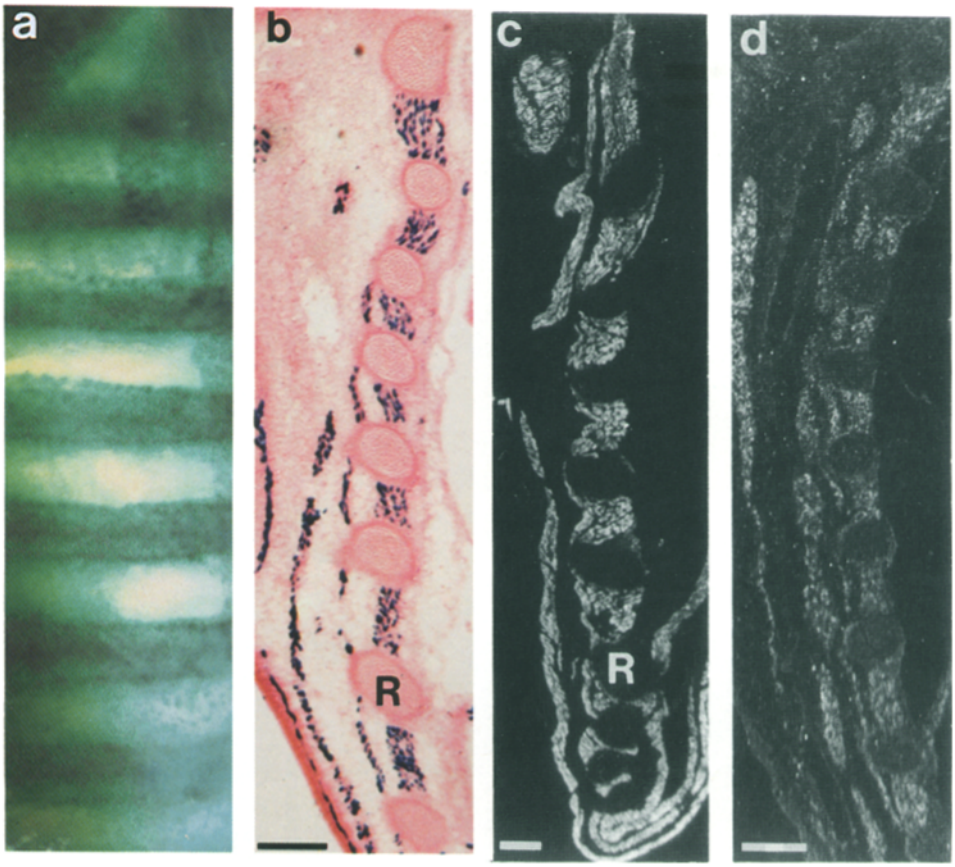

Figure 4. Expression of the 3F-*nlacZ*-E transgene is not graded along the rostrocaudal axis. The thoracic region of a 15.5-d p.c. transgenic embryo (line *2*) was dissected and stained for β-galactosidase activity (*a*); a whole-mount photograph shows a uniform level of β-gal activity in different intercostal muscles (*arrowheads*). Similar results were obtained at 13.5 d p.c. by X-gal coloration of 10-μm cryostat sections (*b*). The level of β-gal message was shown to be uniform in different intercostal muscles of a 16.5-d p.c. embryo by in situ hybridization with an antisense *lacZ* riboprobe (*c*). In situ hybridization with an antisense MLC3F riboprobe (Fig. 1 *a*, probe B) confirmed that the endogenous transcript is not positionally graded (*d*); *R*, rib. Bars, 250 μm. Top is anterior in *a–d*.

IIA MHC in this muscle (Lewis et al., 1982; Donoghue et al., 1991*b*; Wigston and English, 1992). Levels of β-gal activity are also reduced in the plantaris-adjacent region of the lateral gastrocnemius muscle, which in the mouse is also particularly rich in type I fibers (Fig. 3 *c*, *asterisk*; Hallauer et al., 1993). In the diaphragm, transgene expression is strong throughout the muscular component in utero, and it decreases between 2 and 3 wk after birth (Fig. 3, *f* and *g*). At this time, it has been shown that the proportion of MHC I-expressing fibers is increasing in the rat diaphragm (LaFramboise et al., 1991); the adult mouse diaphragm contains a mixture of fibers containing type I, IIA, and IIX(D) MHC, and it is devoid of fibers containing type IIB MHC (Zardini and Parry, 1994). Interestingly, *nlacZ* expression in the diaphragm remains highest in a specific subset of nuclei localized at synaptic and myotendinous junctions (Fig. 3 *g*), comparable with endogenous acetylcholine receptor subunit expression (Hall and Sanes, 1993; Piette et al., 1993). These results support the finding that the *nlacZ* gene is predominantly active in type II fast fibers (with IIB fibers expressing the transgene at a higher level than IIA fibers), and they are comparable with the observations of Donoghue et al. (1991*b*) in 1F-CAT-E transgenic mice. The 3F-*nlacZ*-E construct, therefore, includes the *cis*-acting DNA sequences which restrict β-gal expression to fast muscle fibers in vivo.

Transgene Expression Is Not Graded along the Rostrocaudal Axis

Transgenic mice containing 1.2 kb of rat MLC1F promoter sequences upstream of the CAT reporter gene, plus a 900-bp rat 3′ enhancer fragment downstream of the CAT gene, display a rostrocaudal gradient of reporter gene expression which is distinct from the gradient of somite maturation (Donoghue et al., 1991*a*). To determine whether the regulatory sequences from the MLC1F/3F locus included in the 3F-*nlacZ*-E construct confer a gradient of reporter gene expression, β-gal activity was analyzed in skeletal muscles along the body axis of 3F-*nlacZ*-E mice.

Apart from the rostrocaudal gradient of somite maturation, no positional gradient of transgene expression was observed either during in utero or postnatal development; the absence of a gradient of β-gal activity is demonstrated in the intercostal muscles at 15.5 d p.c. by whole-mount X-gal staining (Fig. 4 *a*), and at 13.5 d p.c. by X-gal staining of cryostat sections (Fig. 4 *b*). At these time points, the MLC1F-CAT transgene clearly displays a positional gradient (Grieshammer et al., 1992). To avoid saturation, β-gal detection was carried out under limiting conditions (reduced time of coloration). Since β-gal activity may not directly reflect *nlacZ* transcription (Gundersen et al., 1993), we confirmed by in situ hybridization that the reporter gene transcript is expressed at a uniform level in intercostal muscles at 16.5 d p.c. (Fig. 4 *c*). Furthermore, we confirmed that the endogenous MLC3F transcript, like the MLC1F transcript (Grieshammer et al., 1992), is expressed uniformly along the rostrocaudal axis (Fig. 4 *d*). These results demonstrate that the mouse MLC3F promoter and core 3′ enhancer sequence do not confer a positional gradient of reporter gene expression in transgenic mice.

Transgene-directed and Endogenous MLC3F Expression in Cardiac Muscle

The MLC3F protein is exclusive to skeletal striated musculature (Barton and Buckingham, 1985). It was, therefore,

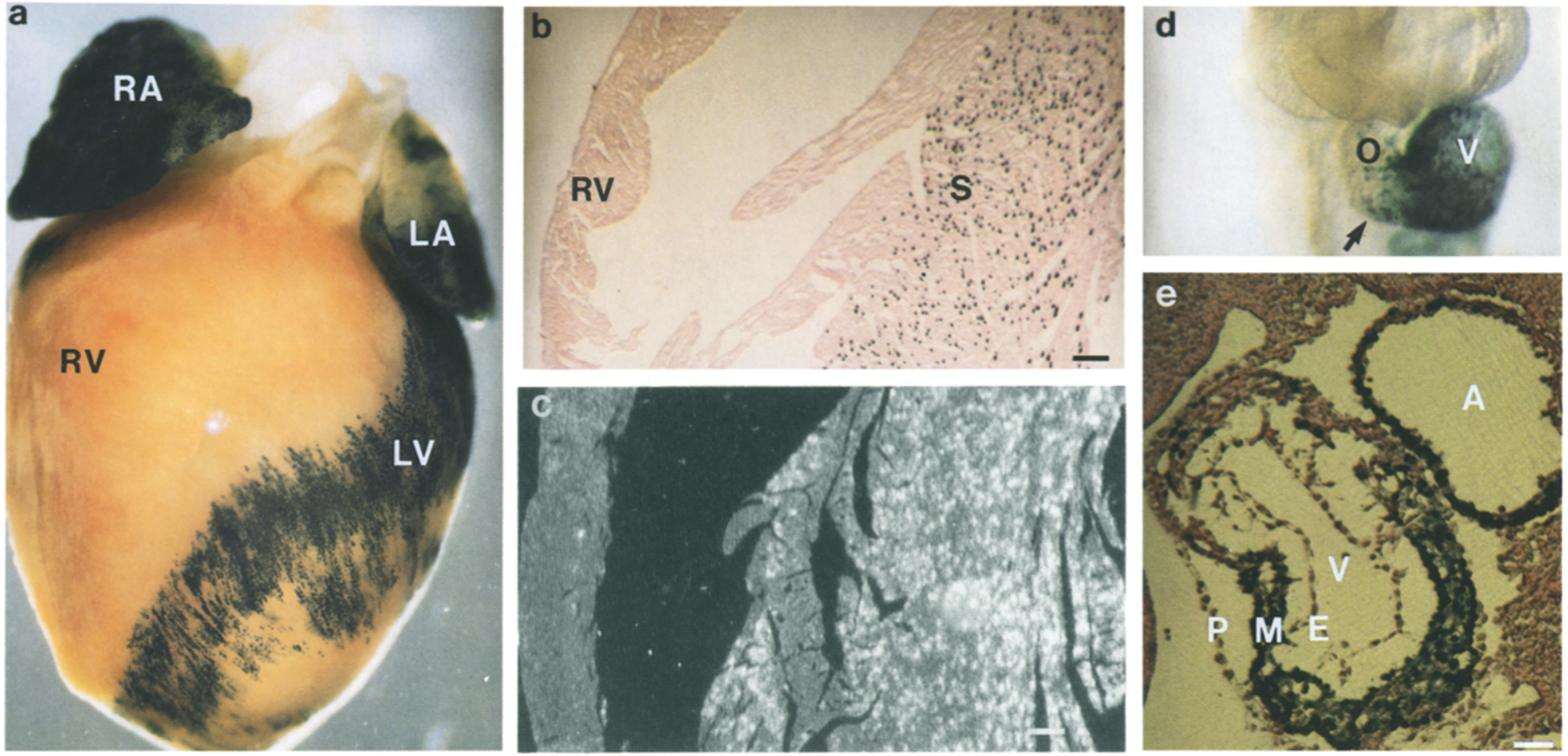

Figure 5. The 3F-*nlacZ*-E transgene is expressed in restricted regions of the heart. Cardiac expression in the adult is confined to the atria and left ventricular myocardium, as shown in a whole-mount photograph of an adult transgenic heart (*a*), and in an X-gal–stained cryostat section, where expression is strong in the interventricular septum, but is excluded from the right ventricular wall (*b*). In situ hybridization using an antisense *lacZ* riboprobe shows that this pattern of β-galactosidase activity reflects differential accumulation of transgene RNA (*c*). The transgene is expressed early in the myocardium of transgenic embryos, showing regionalization as early as 8.5 d p.c. when the outflow tract (*O*) and presumptive right ventricle (*arrow*) are β-gal negative (*d*). At 9.5 d, a cryostat section through the heart shows that β-gal activity is restricted to the atria and presumptive left ventricular myocardium (*e*). *RA*, right atria; *LA*, left atria; *RV*, right ventricle; *LV*, left ventricle; *S*, interventricular septum; *A*, atria; *V*, ventricle; *P*, pericardium; *M*, myocardium; *E*, endocardium. Bar, 100 μm.

unexpected to observe strong *nlacZ* expression in cardiac muscle in both lines of 3F-*nlacZ*-E transgenic mice. In adult mice, cardiac expression of the transgene is regionalized, being largely confined to the left ventricle, interventricular septum, and atria (Fig. 5, *a* and *b*). Transgene expression in the left ventricle and interventricular septum is nonuniform, and regions of nonexpressing myocardiocytes are observed (Fig. 5 *a*); however, no transmural differences in expression are evident. Expression is stronger in the right than left atrium, while only occasional positive myocardiocytes are found in the right ventricular wall. This pattern of 3F-*nlacZ*-E transgene expression was observed by whole-mount X-gal coloration of isolated transgenic hearts (Fig. 5 *a*), by histological detection of β-gal activity in cryostat sections of adult hearts (Fig. 5 *b*), and by in situ hybridization to *nlacZ* transcripts in paraffin sections of adult hearts (Fig. 5 *c*). This pattern of 3F-*nlacZ*-E expression is distinct from all other described sarcomeric, cytoplasmic, and nuclear cardiac markers in the adult myocardium, and, therefore, it defines a previously undescribed restriction of transcriptional activity in the mouse heart.

The developing heart is the earliest site of transgene expression in 3F-*nlacZ*-E mice. At 7.5 d of development, two groups of β-gal–positive cells are observed in the anterior mesoderm. Transgene expression is regionalized early: at 8.5 d p.c., the outflow tract myocardium is β-gal negative (Fig. 5 *d*). By 9.5 d p.c., β-gal expression is localized to the regions of presumptive left ventricle and atria (Fig. 5 *e*); significant expression is excluded from the region of the presumptive right ventricle, outflow tract, and sinus venosus. Right ventricular exclusion of transgene expression therefore precedes ventricular septation; the 3F-*nlacZ*-E transgene thus provides an early marker for specific subregions of the developing myocardium.

The observation that *nlacZ* is expressed in the heart in two lines of 3F-*nlacZ*-E transgenic mice led us to reexamine expression of the endogenous MLC3F gene in cardiac tissue. MLC3F protein and accumulated mRNA were not detected in cardiac muscle by two-dimensional protein gel or Northern analysis in previous studies (Barton et al., 1985*a*, *b*). We demonstrate here, however, using RT-PCR, that the endogenous MLC3F transcript is present in RNA isolated from whole adult mouse hearts, although at a significantly lower level than in RNA from adult skeletal muscle (Fig. 6, *a* and *b*). In contrast, α-cardiac actin is expressed at high levels in cardiac muscle and low levels in skeletal muscle. The myogenic sequence MRF4, which is specific for skeletal muscle, provides a negative control in these experiments (Fig. 6 *b*). The expression of endogenous MLC3F transcripts in the four compartments of the adult heart was analyzed (Fig. 6 *c*). While the endogenous gene is expressed at higher levels in atrial than in ventricular myocardium, no significant differences between left and right ventricles were observed. S16 ribosomal protein transcripts are uniformly expressed in the four cardiac chambers (Fig. 6 *c*).

These findings were confirmed by in situ hybridization; endogenous MLC3F transcripts were detected in the heart of nontransgenic mice at a time before their appearance in developing skeletal muscle (10.5 d p.c., Fig. 7 *b*). The MLC3F antisense in situ probe is derived from the 5′ UTR of the MLC3F transcript (Fig. 1 *a*, probe B), and it does not cross-hybridize to other members of the alkali light chain gene family (Lyons et al., 1990*a*). In agreement with our RT-PCR observations, the endogenous MLC3F gene is expressed at higher levels in atrial than ventricular myocardium (Fig. 7 *b*); restriction of detectable levels of endogenous 3F transcript to the atria occurs between 12.5 and 14.5 d p.c. (Fig. 7, *c* and *d*). The low level of MLC3F ventricular expression at later stages precludes the use of in situ hybridization to analyze whether, like the 3F-*nlacZ*-E transgene, the endogenous 3F transcript shows right ventricular exclusion. Before 14.5 d, however, MLC3F expression is regionalized in the developing ventricles (Fig. 7 *c*). Since the sequence complementary to the MLC3F probe lies within the first intron of the primary MLC1F transcript, the probe can cross-hybridize to unprocessed MLC1F transcripts (see Fig. 1 *a*). Using a second probe complementary to unspliced MLC1F transcripts (within the first intron, 2 kb upstream of the MLC3F promoter; Fig. 1 *a*, probe A), we can differentiate between unprocessed MLC1F and MLC3F transcripts. At 14.5 d p.c., unprocessed MLC1F transcripts are readily detected in skeletal but not in cardiac muscle (Fig. 7, *f* and *g*), while the MLC3F probe hybridizes to transcripts in both muscle types, showing that the signal observed in the heart is specific for the 3F transcription unit (Fig. 7, *d* and *e*). Low levels of processed MLC1F transcripts were in fact detected in adult cardiac muscle by RT-PCR (Fig. 6 *c*); these transcripts, like MLC3F transcripts, were more abundant in

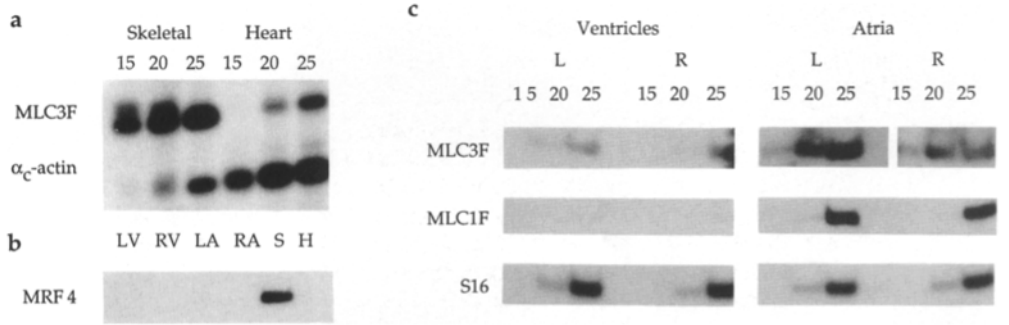

Figure 6. Endogenous MLC3F transcript is expressed in the heart. By RT-PCR, MLC3F transcripts are detected in RNA isolated from adult hearts, although they are present at a lower level than in skeletal muscle RNA (*a*). In contrast, α-cardiac actin is represented at a higher level in cardiac than skeletal muscle RNA. PCR amplifications were carried out for 15, 20, and 25 cycles. Cardiac RNA samples are negative for MRF 4, a marker of skeletal muscle cells, after RT-PCR and Southern blot hybridization with an MRF 4–specific probe (*b*). Expression of the MLC1F and 3F transcripts varies among the four compartments of the heart (*c*); both are present at higher levels in atrial than ventricular RNA. S16 ribosomal protein message, in contrast, is constant in the four compartments. After 15, 20, and 25 cycles of PCR reaction, products were Southern blot hybridized to either 3F or 1F probes, and filters were washed under conditions of high stringency (0.1× SSC). *L*, left; *R*, right; *LV*, left ventricle; *RV*, right ventricle, *LA*, left atria; *RA*, right atria; *S*, skeletal muscle; *H*, cardiac muscle.

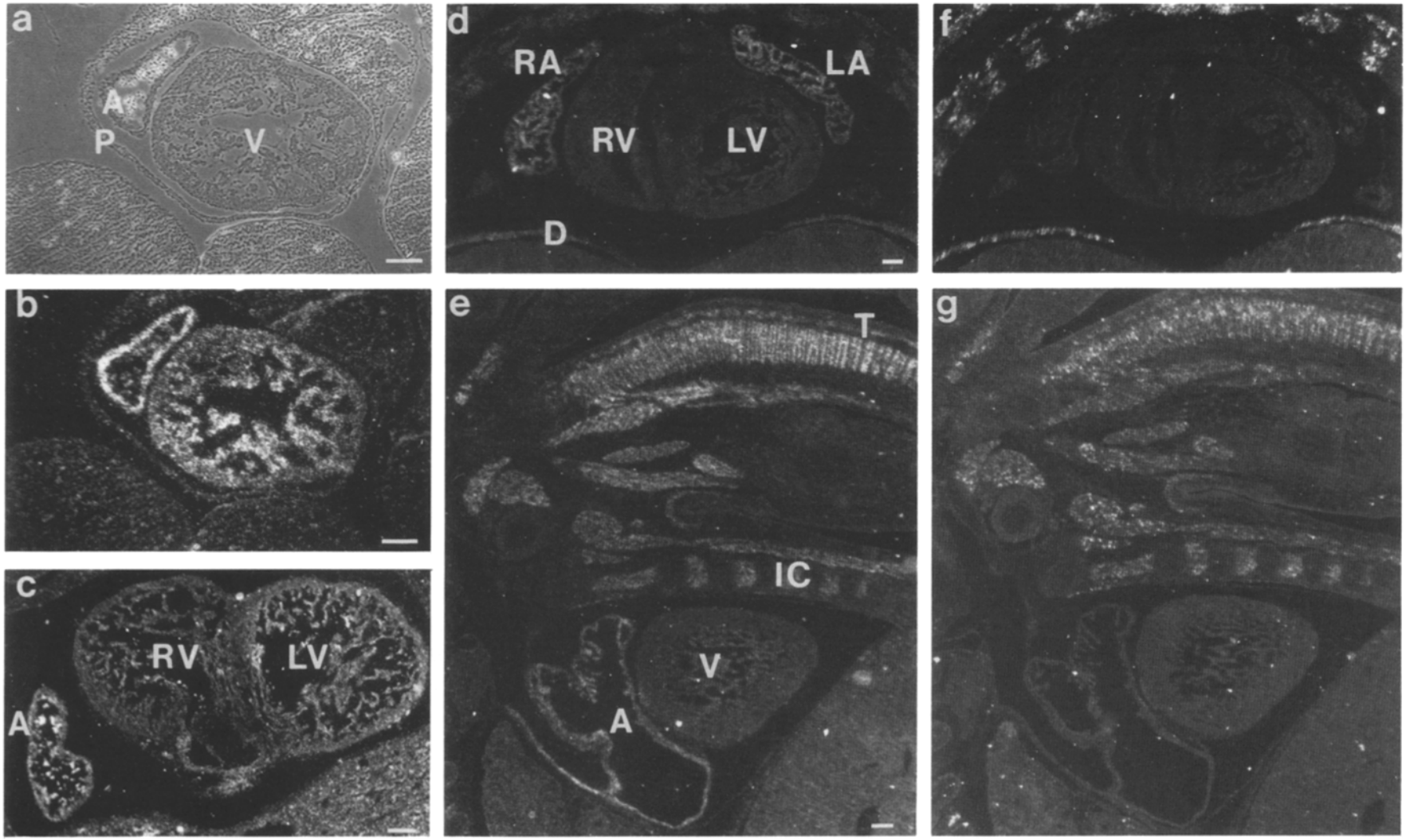

Figure 7. In situ hybridization to endogenous MLC3F transcripts. Bright- (*a*) and dark-field (*b*) micrographs of a section through a nontransgenic mouse heart at 10.5 d after in situ hybridization with an antisense MLC3F riboprobe (Fig. 1 *a*, probe B) showing 3F transcripts in both atrial and ventricular compartments. At 12.5 d p.c., ventricular MLC3F expression is both reduced and regionalized (*c*). By 14.5 d, MLC3F expression in the heart, as detected by in situ hybridization, becomes restricted to both right and left atria (*d* and *e*). An antisense probe complementary to sequences in the first intron of the MLC1F/3F gene (Fig. 1 *a*, probe A) detects abundant unprocessed MLC1F message at 14.5 d p.c. in developing skeletal muscle, but not in cardiac muscle (*f* and *g*). Note the punctate signal obtained with this probe, which hybridizes to unspliced nuclear transcripts. *P*, pericardium; *V*, ventricle; *A*, atria; *RV*, right ventricle; *LV*, left ventricle; *RA*, right atria; *LA*, left atria; *D*, diaphragm; *T*, tongue; *IC*, intercostal muscles. Bars, 100 μm.

atrial than ventricular RNA. By in situ hybridization, however, MLC1F transcripts could only be detected in the heart at early stages of development (before 10.5 d, data not shown).

Discussion

In this study, we demonstrate that the MLC3F promoter and 3′ enhancer are sufficient to confer strong muscle-specific expression and fast fiber-type distribution on a *nlacZ* reporter gene in transgenic mice. Correct temporal activation of the MLC3F promoter, however, is not observed. We have revealed an additional level of regulation at this complex locus, showing that the MLC3F promoter is transcribed in the embryonic and adult mouse heart, describing a novel pattern of cardiac gene activity.

The mouse MLC1F/3F 3′ enhancer drives strong differentiation-specific expression from the mouse MLC3F promoter in cultured muscle cells, confirming in vitro experiments carried out with the rat gene (Garfinkel and Davidson, 1987), and with the human enhancer on the rat promoter (Rosenthal et al., 1990). We subsequently analyzed the expression of the MLC3F promoter/3′ enhancer construct in transgenic mice. During embryogenesis, the 3F-*nlacZ*-E transgene is activated in muscle cells at 9 d p.c., 4–5 d before the endogenous MLC3F transcript first appears (Lyons et al., 1990*a*). In 1F-CAT-E transgenic mice, CAT expression is first detected in the myotome at 9–9.5 d p.c. (Grieshammer et al., 1992), as is the endogenous MLC1F gene. Reporter gene expression also initiates at the same time in the developing premuscle masses of the limb in 1F and 3F transgenic mice (11.5 d, this study and Grieshammer et al., 1992). Thus, while the 3′ enhancer confers correct temporal regulation on the MLC1F promoter in vivo, and is clearly an important element for strong muscle-specific MLC3F transcription in vitro and in transgenic mice, it is not sufficient to activate MLC3F expression in vivo at the correct time. Furthermore, late activation of MLC3F transcription does not appear to be the result of developmental specificity residing in the proximal 3F promoter. Our observations contrast with findings at the β-globin locus, where a major part of the developmental specificity of the β-globin genes resides in the proximal promoters; for example, fetal isoforms are repressed in the adult by factors binding in their promoter regions, the common locus control region subsequently activating distally located adult isoforms (reviewed in Dillon and Grosveld, 1993).

How the late activation of MLC3F transcription is controlled therefore remains unclear, although we envisage two possible mechanisms: (*a*) that the 3′ enhancer does not normally interact with the 3F promoter, and in our transgenic mice may overide MLC3F regulatory signals, perhaps in the absence of a favored interaction with the MLC1F promoter, which is not included in the construct. Note, however, that in the endogenous locus, the MLC1F and 3F promoters are, respectively, 25 and 15 kb from the 3′ enhancer. (*b*) Alternatively, or in combination with (*a*), there may be additional regulatory elements in the MLC1F/3F locus that are required for late MLC3F activation and are not included in the construct. We have identified a region of 800 nucleotides in the first intron of the MLC1F/3F gene that has myotube-specific enhancer activity and interacts preferentially with the MLC3F promoter in vitro. This intronic region is, therefore, a candidate sequence that may be required for correct MLC3F expression in vivo (Kelly, R., S. Alonso, A. Schneider, S. Tajbakhsh, and M. Buckingham. 1994. *J. Cell. Biochem.* 18D:499 [Abstr.]). The analysis of additional transgenic lines to address this question is underway.

Early developmental misregulation of the 3F-*nlacZ*-E transgene is contrasted by later downregulation in muscle types known to contain low levels of MLC3F protein. In adult transgenic mice, low β-gal levels are seen in the soleus and diaphragm, although both muscles express the transgene at high levels in utero. Other muscles, such as the tibialis anterior, continue to express high levels of β-gal in the adult. These changes correlate with the development of adult fiber-type diversity. Biochemical, physiological, and histological properties vary between muscle fibers, which can be subdivided on the basis of which MHC isoform they express (see Pette and Staron, 1990; Schiaffino and Reggiani, 1994). Fiber type content varies among different muscles, such that mouse soleus, for example, contains roughly equal proportions of slow (type I) and fast (type II, A>X) fibers (Lewis et al., 1982; Donoghue et al., 1991*b*), while the tibialis anterior contains >90% fast fibers (Donoghue et al., 1991*b*). The region of the gastrocnemius laterally adjacent to the plantaris muscle, where the transgene is also downregulated, is, like the soleus, rich in fibers containing type I and IIA MHC, and lacks glycolytic fibers containing type IIB MHC. Similarly, the diaphragm is rich in types I, IIA, and IIX(D) fibers, and devoid of type IIB fibers (Zardini and Parry, 1994). These data suggest that (*a*) the 3F-*nlacZ*-E transgene is expressed preferentially in type II (fast) fibers, and (*b*) *nlacZ* expression is higher in a subset of type II fibers (most likely IIB). The 3′ enhancer and MLC3F proximal promoter sequences included in the transgene are therefore sufficient to confer fast fiber-type specificity. Similar results were obtained by Donoghue et al. (1991*b*) in 1F-CAT-E transgenic mice, which express CAT at a higher level in fast than slow fibers; furthermore, CAT levels vary among type II fiber subtypes in the order IIB>IIX(D)>IIA. The core enhancer element common to 1F-CAT-E and 3F-*nlacZ*-E transgenic mice may therefore be involved in selective fiber-type expression. Hallauer et al. (1993) also reported a IIB>IIA>I pattern of expression of a fast quail troponin I-*lacZ* construct in transgenic mice; in this case, the endogenous mouse gene was expressed evenly in all fast (type II) fibers. In contrast, a *lacZ* reporter gene under the control of the *Pgk*-1 promoter is expressed in fast fibers containing IIA or IIX MHC, but not in type IIB fibers or slow type I MHC fibers (McBurney et al., 1994). Distinct *cis*-regulatory elements are thus likely to direct gene expression in different fast fiber types (see Schiaffino and Reggiani, 1994). We are currently comparing the pattern of transcription of the endogenous MLC3F gene with that of the 3F-*nlacZ*-E transgene in particular fiber types at a more detailed cellular level.

The low level of transgene expression observed in the adult diaphragm in 3F-*nlacZ*-E mice (>90% IIA/X(D) fibers, Zardini and Parry, 1994) is consistent with our observations in crural muscles. Interestingly, a subpopulation of nuclei in diaphragm fibers continues to express elevated transgene levels at focal synaptic and terminal myotendinous junctions, both regions being marked by elevated acetylcholinesterase activity (in the myotendinous junction at 20% of levels at the

neuromuscular junction, see Schmalbruch, 1985). The 3F-*nlacZ*-E transgene marker can therefore be added to the list of synaptic membrane, basal laminal, and cytoskeletal proteins that are concentrated at synaptic sites (Hall and Sanes, 1993). At the present time, there is no evidence for elevated endogenous 3F protein at these sites, but this requires further investigation, as does identification of the *cis*-acting sequences in the MLC3F promoter or 3′ enhancer responsible for this expression pattern.

1F-CAT-E transgenic mice exhibit a rostrocaudal gradient of reporter gene expression, which suggests that the transgene responds to intrinsic differences in positional information along the body axis (Donoghue et al., 1991*a*). The endogenous MLC1F gene, however, is not graded in expression, yet the CAT gradient has been shown to be directed by sequences contained within the MLC-derived fragments. It is not dependent on the reporter gene since a gradient of expression is observed when CAT is substituted by either the acetylcholine receptor γ-subunit cDNA (Donoghue et al., 1991*a*) or *lacZ* (Donoghue, M., J. Merlie, and J. Sanes, personal communication). 3F-*nlacZ*-E mice provide a second transgenic mouse strain containing regulatory sequences derived from the 1F/3F locus, and it was thus of interest to determine whether the *nlacZ* gene is expressed in a positionally graded manner in these mice. We have demonstrated in this study that β-gal protein and mRNA levels are not positionally graded in the intercostal muscles of 3F-*nlacZ*-E transgenic embryos, at a stage when the CAT gradient is evident in 1F-CAT-E mice. Our results show that the MLC3F promoter and core 3′ enhancer do not confer a positional gradient of reporter gene expression in transgenic mice, and they suggest that the *cis*-acting sequences responsible for the gradient in 1F-CAT-E mice lie outside the core 3′ enhancer.

The MLC1F and 3F proteins are restricted to skeletal striated muscle (see Barton and Buckingham, 1985). Two other mouse alkali MLC isoforms are expressed in both skeletal and cardiac muscle: MLC1V in slow skeletal fibers and adult ventricle, and MLC1A in embryonic and fetal skeletal muscle and adult atria (Barton et al., 1985*a*, *b*). We have now demonstrated that both transgene directed and endogenous MLC3F transcription occurs in cardiac muscle. Cardiac MLC3F expression is likely to depend on regulatory sequences in the MLC3F promoter region, since, in contrast to the 3F-*nlacZ*-E transgene, the 3′ enhancer does not drive CAT expression in 1F-CAT-E transgenic mouse hearts (Rosenthal et al., 1989). Furthermore, MLC3F transgenic mice lacking the 3′ enhancer express a high level of *nlacZ* in the heart (Kelly, R., S. Alonso, and M. Buckingham, unpublished observations).

3F-*nlacZ*-E transgene expression follows a previously undocumented pattern of cardiac gene expression. As early as 8.5 d of development, at the tubular heart stage, transgene expression is regionalized such that the outflow tract and a region of the common ventricle (future right ventricle) are nonexpressing. The 3F-*nlacZ*-E transgene thus provides an early marker for positional specification in the developing mouse heart, and it should help to address the question of whether such spatial restriction arises from distinct lineages of committed cardiac precursors, or via positional cues. The two major cardiac alkali myosin light chain genes are, in contrast to the 3F-*nlacZ*-E transgene, widely expressed in the myocardium at early stages, and only later in embryogenesis do they become restricted to atrial or ventricular compartments (Lyons et al., 1990*b*). MLC2V, however, the ventricular regulatory myosin light chain, shows earlier regionalization to the ventricular compartment (by 11 d, O'Brien et al., 1993). While many cardiac genes have been documented to be expressed in atria or ventricles, few have been described which demonstrate left/right compartmental differences. Two examples of which we are aware are (*a*) atrial natriuretic peptide, for which low level ventricular expression in the adult (1% of atrial expression levels) is stronger in the left than right ventricle (Gardner et al., 1986); (*b*) muscle creatine kinase (MCK), which shows transient left/right asymmetry with transcripts first appearing in the wall of the right ventricle of the 12.5-d mouse heart; by 15.5 d, MCK transcripts are expressed at a high level in all cardiac myocytes (Lyons, 1994). Our results suggest that transcriptional differences exist between right and left ventricular compartments, and that such differences precede septation (complete by 13 d) and associated secondary physiological constraints. Furthermore, left/right transcriptional differences are mediated by sequences included in the 3F-*nlacZ*-E construct.

By both RT-PCR and in situ hybridization we have shown that our observations in transgenic mice reflect low level transcription of the endogenous MLC3F gene in the mouse heart. MLC3F expression is compartmentalized, with higher transcript levels in atrial than ventricular RNA. Endogenous MLC3F transcripts in embryonic hearts show some ventricular regionalization, but in adult hearts, in contrast to *nlacZ* expression in transgenic mice, no left/right asymmetry is evident; the cause of this difference remains unclear. The low level of MLC3F transcription in the heart is likely to explain why cardiac MLC3F expression was not previously observed (Barton and Buckingham, 1985). Although the low level of MLC1F protein has been reported in embryonic cardiac muscle cells in the chick (Obinata et al., 1983), there is no evidence for the presence of 1F or 3F MLC isoforms in mammalian cardiac muscle (Whalen et al., 1982). Reexamination of MLC protein isoforms present in the adult heart on silver-stained two-dimensional gels revealed no trace of MLC1F or MLC3F proteins either in whole mouse heart or in left and right atrial and ventricular protein preparations (Butler-Browne, G., V. Mouly, F. Edom, R. Kelly, S. Alonso, and M. Buckingham, unpublished data). Therefore, it seems unlikely that MLC3F transcription in the heart is of functional importance since MLC3F protein, if present, is at an extremely low level compared with that of the major cardiac alkali MLC isoforms, MLC1A and MLC1V. It is possible that cardiac MLC3F transcripts are subject to posttranscriptional control. This would not be without precedent: Gorza et al. (1993) have demonstrated that rat cardiac troponin I mRNA accumulates in the ventricle of developing rat hearts from day 11, whereas the protein is not detected in the ventricles before day 18.

While a number of cardiac muscle isoforms are transiently expressed during skeletal muscle development (including MLC1A and α-cardiac actin), there are also examples of skeletal muscle protein isoforms that are transiently expressed in the developing heart: these include α-skeletal actin (Sassoon et al., 1988) and MLC2F (Faerman and Shani, 1993). Our results extend the known overlap in genes activated at early stages of both cardiac and skeletal myogenesis, and

they suggest that transcriptional activation requirements for both striated muscle types are shared by many muscle-specific genes.

Note added in proof. Similar results showing MLC3F expression in the heart have been obtained by Michael McGrew and Nadia Rosenthal (Cardiovascular Research Center, Massachusetts General Hospital, Charlestown, MA), according to a personal communication.

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References

Banerjee-Basu, S., and A. Buonanno. 1993. *Cis*-acting sequences of the rat troponin-I slow gene confer tissue-specific and development-specific transcription in cultured muscle cells as well as fiber type specificity in transgenic mice. *Mol. Cell. Biol.* 13:7019–7028.

Barton, P. R. J., and M. E. Buckingham. 1985. The myosin alkali light chain proteins and their genes. *Biochem. J.* 231:249–261.

Barton, P. R. J., A. Cohen, B. Robert, M. Y. Fizman, F. Bonhomme, J. Guenet, D. P. Leader, and M. E. Buckingham. 1985*a*. The myosin alkali light chains of mouse ventricular and slow skeletal muscle are indistinguishable and are encoded by the same gene. *J. Biol. Chem.* 260:8578–8584.

Barton, P. R. J., B. Robert, M. Y. Fiszman, D. P. Leader, and M. E. Buckingham. 1985*b*. The same myosin alkali light chain gene is expressed in adult cardiac atria and in fetal skeletal muscle. *J. Muscle Res. Cell Motil.* 6:461–475.

Barton, P. J., A. J. Harris, and M. E. Buckingham. 1989. Myosin light chain gene expression in developing and denervated fetal muscle in the mouse. *Development (Camb.).* 107:819–824.

Biben, C., B. J. Kirschbaum, I. Garner, and M. Buckingham. 1994. Novel muscle-specific enhancer sequences upstream of the cardiac actin gene. *Mol. Cell. Biol.* 14:3504–3513.

Bottinelli, R., R. Betto, S. Schiaffino, and C. Reggiani. 1994. Both myosin heavy chain and alkali light chain isoforms determine unloaded shortening velocity in rat muscle fibers. *J. Physiol.* 478:341–349.

Brennan, T. J., D. G. Edmondson, and E. N. Olson. 1990. Aberrant regulation of MyoD1 contributes to the partially defective myogenic phenotype of BC3H1 cells. *J. Cell Biol.* 110:929–937.

Buckingham, M. 1992. Making muscle in mammals. *Trends Genet.* 8:144–149.

Cox, R. D., and M. E. Buckingham. 1992. Actin and myosin genes are transcriptionally regulated during mouse skeletal muscle development. *Dev. Biol.* 149:228–234.

Dillon, N., and F. Grosveld. 1993. Transcriptional regulation of multigene loci: multilevel control. *Trends Genet.* 9:134–137.

Daubas, P., A. Klarsfeld, I. Garner, C. Pinset, R. Cox, and M. Buckingham. 1988. Functional activity of the two promoters of the myosin alkali light chain gene in primary muscle cell cultures: comparison with other muscle gene promoters and other culture systems. *Nucleic Acids Res.* 16: 1251–1271.

Donoghue, M., H. Ernst, B. Wentworth, B. Nadal-Ginard, and N. Rosenthal. 1988. A muscle-specific enhancer is located at the 3′ end of the myosin light chain 1/3 gene locus. *Genes Dev.* 2:1779–1790.

Donoghue, M., J. P. Merlie, N. Rosenthal, and J. R. Sanes. 1991*a*. A rostrocaudal gradient of transgene expression in adult skeletal muscle. *Proc. Natl. Acad. Sci. USA.* 88:5847–5851.

Donoghue, M. J., J. D. Alvarez, J. P. Merlie, and J. R. Sanes. 1991*b*. Fiber type- and position-dependent expression of a myosin light chain-CAT transgene detected with a novel histochemical stain for CAT. *J. Cell Biol.* 115:423–434.

Donoghue, M. J., R. Morris-Valero, Y. R. Johnson, J. P. Merlie, and J. R. Sanes. 1992*a*. Mammalian muscle cells bear a cell-autonomous, heritable memory of their rostrocaudal position. *Cell.* 69:67–77.

Donoghue, M. J., B. L. Patton, J. R. Sanes, and J. P. Merlie. 1992*b*. An axial gradient of transgene methylation in murine skeletal muscle: genomic imprint of rostrocaudal position. *Development (Camb.).* 116:1101–1112.

Faerman, A., and M. Shani. 1993. The expression of the regulatory myosin light chain 2 gene during mouse embryogenesis. *Development (Camb.).* 118:919–929.

Gardner, D. G., C. F. Deschepper, W. F. Ganong, S. Hane, J. Fiddes, J. D. Baxter, and J. Lewicki. 1986. Extra-atrial expression of the gene for atrial natriuretic factor. *Proc. Natl. Acad. Sci. USA.* 83:6697–6701.

Garfinkel, L. I., and N. Davidson. 1987. Developmentally regulated expression of a truncated myosin light-chain 1F/3F gene. *Mol. Cell. Biol.* 7:3826–3829.

Gauthier, G. F., S. Lowey, P. Benfield, and A. W. Hobbs. 1982. Distribution and properties of myosin isoenzymes in developing avian and mammalian skeletal muscle fibers. *J. Cell Biol.* 92:471–484.

Gorza, L., S. Ausoni, N. Merciai, K. E. Hastings, and S. Schiaffino. 1993. Regional differences in troponin I isoform switching during rat heart development. *Dev. Biol.* 156:253–264.

Grieshammer, U., D. Sassoon, and N. Rosenthal. 1992. A transgene target for positional regulators marks early rostrocaudal specification of myogenic lineages. *Cell.* 69:79–93.

Gundersen, K., J. R. Sanes, and J. P. Merlie. 1993. Neural regulation of muscle acetylcholine receptor ε- and α-subunit gene promoters in transgenic mice. *J. Cell Biol.* 123:1535–1544.

Hall, Z. W., and J. R. Sanes. 1993. Synaptic structure and development: the neuromuscular junction. *Cell.* 72:99–121.

Hallauer, P. L., H. L. Bradshaw, and K. E. M. Hastings. 1993. Complex fiber-type expression of fast skeletal troponin I gene constructs in transgenic mice. *Development (Camb.).* 119:691–701.

Hogan, B., F. Costantini, and E. Lacy. 1986. Manipulating the Mouse Embryo: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 332 pp.

Hoh, J. F. Y. 1979. Developmental changes in chicken skeletal myosin isoenzymes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 98:267–270.

Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith. 1984. A short amino-acid sequence able to specify nuclear location. *Cell.* 39: 499–509.

LaFramboise, W. A., M. J. Daood, R. D. Guthrie, S. Schiaffino, P. Moretti, B. Brozanski, M. P. Ontell, G. S. Butler-Brown, R. G. Whalen, and M. Ontell. 1991. Emergence of the mature myosin phenotype in the rat diaphragm muscle. *Dev. Biol.* 144:1–15.

Laird, P. W., A. Zijderveld, K. Linders, M. A. Rudnicki, R. Jaenisch, and A. Berns. 1991. Simplified mammalian DNA isolation procedure. *Nucleic Acids Res.* 19:4293.

Lee, K. J., R. S. Ross, H. A. Rockman, A. N. Harris, T. X. O'Brien, M. van Bilsen, H. E. Shubeita, R. Kandolf, G. Brem, J. Price, et al. 1992. Myosin light chain-2 luciferase transgenic mice reveal distinct regulatory programs for cardiac and skeletal muscle-specific expression of a single contractile protein gene. *J. Biol. Chem.* 267:15875–15885.

Lewis, D. M., D. J. Parry, and A. Rowlerson. 1982. Isometric contractions of motor units and immunohistochemistry of mouse soleus muscle. *J. Physiol.* 325:393–401.

Li, Z., P. Marchand, J. Humbert, C. Babinet, and D. Paulin. 1993. Desmin sequence elements regulating skeletal muscle-specific expression in transgenic mice. *Development (Camb.).* 117:947–959.

Lowey, S., G. S. Waller, and K. M. Trybus. 1993. Skeletal muscle myosin light chains are essential for physiological speeds of shortening. *Nature (Lond.).* 365:454–456.

Lyons, G. E., M. Ontell, R. Cox, D. Sassoon, and M. Buckingham. 1990*a*. The expression of myosin genes in developing skeletal muscle in the mouse embryo. *J. Cell Biol.* 111:1465–1476.

Lyons, G. E., S. Schiaffino, D. Sassoon, P. Barton, and M. Buckingham. 1990*b*. Developmental regulation of myosin gene expression in mouse cardiac muscle. *J. Cell Biol.* 111:2427–2436.

Lyons, G. E. 1994. In situ analysis of the cardiac gene program during embryogenesis. *Trends Cardiovasc. Med.* 4:70–77.

McBurney, M. W., W. A. Staines, K. Boekelheide, D. Parry, K. Jardine, and L. Pickavance. 1994. Murine *Pgk*-1 promoter drives widespsread but not uniform expression in transgenic mice. *Dev. Dynam.* 200:278–293.

Merrifield, P. A., and I. R. Konigsberg. 1987. Nerve-dependent accumulation of myosin light chain 3 in developing limb musculature. *Development (Camb.).* 101:673–684.

Nabeshima, Y., Y. Fujii-Kuriyama, M. Muramatsu, and K. Ogata. 1984. Alternative transcription and two modes of splicing results in two myosin light chains from one gene. *Nature (Lond.).* 308:333–338.

Obinata, T., T. Masaki, H. Takano-Ohmuro, T. Tanaka, and N. Shimizu. 1983. Coexistence of cardiac-type and fast skeletal-type light chains in embryonic chicken cardiac muscle. *J. Biochem.* 94:1025–1028.

O'Brien, T. X., K. J. Lee, and K. R. Chien. 1993. Positional specification of ventricular myosin light chain 2 expression in the primitive murine heart tube. *Proc. Natl. Acad. Sci. USA.* 90:5157–5161.

Pajak, L., M. Mariappan, and D. F. Wieczorek. 1991. Reprogramming of myosin light chain 1/3 expression in muscle heterokaryons. *Dev. Biol.* 145:28–39.

Pette, D., and R. S. Staron. 1990. Cellular and molecular diversities of mammalian skeletal muscle fibers. *Rev. Physiol. Biochem. Pharmacol.* 116:1–76.

Periasamy, M., E. Strehler, L. Garfinkel, R. Gubits, N. Ruiz-Opazo, and B. Nadal-Ginard. 1984. Fast skeletal muscle myosin light chains 1 and 3 are produced from a single gene by a combined process of differential RNA transcription and splicing. *J. Biol. Chem.* 259:13595–13604.

Piette, J., M. Huchet, D. Houzelstein, and J. P. Changeux. 1993. Compartmentalized expression of the alpha- and gamma-subunits of the acetylcholine receptor in recently fused myofibers. *Dev. Biol.* 157:205–213.

Robert, B., P. Daubas, M.-A. Akimenko, A. Cohen, I. Garner, J.-L. Guenet, and M. E. Buckingham. 1984. A single locus in the mouse encodes both myosin light chains 1 and 3, a second locus corresponds to a related pseudogene. *Cell.* 39:129–140.

Rosenthal, N. 1989. Muscle cell differentiation. *Curr. Opin. Cell Biol.* 1:1094–1101.

Rosenthal, N., J. M. Kornhauser, M. Donoghue, K. M. Rosen, and J. P. Merlie. 1989. Myosin light chain enhancer activates muscle-specific, developmentally regulated gene expression in transgenic mice. *Proc. Natl. Acad. Sci. USA.* 86:7780–7784.

Rosenthal, N., E. B. Berglund, B. M. Wentworth, M. Donoghue, B. Winter, E. Bober, T. Braun, and H.-H. Arnold. 1990. A highly conserved enhancer downstream of the human MLC1/3 locus is a target for multiple myogenic determination factors. *Nucleic Acids Res.* 18:6239–6246.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanes, J. R., J. L. R. Rubenstein, and J.-F. Nicolas. 1986. Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:3133–3142.

Sassoon, D., I. Garner, and M. Buckingham. 1988. Transcripts of α-cardiac and α-skeletal actins are early markers for myogenesis in the mouse embryo. *Development (Camb.).* 104:155–164.

Sassoon, D., and N. Rosenthal. 1993. Detection of messenger RNA by in situ hybridization. *Methods Enzymol.* 225:384–404.

Schiaffino, S., and C. Reggiani. 1994. Myosin isoforms in mammalian skeletal muscle. *J. Appl. Physiol.* 77:493–501.

Schmalbruch, H. 1985. The myotendinous junction. Skeletal Muscle. Springer-Verlag, Berlin. pp. 139–142.

Seidel, U., and H. H. Arnold. 1989. Identification of the functional promoter regions in the human gene encoding the myosin alkali light chains MLC1 and MLC3 of fast skeletal muscle. *J. Biol. Chem.* 264:16109–16117.

Strehler, E. E., M. Periasamy, M.-A. Strehler-Page, and B. Nadal-Ginard. 1985. Myosin light-chain 1 and 3 gene has two structurally distinct and differentially regulated promoters evolving at different rates. *Mol. Cell. Biol.* 5:3168–3182.

Tajbakhsh, S., E. Vivarelli, G. Cusella-De Angelis, D. Rocancourt, M. Buckingham, and G. Cossu. 1994. A population of myogenic cells derived from the mouse neural tube. *Neuron.* 13:813–821.

Wada, M., and D. Pette. 1993. Relationships between alkali light-chain complement and myosin heavy-chain isoforms in single fast-twitch fibers of rat and rabbit. *Eur. J. Biochem.* 214:157–161.

Weintraub, H. 1993. The MyoD family and myogenesis—redundancy, networks, and thresholds. *Cell.* 75:1241–1244.

Wentworth, B. M., M. Donoghue, J. C. Engert, E. B. Berglund, and N. Rosenthal. 1991. Paired MyoD-binding sites regulate myosin light chain gene expression. *Proc. Natl. Acad. Sci. USA.* 88:1242–1246.

Whalen, R. G., S. M. Sell, A. Eriksson, and L.-E. Thornell. 1982. Myosin subunit types in skeletal and cardiac tissues and their developmental distribution. *Dev. Biol.* 91:478–484.

Wigston, D. J., and A. W. English. 1992. Fiber-type proportions in mammalian soleus muscle during postnatal development. *J. Neurobiol.* 23:61–70.

Yu, Y.-T., R. E. Breitbart, L. B. Smoot, Y. Lee, V. Mahdavi, and B. Nadal-Ginard. 1992. Human myocyte-specific enhancer factor 2 comprises a group of tissue-restricted MADS box transcription factors. *Genes Dev.* 6: 1783–1798.

Zardini, D. M., and D. J. Parry. 1994. Identification, distribution and myosin subunit composition of type IIX fibers in mouse muscles. *Muscle and Nerve.* 17:1308–1316.