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Embryonic and Fetal Myogenic Programs Act through Separate Enhancers at the MLC1F/3F Locus

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Embryonic and fetal stages of skeletal muscle development are characterized by the differential expression of a number of muscle-specific genes. These include the products of independent promoters at the fast myosin light chain 1F/3F locus. In the mouse embryo MLC1F transcripts accumulate in embryonic skeletal muscle from E9, 4–5 days before high-level accumulation of MLC3F transcripts. A 3′ enhancer can activate MLC1F and MLC3F promoters in differentiated muscle cells *in vitro* and in transgenic mice; both promoters, however, are activated at the time of MLC1F transcript accumulation. We now demonstrate the presence of a second muscle-specific enhancer at this locus, located in the intron separating the MLC1F and MLC3F promoters. Transgenic mice containing the intronic, but lacking the 3′ enhancer, express high levels of an *nlacZ* reporter gene from the MLC3F promoter in adult fast skeletal muscle fibers. In contrast to the 3′ enhancer, the intronic element is inactive both in embryonic muscle cells *in vivo* and in embryonic myocyte cultures. The intronic enhancer is activated at the onset of fetal development in both primary and secondary muscle fibers, at the time of endogenous MLC3F transcript accumulation. Late-activated MLC3F transgenes thus provide a novel *in toto* marker of fetal myogenesis. These results suggest that temporal regulation of transcription at the MLC1F/3F locus is controlled by separate enhancers which are differentially activated during embryonic and fetal development.

INTRODUCTION

Coordinated development requires precise temporal and spatial control of gene expression, regulating the properties of diverse cell types during embryogenesis. Striated skeletal muscle provides a model system for analysis of the genetic control of these processes. Adult skeletal muscle is a heterogeneous tissue composed of several cell-types with distinct biochemical and contractile properties (Kelly and Rubinstein, 1994). In addition, muscles differ along the body axis, defining the spatial and functional identity of muscle groups and individual muscles throughout the body (Donoghue and Sanes, 1994); this diversity originates in the embryo where muscle genes are expressed in a dynamic pattern during embryonic and fetal myogenesis (Lyons and Buckingham, 1992; Stockdale, 1992). A major regulatory step in the generation of cell-type, spatial and temporal diversity in skeletal muscle is control at the level of transcription. Dissection of the *in vivo* role of skeletal muscle enhancers and promoters in transgenic mice has resulted in the definition of *cis*-acting elements which control fiber-type-specific expression or confer distinct transcriptional properties on rostral, caudal, and individual muscles (Concordet *et al.*, 1993; Hallauer *et al.*, 1993; Banerjee-Basu and Buonanno, 1993; Levitt *et al.*, 1995; Donoghue *et al.*, 1991; Patapoutian *et al.*, 1993). In this study we define a developmentally regulated enhancer which is active in adult and fetal, but not embryonic, skeletal muscle.

The expression profiles of members of the alkali myosin light chain (MLC) gene family illustrate the diverse patterns of muscle-specific gene expression *in vivo* (Barton and Buckingham, 1985). In birds and mammals, the MLC1F/3F gene encodes two MLC isoforms found in developing and adult fast muscle fibers, MLC1F and MLC3F, which differ in the N-terminal region due to the use of two promoters and a subsequent alternative splicing event (Nabeshima *et al.*, 1984; Periasamy *et al.*, 1984; Robert *et al.*, 1984). MLC3F

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transcripts are also found in cardiac muscle (Kelly *et al.*, 1995). In skeletal muscle, MLC1F and MLC3F isoforms appear at distinct times during embryogenesis: in the mouse, MLC1F transcripts accumulate from E9 when embryonic skeletal muscle first differentiates in the myotomal compartment of somites, whereas MLC3F transcripts accumulate at a high level in fetal skeletal muscle from E13.5 to E15 (Lyons *et al.*, 1990; Ontell *et al.*, 1993). This temporal difference in mRNA accumulation is due to uncoupled transcription at the MLC1F and MLC3F promoters, suggesting that they respond to different developmental cues (Cox and Buckingham, 1992). It has been shown that the two promoters are functionally independent in muscle cells in culture (Strehler *et al.*, 1985; Daubas *et al.*, 1988; Seidel and Arnold, 1989; Pajak *et al.*, 1991), and that MLC1F and MLC3F gene products accumulate differently in response to innervation (Merrifield and Konigsberg, 1987; Barton *et al.*, 1989).

The MLC1F/3F gene is therefore of interest in defining *cis*-acting sequences which regulate the transcriptional control of skeletal muscle diversity. An enhancer 3′ to the MLC1F/3F gene confers high-level expression from the MLC1F promoter in skeletal muscle cells in culture and in transgenic mice, where it is active by E9.5 in the myotome, and E11.5 in forming limb muscles, coincident with endogenous MLC1F transcript accumulation (Donoghue *et al.*, 1988; Rosenthal *et al.*, 1989; Grieshammer *et al.*, 1992). We previously addressed the question of how a single enhancer can regulate two independent promoters by investigating the role of this element on transcription from the MLC3F promoter in transgenic mice. The 3′ enhancer precociously activates MLC3F transcription in skeletal muscle at the time of MLC1F promoter activation, and therefore does not confer correct developmental specificity on the MLC3F promoter *in vivo*, nor does this specificity reside in the promoter itself (Kelly *et al.*, 1995).

How, then, is temporal regulation of MLC3F transcription controlled? We report here the identification of a second skeletal muscle-specific enhancer within the first intron of the mouse MLC1F/3F gene. In transgenic mice both MLC1F/3F enhancers are active in adult skeletal muscle; this novel element, however, does not function in embryonic muscle cells (E9–E13.5), and activates transgene expression during fetal myogenesis (E13.5–birth), coincident with high-level accumulation of the endogenous MLC3F transcript. These results demonstrate that distinct spatiotemporal patterns of skeletal muscle gene expression during development can be mediated by different enhancers at one locus and provide novel insights into skeletal muscle diversification during development.

METHODS

Plasmid Constructions

(i) 3F-nlacZ constructs. Plasmid(p) p3F-*nlacZ*-2 (Kelly *et al.*, 1995) contains 2 kb upstream of the MLC3F transcriptional start site, *nlacZ*-SV40poly(A) in frame in the second MLC3F-specific exon and 1 kb of MLC3F sequence 3′ of *nlacZ* in pBluescript (Stratagene). p3F-*nlacZ*-2E (Kelly *et al.*, 1995) contains the 3′ enhancer at the 3′ end of the insert of p3F-*nlacZ*-2 (in the genomic orientation relative to the MLC3F promoter). p3F-*nlacZ*-2 was extended to −9 kb: pH1-BH was derived from pH1 containing the first intron and MLC1F promoter region of the mouse MLC1F/3F gene (Robert *et al.*, 1984) by cloning intronic sequences from a *Bam*HI site 3′ to exon 1 to the *Hin*dIII site at −2 kb (7 kb) into pBluescript; p3F-*nlacZ*-2 was digested with *Kpn*I and partially with *Hin*dIII. The largest (7 kb) *Kpn*I–*Hin*dIII fragment was ligated into *Kpn*I–*Hin*dIII-digested pH1-BH to generate p3F-*nlacZ*-9. Deletions between −9 and −2: the 14-kb insert of p3F-*nlacZ*-9 was isolated on a *Sac*II–*Kpn*I fragment and digested partially with Sau3A to generate p3F-*nlacZ*-5.8 and p3F-*nlacZ*-5. p3F-*nlacZ*-5 was digested with *Not*I and *Sal*I, blunt-ended, and religated to generate p3F-*nlacZ*-4.2. The region between −4.2 and −5 kb upstream of the MLC3F transcription initiation site (p800) was subcloned from p3F-*nlacZ*-5 by digestion with *Sal*I and *Sma*I, filling in with Klenow polymerase, dilution, and religation, to delete all insert sequences 3′ of −4.2 kb; this plasmid was digested with *Kpn*I and ligated to the 7-kb *Kpn*I insert of p3F-*nlacZ*-2 to generate p3F-*nlacZ*-2I. p3F-*nlacZ*-9 was digested with *Pst*I and a 13.8-kb fragment religated to generate p3F-*nlacZ*-9Δ(−6:−2.8). The 13.8-kb fragment was ligated to a 2.7-kb *Pst*I fragment containing the sequence between −5.5 and −2.8 kb to generate p3F-*nlacZ*-9Δ(−6:−5.5) (containing one copy of the sequence between −5.5 and −2.8 in the genomic orientation), and p3F-*nlacZ*-9Δ(−6:−5.5)rev (containing this sequence in the reverse orientation).

(ii) TK-CAT constructs. The 800-bp intronic enhancer was subcloned in forward and reverse orientations upstream of the herpes simplex virus thymidine kinase (TK) promoter in pBLCAT2 (Luckow and Schutz, 1987). The *Sal*I–*Kpn*I insert of pBLCAT2 was subcloned into p3F-*nlacZ*-5 digested with *Sal*I and *Kpn*I to generate pI-TK-CAT. An 800-bp *Sal*I–*Sac*II fragment from p3F-*nlacZ*-5 was digested with Sau3A and ligated into *Sal*I/*Bam*HI-digested pBLCAT2 to generate pIrev-TK-CAT.

Cell Culture and Transfections

C2/7 cells were cultured and transfected at the myoblast stage as described in Kelly *et al.* (1995). Myoblasts were harvested 24 hr posttransfection and myotubes 2–3 days after switching the cells to differentiation medium (2% fetal calf serum). NIH 3T3 fibroblasts were cultured in 7% fetal calf serum in DMEM and transfected as for C2/7 cells, except at elevated confluency (approximately 75%). Primary embryonic cultures were prepared from E11.5 limb buds as described by Cusella-De Angelis *et al.* (1994). β-Galactosidase assays were performed on 0.2–7% of cell extract (from a 6-cm culture dish) as described in Sambrook *et al.* (1989), and using a chemiluminescent reporter assay (GalactoLight, Tropix, Bedford, MA) following the manufacturer's instructions (Jain and Magrath, 1991). Luminescence was measured on a Berthold luminometer (Model LB-9501). CAT assays were performed on 0.2–5% of cell extract (Biben *et al.*, 1994). β-Galactosidase and CAT values were normalized for transfection efficiency by evaluating luciferase activity from a cotranfected RSVluciferase vector (10–20% cell extract; Biben *et al.*, 1994). Values shown represent the mean plus standard error of the mean (SEM) of at least four experiments with more than one plasmid preparation for each construct. Student's *t* test was used to evaluate whether differences between mean values were significant.

Generation of Transgenic Mice

3F-*nlacZ*-2E transgenic mice are described in (Kelly *et al.*, 1995). The insert of p3F-*nlacZ*-9 was excised as a *Kpn*I–*Sac*II or *Bam*HI fragment; the inserts of p3F-*nlacZ*-2, and p3F-*nlacZ*-2I were excised as *Kpn*I and *Sac*II–*Bam*HI fragments, respectively. Fragments for microinjection were purified by gel electrophoresis and passage through an Elutip column (Schleicher and Schuell). Transgenic mice were generated by microinjection of purified DNA fragments into fertilized (C57BL/6J × SJL) F_2 eggs at concentrations of 700–1000 copies/pl using standard techniques (Hogan *et al.*, 1994). Injected eggs were reimplanted into pseudopregnant (C57BL/6J × CBA) F_1 foster mothers.

Identification and Analysis of Transgenic Mice

Transgenic mice were identified as described in Kelly *et al.* (1995); all except 3F-*nlacZ*-2 lines could be identified by *in toto* X-gal staining of skinned tails. Transgene expression was analyzed as described (Kelly *et al.*, 1995). Anti-fast and neonatal myosin heavy chain (MHC) (MY32, Sigma), anti-sarcomeric MHC (MF20; Bader *et al.*, 1982), and anti-β-galactosidase antibodies were applied to cryostat sections as described in Tajbakhsh and Buckingham (1995). Transgene copy number was determined using a Phosphorimager (Molecular Dynamics). β-Galactosidase activity in transgenic mice was quantified as follows: adult tissues were dissected into 300 μl of TGD (0.25 *M* Tris–HCl (pH 7.5), 15% glycerol, 10 m*M* DTT) on ice and homogenized for 15 sec using a polytron Tissumizer at 80% maximum power (Tekmar, Cincinnati, OH). Three hundred microliters of lysis buffer (GalactoLight, Tropix) plus 1 m*M* DTT, 0.2 m*M* PMSF, and 5 μg/ml leupeptin was added and the samples centrifuged for 5 min at 15,000 rpm. The supernatant was incubated at 48°C for 1 hr to inactivate endogenous β-galactosidase activity, the protein concentration was measured using a Bio-Rad protein assay (Bio-Rad, Munich, Germany), and 1 μg of extract was assayed in duplicate for β-galactosidase activity for 60 min at room temperature using the chemiluminescent reporter assay described above. Activity was expressed as relative light units (rlu)/60 min/μg of extract. A standard concentration series of purified β-galactosidase (714 U/mg, Sigma) was routinely included to evaluate assay linearity and reproducibility.

In Situ Hybridization

RNA–RNA hybridization was performed as described in Kelly *et al.* (1995). Antisense riboprobes used were from the first MLC1F/3F intron (Kelly *et al.*, 1995), the MLC1F 5′UTR (Lyons *et al.*, 1990), and a 61-nt MLC3F antisense riboprobe containing 25 nt complementary to each MLC3F-*nlacZ*-specific exon (2 and 3, Fig. 1a), and 11-nt complementary to exon 4 (details to be published elsewhere).

DNA Sequence Analysis

The 800-bp enhancer element from the MLC1F/3F first intron was sequenced using an Auto Read sequencing kit (Pharmacia, Uppsala, Sweden) using oligonucleotide primers 1–4 in addition to M13 forward and reverse primers; reaction products were separated using an automated ALF DNA sequencer (Pharmacia).

Oligonucleotides

(1) 5′-TGTCTGTAGAAGTCATTC-3′; (2) 5′-AAGGAGTAA-AGTAGGTG-3′; (3) 5′-TTTTCTCGCCCAACCAGAA-3′; (4) 5′-GTCTCTGGCACAGATTTAATTA-3′.

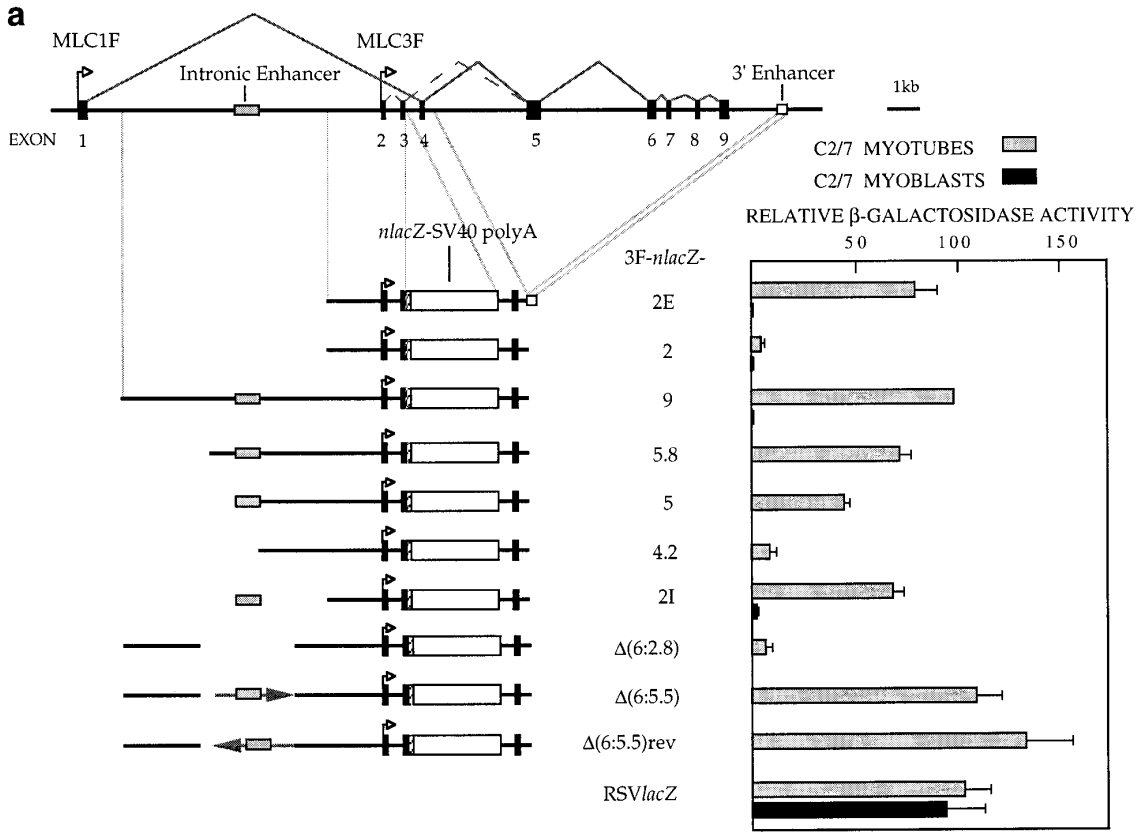
RESULTS

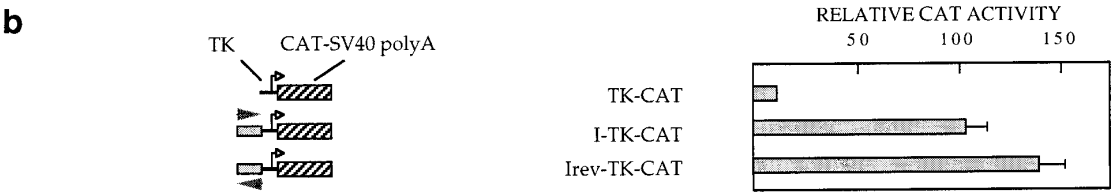
A Second Muscle-Specific Enhancer Element at the Mouse MLC1F/3F Locus

In transgenic mice the 3′ enhancer activates both MLC1F and MLC3F promoters from E9, 4 days before high-level transcription from the endogenous MLC3F promoter (Grieshammer *et al.*, 1992; Kelly *et al.*, 1995). We initiated a search for additional *cis*-acting DNA elements which might regulate late transcription from the MLC3F promoter. A series of plasmid constructs were generated containing mouse MLC1F/3F sequences including the MLC3F promoter upstream of an *Escherichia coli lacZ* gene with a nuclear localization signal (*nlacZ*) fused to the first 8 amino acids of the MLC3F coding sequence (Fig. 1a; Kelly *et al.*, 1995). Of particular interest was the 10-kb intron separating the MLC1F and MLC3F promoters, since an analysis of DNase I-hypersensitive sites at the MLC1F/3F locus in C2/7 muscle cells had revealed the presence of at least one differentiation-specific DNase I-hypersensitive site within this intron, in addition to a site at the 3′ end of the gene (data not shown). On transient transfection into C2/7 cells, a construct containing 9 kb of intronic sequence upstream of the MLC3F transcriptional start site (3F-*nlacZ*-9) was 25-fold more active in differentiated myotubes than a −2 kb MLC3F promoter (3F-*nlacZ*-2; Fig. 1a). This result suggests that there is a second MLC1F/3F enhancer within this 7-kb intronic sequence.

In order to delimit this regulatory element, sequences between −9 and −2 kb upstream of the first MLC3F exon were sequentially deleted and reporter gene activity assayed in C2/7 myotubes. Activity dropped to 50% that of 3F-*nlacZ*-9 on progressive deletion from −9 to −5 kb (Fig. 1a); activity then dropped sharply to the level of the 3F-*nlacZ*-2 on deletion to −4.2 kb (compare 3F-*nlacZ*-5 and −4.2), suggesting that at least 50% of the activity of the intron lies in the 800-bp sequence from −4.2 to −5 kb. When placed upstream of the −2-kb promoter, the 800-bp sequence enhances transcription with approximately 60% of the activity of the entire intron (3F-*nlacZ*-2I, Fig. 1a). Deletion of a 3.2-kb fragment within the first intron, including the −5 to −4.2 kb region, reduces activity to that of 3F-*nlacZ*-2; reintroduction of a 2.7-kb fragment containing the 800-bp sequence in either orientation restores enhancer activity (Fig. 1a). The intronic element is therefore able to enhance transcription from the MLC3F promoter in a position- and orientation-independent manner.

Transient transfections into C2/7 myoblasts and NIH 3T3 fibroblasts were carried out to assess the cell-type specificity of the intronic enhancer. Reporter gene expression





c

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1                                                                    80
GATCTTAGGG AAGAAATAAA AACGTATTTC CCTATGGGTC AGCATCAGTA ATGTCTTTTG TCTGTTACAT TGTCTATACT
81                                                                  160
GTGGGAGAAC ACAGCAGCTG AAATGGAATG GAGAAACGTT GAGGGGTAAA GGCTGGAACT CTCCCCATAG CGACATTTTG
161                                                                 240
AAGTGTATAT AACTGAACAA GGGTCTTTGC AAAGTATCAC ACAGTTTGCA AAGAAAGACA CGCCTTTACC GACTAAAATG
241                                                                 320
TGAAAACTGC TTTTCATCTA AAGATAATTA AATCTGTGCC AGACGAGGAG ACCATAAAGT GACAGGGCTG GATTTAGCCC
321                                                                 400
TTGACCTTTC AGTTCTGCAA ATGAAACCCC TGGCAAGTTG TCTGTAGAAG TCATTCCATA ATTGCCCAGC CTTAAGCACC
401                                                                 480
ACCTGACGGG TGGGAATTGT GGGAAGGGTT ATATTTAGTC TGCTGAGACT GCACATTCTT CTCTTCCAAC TGAGCGGCCT
481                                                                 560
TTTGTATGAA GATAGGATGT CACCTACTTT ACTCCTTCAT CAATGCTACC ACTTCTGTTT TCTCGCCCAA CCAGAATCCC
561                                                                 640
GACAACAAAA CCCGCTGTAT AAAAGTATCA GTGGCTTCAG AATATGCTAG TTAAATTTTA AATCATGTGG GTGATAAGTC
641                                                                 720
CATTTGTTCT AGTCAGAAAC TAATAATAGT GACTTGAAGC TAGGGAGGAA AGGTTTCTCT TGGAACATTT TTCCTTCTGT
721
CCTTGGGAGT GAGCTGTTTC TTAGCTACTG TGCGCGCCCA ACAGTGTATT ATCTCCAGCT CATAAAAATC CAGTCGAC
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levels were compared to that of a Rous sarcoma virus promoter (RSV*lacZ*). Whereas the activity of 3F-*nlacZ*-9 is approximately equivalent to that of RSV*lacZ* in myotubes, it is approximately 200 times less than RSV*lacZ* in myoblasts (Fig. 1a) and 40 times less in NIH 3T3 fibroblasts (data not shown). Similar results were obtained with the −2 kb promoter plus either the intronic enhancer (3F-*nlacZ*-2I) or the 3′ enhancer (3F-*nlacZ*-2E) (Fig. 1a), demonstrating that both enhancers are active at a high level specifically in differentiated muscle cells. The activity of the 800-bp sequence was tested on a heterologous promoter, the HSV thymidine kinase (TK) promoter driving expression of a CAT reporter gene. The intronic enhancer, in either orientation upstream of the TK promoter, increases CAT activity approximately 10-fold over the promoter alone (Fig. 1b), showing that the activity of the intronic enhancer is not promoter specific. The DNA sequence of the 800-bp intronic enhancer is presented in Fig. 1c. This element contains a number of consensus binding motifs for transcription factors found in other muscle-specific regulatory elements, including 6 E-boxes, the target sites of myogenic basic-helix–loop–helix (bHLH) transcription factors (Weintraub *et al.*, 1991), and several A/T-rich stretches (Fig. 1c).

Comparative Analysis of the Intronic and 3′ Enhancers in Transgenic Mice

The above results establish the presence of a second enhancer at the mouse MLC1F/3F locus which drives high-level MLC3F transcription *in vitro*. What is the role of this element *in vivo*? Transgenic mice containing the intronic element upstream of the MLC3F promoter and *nlacZ* reporter gene (3F-*nlacZ*-9 and 3F-*nlacZ*-2I) were generated and the β-galactosidase expression pattern was compared with that of transgenes containing the MLC3F promoter plus 3′ enhancer (3F-*nlacZ*-2E) or the MLC3F promoter alone (3F-*nlacZ*-2). Transgene constructs and expression profiles are summarized in Table 1; for each construct at least two independent lines were analyzed. We present our findings with respect to (i) the level and cell-type specificity of transgene expression in adult muscle and (ii) the spatiotemporal pattern of transgene expression during embryonic and fetal myogenesis.

The 3′ enhancer has been previously demonstrated to be a strong positive regulatory element in adult skeletal muscle (Rosenthal *et al.*, 1989; Kelly *et al.*, 1995). Transgenic mice carrying the intronic enhancer also display a high level of reporter gene expression in adult muscle: 3F-*nlacZ*-9 and 3F-*nlacZ*-2I transgenic mice exhibit a comparable level and cell-type specificity of β-galactosidase activity to 3F-*nlacZ*-2E mice on whole-mount X-gal colouration of individual muscles (Fig. 2a). Quantitative analysis of transcriptional activity per microgram of tissue extract using a chemiluminescent assay (Jain and Magrath, 1991; Shaper *et al.*, 1994) showed that transgenic mice containing either the intronic (3F-*nlacZ*-9) or the 3′ enhancer (3F-*nlacZ*-2E) have high β-galactosidase activities in adult skeletal muscle (quantified in extensor digitorum longus (EDL), soleus and masseter), and background activities in a control tissue, kidney (Figs. 2b and 2c). These transgenes are also expressed in the myocardium (left ventricle), a site of low-level endogenous MLC3F expression (Kelly *et al.*, 1995). In contrast, only two of four lines of 3F-*nlacZ*-2 transgenic mice containing the −2-kb MLC3F promoter express *nlacZ* (lines 5 and 8), and in these mice expression is confined largely to the heart (Fig. 2d), where activity is comparable to that seen with either enhancer. X-gal treatment of whole-mount muscles from 3F-*nlacZ*-2 mice reveals extremely low-level and patchy β-galactosidase activity in skeletal muscle fibers, although higher levels of expression are observed in a subset of muscles of the head and neck, including regions of the masseter (data not shown). These data demonstrate (i) that the intronic enhancer confers high level transgene expression in adult skeletal muscle in the absence of the 3′ enhancer, and (ii) that while MLC1F/3F sequences included in 3F-*nlacZ*-2 effect transcription in cardiac muscle, the MLC3F promoter does not support significant transgene expression in the majority of skeletal muscles *in vivo*.

The MLC3F isoform is specific to fast fibers in adult skeletal muscle. β-Galactosidase expression was assayed in two hindlimb muscles of adult transgenic mice: the EDL, which contains fast glycolytic type IIB fibers, and the soleus, which contains slow type I and fast oxidative type IIA fibers (Schiaffino and Reggiani, 1996). The level of transgene expression in the soleus of 3F-*nlacZ*-9 transgenic mice is 8- to 10-fold lower than in the EDL (Fig. 2b), and we therefore conclude

FIG. 1. Two skeletal muscle enhancers at the mouse MLC1F/3F locus. (a) Structure of the locus showing 3F-*nlacZ* reporter gene constructs and activity in C2/7 myotubes and myoblasts. Independent MLC1F and MLC3F promoters generate transcripts with differential 5′ splicing patterns; the positions of the 3′ (E, open box) and intronic enhancers (I, shaded box) are indicated. Construct nomenclature refers to the 5′ limit of each construct with respect to the MLC3F transcription start site. Mean β-galactosidase activities plus SEM are expressed relative to that of 3F-*nlacZ*-9 (set arbitrarily at 100 for myotubes). (b) Activity of the intronic enhancer on transcription from a heterologous promoter (TK) in C2/7 myotubes; CAT activities (mean plus SEM) are presented relative to TK-CAT (set arbitrarily at 10). (c) DNA sequence of the 800-bp intronic enhancer. The sequence extends from −5 to −4.2 kb upstream of the MLC3F transcriptional start site (EMBL Accession No. Y08458). E-box target motifs for myogenic bHLH proteins (boxed) and an A/T-rich sequence containing a single mismatch with a MEF2 site in the 5′ enhancer of the M-creatine kinase gene (shaded box) are indicated (Gosset *et al.*, 1989). Open arrows, transcriptional start sites; —, MLC1F and – –, MLC3F splice patterns; rev, intronic enhancer in reverse orientation; Δ(6:2.8), internal intronic deletion from −6 to −2.8 kb upstream of the first MLC3F exon; Δ(6:5.5), internal intronic deletion from −6 to −5.5 kb.

TABLE 1
MLC3F Transgenes: Expression in Striated Muscle

Construct	Transgene +ve F_0 mice	Lines	Transgene copy number	Embryonic (E12.5)		Fetal (E16.5)		Adult	
				Skeletal	Cardiac	Skeletal	Cardiac	Skeletal	Cardiac
3F-*nlacZ*-9	1[a]	9-a	2	−	+	+	+	+	+
		9-b	4	−	+	+	+	+	+
3F-*nlacZ*-21	2	2I-1	nd	−	+	+	+	+	+
		2I-27	nd	−	+	+	+	+	+
3F-*nlacZ*-2E	2	2E-1	7	+	+	+	+	+	+
		2E-2	7	+	+	+	+	+	+
3F-*nlacZ*-2	5[b]	2-5	8	−	+	−	+	−[c]	+
		2-8	18	−	+	−	+	−[c]	+
		2-10	50	−	−	−	−	−	−
		2-11	30	−	−	−	−	−	−

Note. nd, not determined.

[a] Lines 9-a and 9-b were derived from a single 3F-*nlacZ*-9 F_0 mouse (transmission frequencies: 9-a, 49/79 F_1 mice; 9-b, 4/79 F_1 mice). Two independent F_0 3F-*nlacZ*-9 embryos were analyzed at E14.5 and showed an expression profile comparable to that in Fig. 5d.

[b] One 3F-*nlacZ*-2 F_0 mouse did not transmit the transgene (0/38 F_1 mice); in this mouse no transgene expression was observed in adult tissues.

[c] Significant β-galactosidase activity was observed in these lines only in a subset of muscles of the head and neck (data not shown).

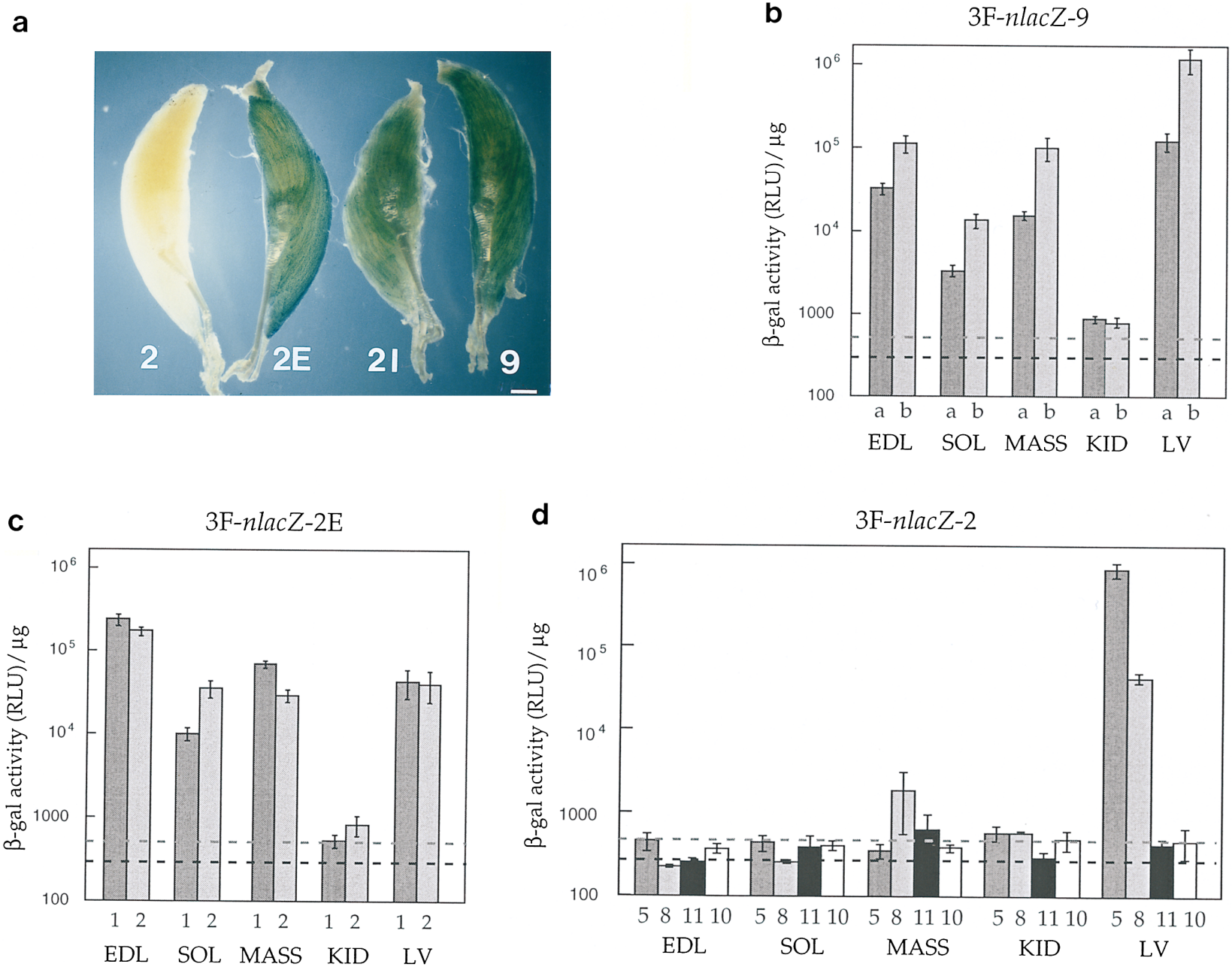
that the transgene is expressed at a higher level in fast IIB than in IIA and slow fibers. 3F-*nlacZ*-2I mice display a similar fiber-type distribution, as does the 3F-*nlacZ*-2E transgene which is down-regulated in muscles with reduced contributions of fast IIB fibers (Fig. 2c; Kelly *et al.*, 1995). Since both the intronic and 3′ enhancers confer a similar fiber-type distribution on the MLC3F promoter, fiber-type restrictive elements may be present in the −2-kb promoter region.

A rostrocaudal gradient of CAT activity has been observed in 1F-CAT-E and 1F-*lacZ*-E transgenic mice (Donoghue *et al.*, 1991; Rao *et al.*, 1996). No positional gradient of *nlacZ* transcripts was observed in 3F-*nlacZ*-2E transgenic mice (Kelly *et al.*, 1995). McGrew *et al.* (1996), however, reported graded reporter gene expression in 3F-CAT-E and 3F-CAT transgenic mice, suggesting that redundant *cis* elements at this locus may contribute to graded expression. We therefore investigated whether *nlacZ* expression was positionally graded in transgenic mice containing the intronic enhancer. The masseter, a rostral muscle with low-level reporter gene expression in 1F-CAT-E mice (100-fold less than EDL, Donoghue *et al.*, 1991), expresses *nlacZ* at a high level in 3F-*nlacZ*-9 and 3F-*nlacZ*-2E mice, suggesting that these constructs do not confer a gradient of transgene activity (Figs. 2b and 2c). Furthermore, no gradient of reporter gene activity was observed across the intercostal muscles of MLC3F transgenic mice containing either enhancer (constructs 3F-*nlacZ*-2E, 3F-*nlacZ*-9, and 3F-*nlacZ*-2I; data not shown). Elements responsible for graded expression are therefore likely to lie outside the sequences included in these 3F-*nlacZ* constructs.

The 3′ but Not the Intronic Enhancer Is Active in Embryonic Skeletal Muscle

Myogenesis *in utero* occurs in two waves: embryonic (E9–E13.5) and fetal (E13.5–birth), which result in the formation of primary and secondary myotubes, respectively (Kelly and Zacks, 1969; Ontell and Kozeka, 1984; Ross *et al.*, 1987). The first embryonic skeletal muscle cells arise in the myo-

FIG. 2. MLC3F transgene expression in adult tissues. (a) Representative X-gal-treated extensor digitorum longus (EDL) muscles from adult MLC3F transgenic mice containing no enhancer (3F-*nlacZ*-2), the 3′ enhancer (3F-*nlacZ*-2E), or the intronic enhancer (3F-*nlacZ*-2I and 3F-*nlacZ*-9); dissected muscles were fixed and stained for β-galactosidase activity in parallel. Mean β-galactosidase activities plus SEM are shown for various adult tissues of transgenic lines containing constructs 3F-*nlacZ*-9 (b), 3F-*nlacZ*-2E (c), and 3F-*nlacZ*-2 (d). Activities are expressed on a logarithmic scale as relative light units (rlu) per microgram of protein extract purified from skeletal muscle (EDL, soleus (SOL) and superficial masseter (MASS)), kidney (KID), and left ventricular cardiac muscle (LV). At least four adult (2–6 months) heterozygotes were assayed per line (two adults for 3F-*nlacZ*-2 lines). EDL and SOL values were determined in duplicate for each animal and represent the mean and SEM of at least 8 muscles (4 for 3F-*nlacZ*-2 lines). Background β-galactosidase activities in nontransgenic adult mice are indicated by broken lines and represent high and low extremes (KID and EDL, respectively). 2, 3F-*nlacZ*-2; 2E, 3F-*nlacZ*-2E; 2I, 3F-*nlacZ*-2I; 9, 3F-*nlacZ*-9. Scale bar: 1 mm.



tomal compartment of the somite at E9. The 3F-*nlacZ*-2E transgene is expressed in the myotome at this stage, 4 days before high-level transcription from the endogenous MLC3F promoter, and at the time of activation of the MLC1F promoter (Fig. 3b; Kelly *et al.*, 1995). The 3F-*nlacZ*-2E transgene is also expressed in the developing heart. At E12.5 3F-*nlacZ*-2E transgene expression extends throughout the embryonic skeletal musculature (Figs. 3e and 3h). Strikingly, despite the presence of the intronic enhancer which is active in adult skeletal muscle, the reporter gene in 3F-*nlacZ*-9 mice is not transcribed in embryonic skeletal muscle; at E10.5 the 3F-*nlacZ*-9 transgene is expressed in the heart, but not in differentiated skeletal muscle cells (Fig. 3a). The 3F-*nlacZ*-9 transgene is also expressed ectopically in the nervous system, notably in dorsal root ganglia which flank the neural tube along the axis of the embryo, adjacent to, but distinct from, the myotome (Figs. 3a, 3d, and 3g). Two days later (E12.5) there is still no detectable expression in embryonic skeletal muscle, although transgene activity persists in the heart and nervous system (Figs. 3d and 3g). Similar results are observed with the 3F-*nlacZ*-2I transgene (results not shown). In the absence of either enhancer, the MLC3F promoter is not expressed in embryonic skeletal muscle (Figs. 3c, 3f, and 3i). Several sites of ectopic expression are seen in MLC3F transgenic mice: 3F-*nlacZ*-9 and 3F-*nlacZ*-2I transgenic mice express *nlacZ* in the roof plate of the neural tube and choroid plexus (Figs. 3a, 3d, and 5a). Transgene-expressing 3F-*nlacZ*-2 lines have β-galactosidase activity in dorsal mesenchymal cells in the forelimb region, and at a low level in dorsal root ganglia (Figs. 3c, 3f, and 5b); expression persists postnatally in the fascia of certain forelimb muscles. In addition, all MLC3F transgenic lines analyzed express β-galactosidase in the dorsal otic vesicle (Kelly *et al.*, 1995). Elements responsible for expression at sites shared by independent transgenic lines are presumably uncovered in the transgenes, and silenced at the MLC1F/3F locus, since none are sites of endogenous MLC3F expression (as assessed by *in situ* hybridization, data not shown). MLC3F transgene expression profiles in embryos prior to E13 thus demonstrate that of the two enhancers which activate the MLC3F promoter in adult skeletal muscle, only the 3′ enhancer is functional in embryonic muscle. We conclude that activity of the intronic enhancer is temporally regulated during skeletal muscle development.

Embryonic myoblasts can be cultured *in vitro* where they differentiate as mono- or oligonucleated myocytes, and have distinct properties from myoblasts isolated at later stages of development (Vivarelli *et al.*, 1988; Smith and Miller, 1992). We investigated whether the intronic enhancer would be inactive in embryonic myocytes when they differentiate outside the embryonic environment. Primary myoblast cultures were prepared from limb buds of E11.5 transgenic embryos and transgene expression analyzed after myocyte differentiation. After 48 hr cultures were fixed and assayed for myosin heavy chain (MHC) and β-galactosidase expression by immunocytochemistry. Numerous differentiated MHC-positive myocytes were observed in all cases. In cultures isolated from 3F-*nlacZ*-9 limb buds, myocyte nuclei were β-galactosidase negative (Figs. 4a and 4b). Weakly positive nuclei were occasionally detected in these cultures after 4–5 days of differentiation. In contrast, nuclei of 3F-*nlacZ*-2E myocytes invariably contained β-galactosidase positive nuclei after 48 hr (Figs. 4c and 4d). The intronic enhancer therefore remains inactive in embryonic muscle cells cultured *in vitro;* MLC3F transgene expression in these cells appears to be dependent on the 3′ enhancer.

The Intronic Enhancer Is Activated during Fetal Development

The experiments described above demonstrate that the intronic enhancer activates transgene expression in adult but not embryonic skeletal muscle. The expression profiles of different transgenic lines were subsequently analyzed during the second, fetal, phase of *in utero* myogenesis. The intronic enhancer becomes active in skeletal muscle during this stage of development; expression of 3F-*nlacZ*-9 and 3F-*nlacZ*-2I transgenes in skeletal muscle is observed from E13.5, appearing initially at a low level in particular muscles, including the cervical and thoracic domains of the developing trapezius muscle (Fig. 5a). In 3F-*nlacZ*-9 line 9-b mice (which express the transgene at a higher level than line 9-a mice) low-level expression is already detectable in a triangular (presumed forming trapezius) muscle in the cervical region at E12.5 (data not shown). In contrast, no skeletal muscle expression is observed in transgene expressing 3F-*nlacZ*-2 lines at E13.5 (Fig. 5b). 3F-*nlacZ*-9 and 3F-*nlacZ*-2I transgene expression increases rapidly from E13.5, initially in muscles adjacent to the developing trapezius including the deltoid muscle, extensor muscles of the forelimb, and the latissimus dorsi (Figs. 5d and 5e). The 3′ enhancer is active in the majority of skeletal muscles at the same stage (Figs. 5c and 5f). A similar profile of late transgene activation was observed in two F_0 3F-*nlacZ*-9 mice analyzed at E14.5 (data not shown). Histological analysis confirms that 3F-*nlacZ*-9 transgenes are expressed in only a subset of muscles which express 3F-*nlacZ*-2E transgenes at E14.5 (Figs. 6a and 6b). Endogenous MLC3F and MLC1F transcripts were localized in fetal skeletal muscles by *in situ* hybridization. MLC3F transcripts are detectable at a low level in the majority of skeletal muscles at E15.5, but accumulate at a higher level in particular muscles which are also those where the intronic enhancer is first activated (Fig. 6c); MLC1F transcripts, in contrast, are present at an equivalent level in different muscles (Fig. 6d). Differential activation of MLC3F transgenes in the presence of either the 3′ or intronic enhancer therefore reflects the differential accumulation of endogenous MLC1F and MLC3F transcripts. In the next 4 days of fetal development the majority of skeletal muscles activate the 3F-*nlacZ*-9 transgene such that by E18.5 expression is comparable to that of 3F-*nlacZ*-2E mice (Fig. 7). Whole-mount X-gal staining of 3F-*nlacZ*-9 and 3F-*nlacZ*-2I transgenes thus reveals

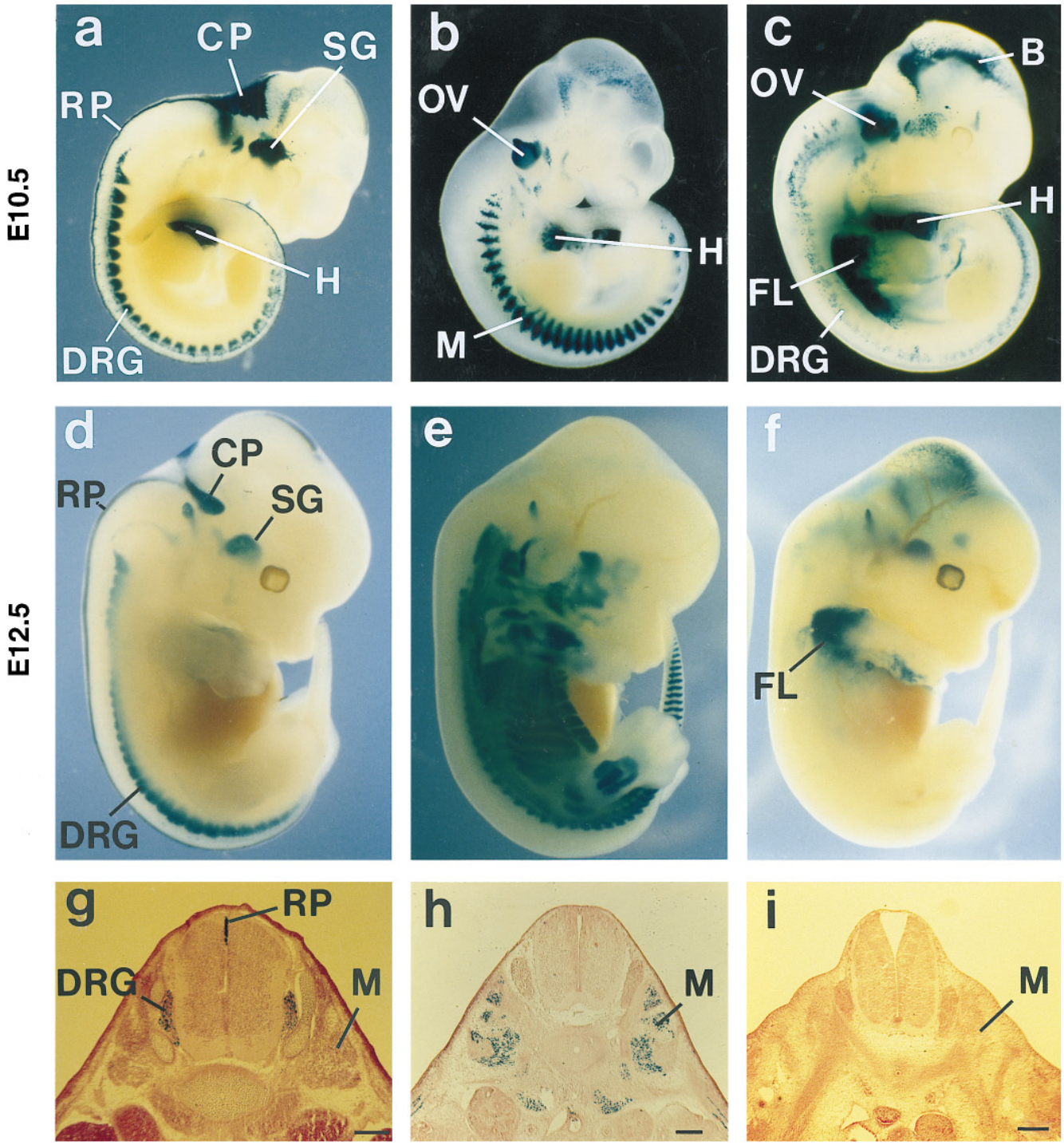


FIG. 3. The intronic enhancer is inactive in embryonic muscle *in vivo*. Representative transgenic embryos stained in whole-mount with X-gal for constructs 3F-*nlacZ*-9 (a and d), 3F-*nlacZ*-2E (b and e) and 3F-*nlacZ*-2 (c and f). At E10.5 only the 3F-*nlacZ*-2E transgene (b) is active in the myotomal compartment (M) of the developing somites. The 3F-*nlacZ*-9 (a) and 3F-*nlacZ*-2 (c) transgenes are expressed in myocardium (H) and ectopically in the nervous system, but not in the myotome. At E12.5 3F-*nlacZ*-9 (d) and 3F-*nlacZ*-2 mice (f) still do not express the transgene in skeletal muscle, whereas the 3F-*nlacZ*-2E (e) transgene is widely expressed in embryonic muscle. Transverse cryostat sections through representative E12.5 embryos show β-galactosidase expression in skeletal muscle (M) of 3F-*nlacZ*-2E mice (h) but not 3F-*nlacZ*-9 (g) or 3F-*nlacZ*-2 mice (i), although the 3F-*nlacZ*-9 transgene is expressed in dorsal root ganglia (DRG) and the roof-plate of the neural tube (RP). CP, forming choroid plexus; SG, semilunar ganglia; FL, *nlacZ*-expressing mesenchymal cells in the forelimb region of 3F-*nlacZ*-2 mice; B, brain; OV, otic vesicle. Scale bars: 200 μm.

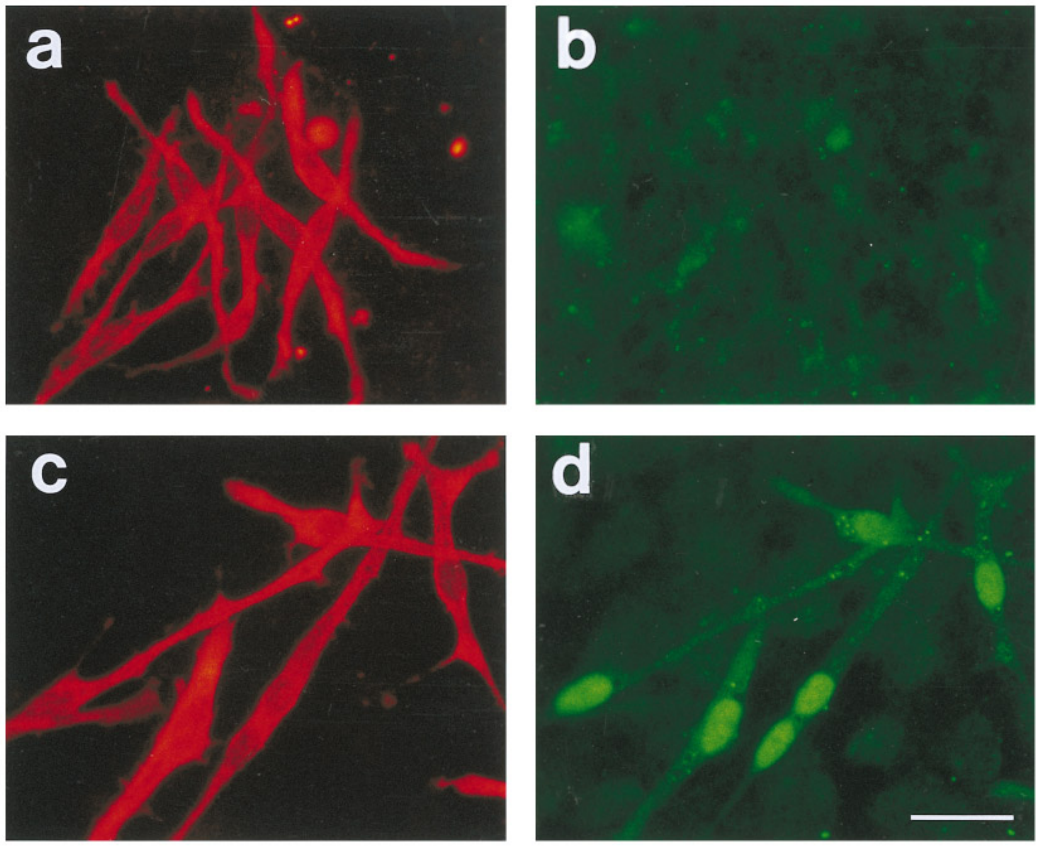
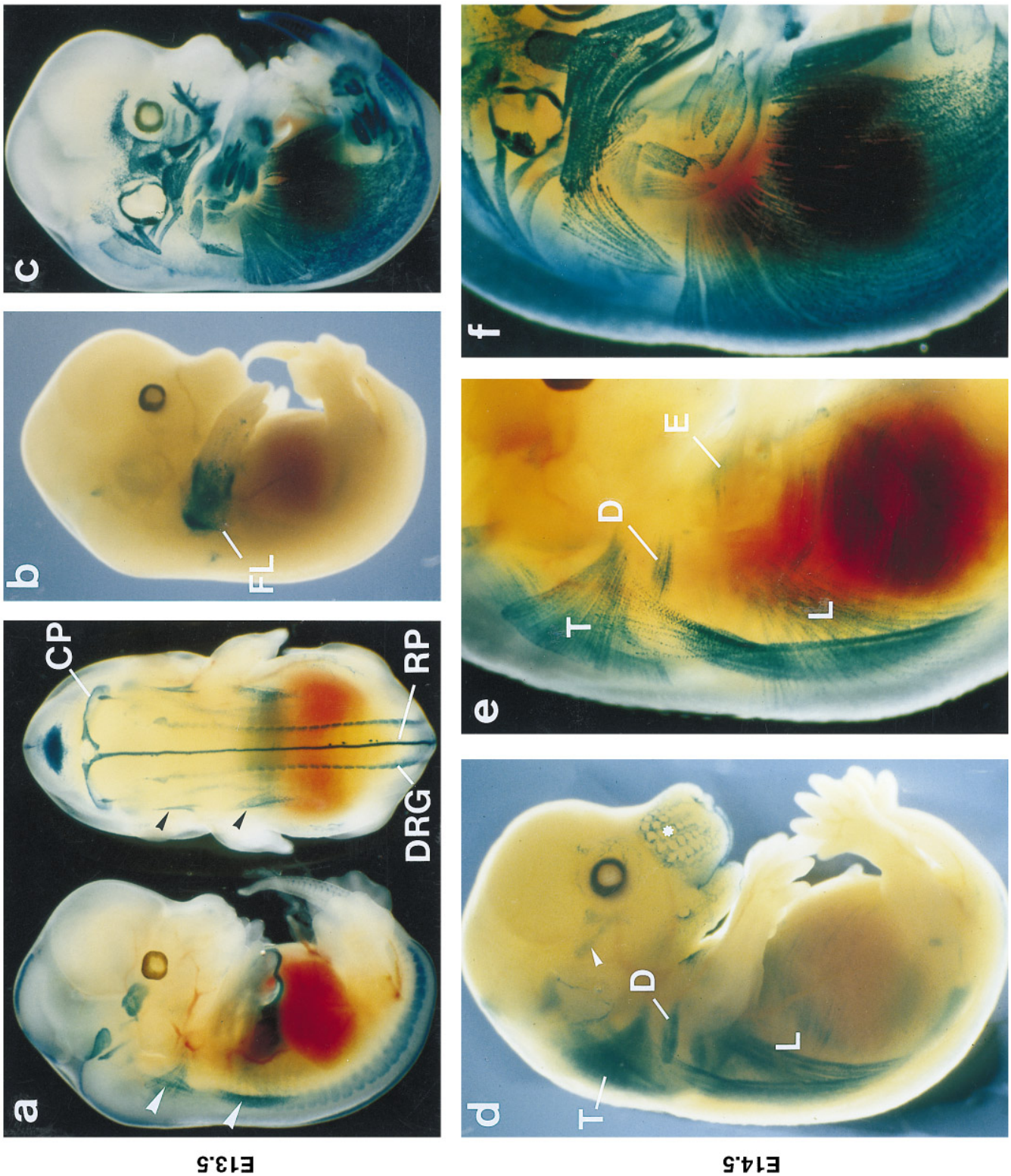


FIG. 4. The intronic enhancer is inactive in embryonic myocytes in culture. Double immunofluoresence analysis of embryonic myocytes derived from myoblasts isolated from E11.5 limb buds of 3F-*nlacZ*-9 (a and b) and 3F-*nlacZ*-2E (c and d) transgenic embryos. After 48 hr in culture cells were fixed and stained with antibodies to detect sarcomeric myosin heavy chain (MHC) (a and c) and β-galactosidase (b and d). Whereas all 3F-*nlacZ*-2E-derived myocytes express the transgene, no β-galactosidase-positive nuclei are observed in 3F-*nlacZ*-9 myocytes. Scale-bar: 50 μm.

a dynamic profile of skeletal muscle gene activation during fetal development.

3F-*nlacZ*-9 and 3F-*nlacZ*-2I transgene activation during fetal myogenesis is coincident with extensive secondary myotube formation. We investigated whether fetal transgene activation was exclusive to secondary myofibers using antibodies to detect β-galactosidase and perinatal and adult fast myosin heavy chain (MHC) isoforms. In the tibialis anterior at E17.5 small secondary fibers can be observed surrounding large centrally nucleated primary fibers. β-Galactosidase-positive nuclei could be detected in both primary and secondary fibers of 3F-*nlacZ*-9 mice (Fig. 8), showing that the intronic enhancer is activated in both myotube populations at the time of secondary fiber formation.

FIG. 5. The intronic enhancer is activated during fetal development. Representative transgenic embryos stained in whole-mount with X-gal for constructs 3F-*nlacZ*-9 (a and e), 3F-*nlacZ*-2 (b), 3F-*nlacZ*-2E (c and f), and 3F-*nlacZ*-2I (d). 3F-*nlacZ*-9 transgene expression initiates in the developing trapezius muscle at E13.5 (a, arrowheads); ectopic expression persists in the nervous system. At E13.5 the 3F-*nlacZ*-2 transgene is inactive in skeletal muscle (b), whereas the 3F-*nlacZ*-2E transgene is expressed throughout the embryonic musculature (c). At E14.5 MLC3F transgenes containing the intronic enhancer are more extensively expressed in skeletal muscles (d, 3F-*nlacZ*-2I; e, 3F-*nlacZ*-9), including the forming trapezius (T), lattisimus dorsi (L), deltoid (D), and forelimb extensor muscles (E). The majority of skeletal muscles express the 3F-*nlacZ*-2E transgene at E14.5 (f). DRG, dorsal root ganglia; RP, roof plate; CP, choroid plexus; arrowhead (d), 3F-*nlacZ*-2I transgene expression initiating in the temporalis muscle; asterisk, ectopic activation of the 3F-*nlacZ*-2I transgene in ectoderm in the nasal region (d); FL, forelimb region showing ectopic activation of the 3F-*nlacZ*-2 transgene (b).



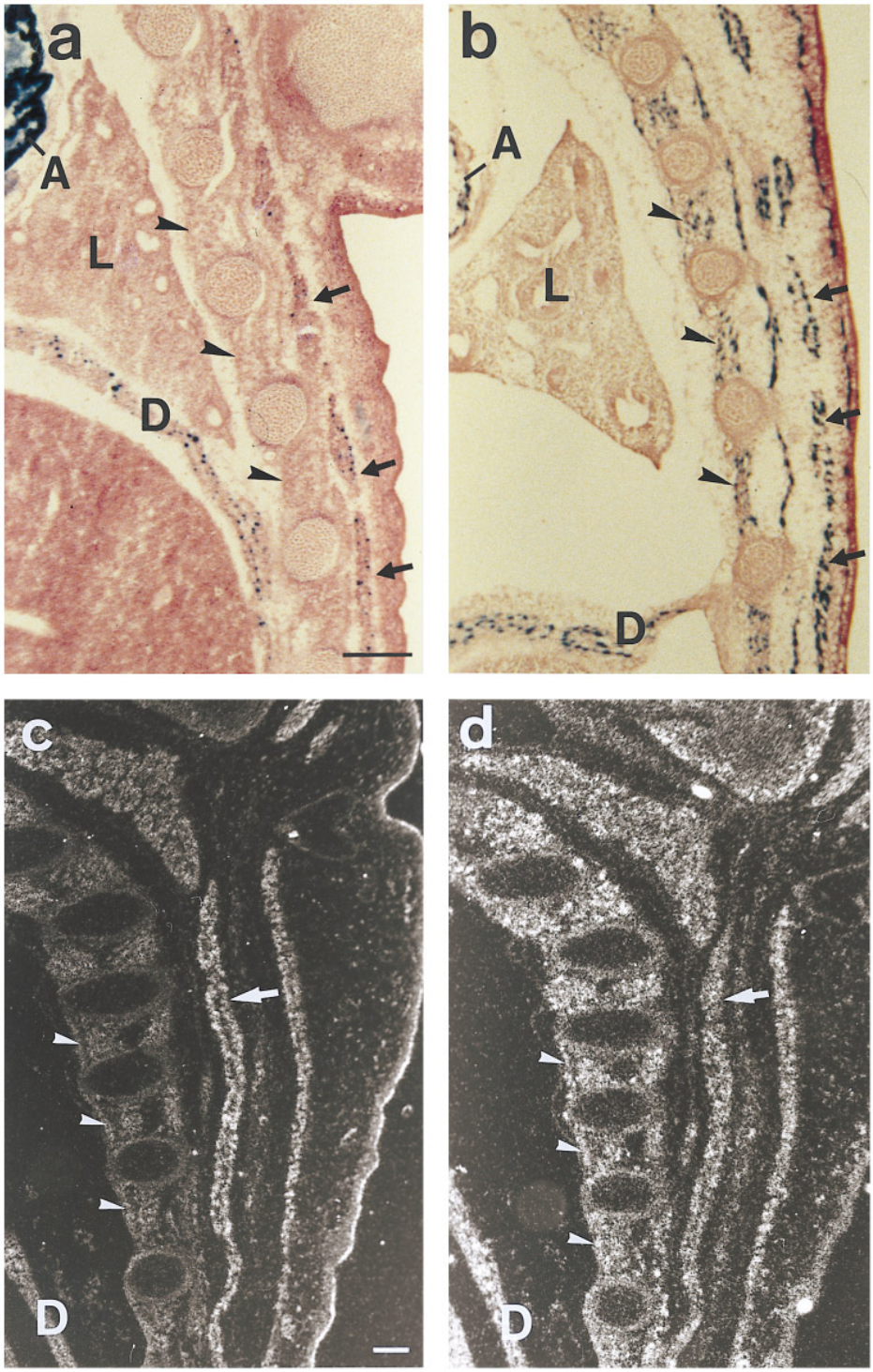


FIG. 6. Fetal activation of MLC3F transgenes. (a) Histological analysis of transgene expression in skeletal muscle of a 3F-*nlacZ*-9 fetus at E14.5. Transgene expression is detected after X-gal treatment in a subset of skeletal muscles at this stage (diaphragm, D; body-wall muscles, arrows) whereas others are β-galactosidase negative (intercostal muscles, arrowheads). The 3F-*nlacZ*-9 transgene is expressed at a high level in myocardial cells (A, atria). (b) The 3F-*nlacZ*-2E transgene is expressed in all skeletal muscles at E14.5. *In situ* hybridization to endogenous MLC3F transcripts (c) and MLC1F transcripts (d) at E15.5; the MLC3F probe detects only processed MLC3F transcripts, whereas the MLC1F probe recognizes unspliced MLC1F transcripts resulting in a nuclear signal. Higher levels of MLC3F transcripts are detected in those muscles which first activate the 3F-*nlacZ*-9 transgene. L, lung. Scale bar: 100 μm.

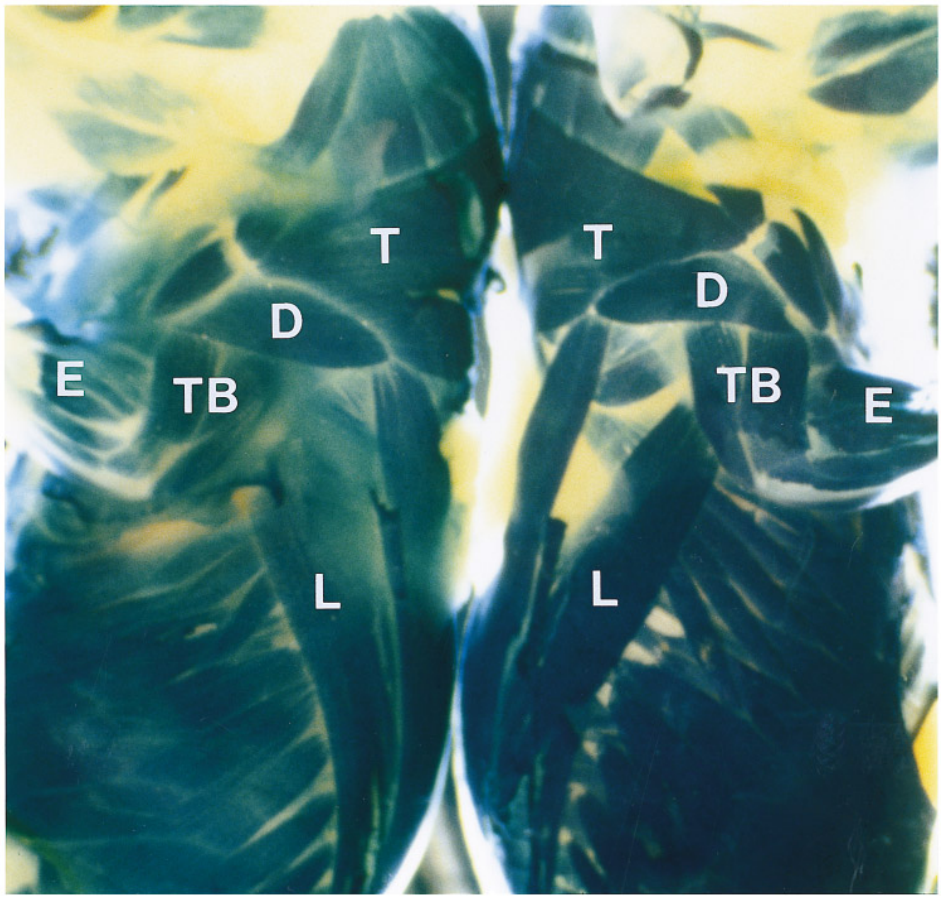


FIG. 7. MLC3F transgene expression at late fetal stages. X-gal-stained skinned fetuses of 3F-*nlacZ*-9 (left) and 3F-*nlacZ*-2E (right) transgenic lines at E18.5. At this stage the majority of skeletal muscles express both transgenes. T, trapezius (cervical part); D, deltoid; TB, triceps brachii; E, forelimb extensor muscles; L, latissimus dorsi.

DISCUSSION

The MLC1F/3F gene contains two independent promoters, MLC1F and MLC3F, which are activated at a high level during embryonic and fetal stages of myogenesis, respectively. In this study we demonstrate that, in addition to a 3′ enhancer active in embryonic, fetal, and adult skeletal muscle, there is a second enhancer in the first intron which is active in adult but not embryonic skeletal muscle. MLC3F transgenes under transcriptional control of this enhancer are activated during fetal development in a dynamic profile of skeletal muscle gene expression which provides an *in toto* image of fetal myogenesis. Embryonic and fetal myogenic programs therefore activate MLC1F/3F transcription through distinct regulatory elements.

Our results show that high-level MLC3F transgene expression in adult skeletal muscle can be mediated by one of two alternative enhancers. These elements correspond to the two DNase I-hypersensitive sites which we had identified at the MLC1F/3F locus. The intronic element eluded detection in the enhancer-trap screen of DNA fragments from the rat MLC1F/3F locus which led to the identification of the 3′ enhancer (Donoghue *et al.*, 1988), possibly because the intronic fragments used did not contain the complete 800-bp element; furthermore, we have observed that the activity of the intronic enhancer is greater on the MLC3F promoter than on the MLC1F promoter used in the enhancer-trap assay (unpublished data). While the 3′ enhancer is clearly an important regulatory element at this locus, our observations suggest that it is not essential for robust skeletal muscle-specific transcription from the MLC3F promoter *in vivo*. Indeed, the 3′ and intronic enhancers confer similar transgene expression profiles in adult muscle. Both enhancers are strong regulatory elements, since all transgene-positive mice express β-galactosidase; the strength of these enhancers is likely to contribute to the low variability in expression levels observed between lines (see Rao *et al.* (1996) for a compar-

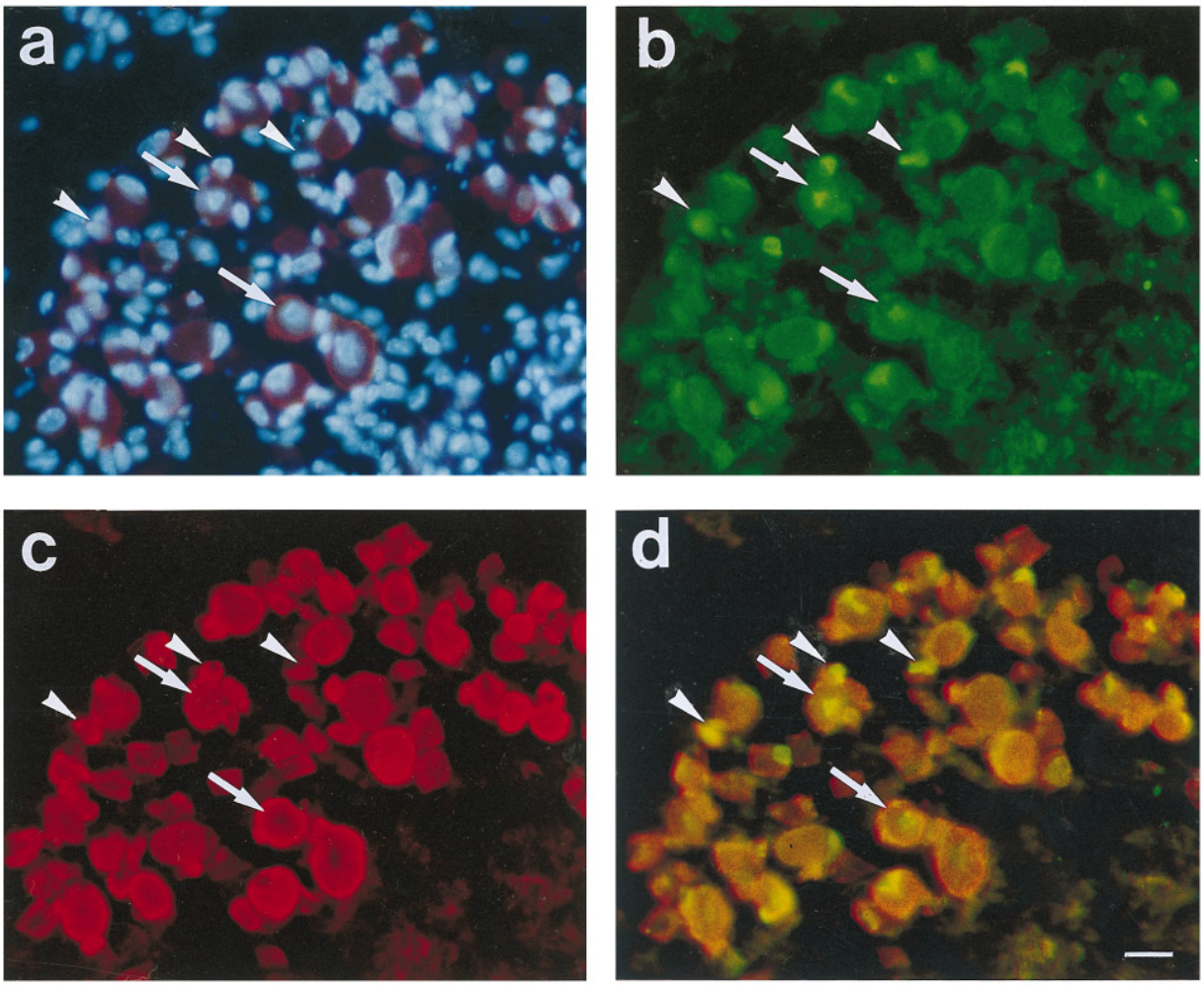


FIG. 8. MLC3F transgene activation in primary and secondary fibers during fetal myogenesis. Double immunofluorescence analysis of cryosections through the tibialis anterior of an E17.5 3F-*nlacZ*-9 fetus. (a) All nuclei in the field are identified by Hoechst stain; (b) nlacZ-positive nuclei are detected with an anti-β-galactosidase antibody (green). (c) Anti-perinatal (fast) myosin heavy chain antibody (red) identifies small secondary and large primary myotubes. (d) Double exposure of the images in (b) and (c) shows that the MLC3F-*nlacZ*-9 transgene is expressed both in the central nuclei of primary fibers (arrows) and peripheral nuclei of secondary fibers (arrowheads). Scale bar: 20 μm.

ison of the strength of the 3′ enhancer with other muscle-specific enhancers). Both enhancers confer a higher level of transgene expression in fast IIB than IIA and slow fibers, reflecting the endogenous MLC3F distribution (Wada and Pette, 1993), and suggesting that *cis*-acting elements which restrict fiber-type distribution may be present in the 2-kb promoter region. Finally, both enhancers confer a high level of β-galactosidase expression in rostral muscles, in contrast to 1F-CAT-E transgenic mice (Donoghue *et al.*, 1991), suggesting that neither the 3′ nor intronic enhancer elements included in the 3F-*nlacZ* constructs confer a rostrocaudal positional gradient on MLC3F transcription. This observation is consistent with the identification of regions within the MLC1F promoter which effect position-dependent expression of the 1F-CAT-E transgene (Rao *et al.*, 1996).

In the absence of either enhancer the 2-kb MLC3F promoter supports only extremely low-level reporter gene expression in the majority of skeletal muscles. Significant levels of expression in particular head and neck muscles of 3F-*nlacZ*-2 transgenic mice, including subregions of the masseter (R. Kelly, P. Zammit, and M. Buckingham, unpublished data), suggest that transcriptional control in anterior muscles may differ from trunk muscles (see Tajbakhsh *et al.*, 1997), consistent with studies in avian embryos demonstrating distinct embryological origins of these muscle groups

(Noden, 1984; Wachtler and Jacob, 1986; Couly *et al.*, 1992). In contrast to skeletal muscle, the 2-kb MLC3F promoter region can effect high-level reporter gene expression in cardiac striated muscle from early embryonic stages (in two of four transgenic lines). *cis*-acting elements conferring cardiac MLC3F expression therefore lie in this extended MLC3F promoter region. This conclusion is in agreement with the observations of McGrew *et al.* (1996) in 3F-CAT and 3F-CAT-E transgenic mice containing a 628-bp rat MLC3F promoter. Although these authors detected CAT expression in pectoral and some limb muscles of mice from individual lines with the promoter alone, skeletal muscle expression was markedly augmented when the 3′ enhancer was present (McGrew *et al.*, 1996).

The major difference in reporter gene activity between mice containing the intronic and 3′ enhancers is the time at which the transgene is activated in skeletal muscle *in utero*. 3F-*nlacZ*-2E transgenic mice activate *nlacZ* from E9, whereas 3F-*nlacZ*-9 and 3F-*nlacZ*-2I mice do not activate the transgene in skeletal muscle prior to E13.5. Significantly, the stages at which the two enhancers are activated correspond to the times at which MLC1F and MLC3F endogenous transcripts accumulate. We therefore conclude that the two enhancers at this locus mediate the differential response to developmental cues resulting in early MLC1F and late MLC3F transcript accumulation. The DNA sequence of the 800-bp intronic element reveals the presence of six target motifs for bHLH proteins, transcription factors which include the myogenic regulatory proteins MyoD, myogenin, myf-5, and MRF4. These proteins have been shown to be key activators of skeletal muscle-specific transcription (Weintraub *et al.*, 1991). The 3′ enhancer also contains E-box motifs which contribute to enhancer activity *in vitro* and *in vivo* (Wentworth *et al.*, 1991; Rao *et al.*, 1996). Myogenic bHLH expression profiles, however, suggest that these regulatory factors alone are unlikely to mediate differential activation of the two enhancers (Lyons and Buckingham, 1992). Experiments are in progress to characterize nuclear factors which interact with the intronic enhancer.

It has been demonstrated that embryonic and fetal muscle cells follow distinct myogenic programs in primary cultures of avian, rodent, and human myoblasts (Haushka, 1974; Stockdale and Miller, 1987; Vivarelli *et al.*, 1988; Smith and Miller, 1992; Stockdale, 1992; Pin and Merrifield, 1993). These studies have shown that embryonic and fetal myoblasts have different morphological properties and growth requirements, and differentiate *in vitro* to generate myotubes with distinctive MHC phenotypes. Embryonic and fetal myoblasts are thus likely to represent distinct lineages predisposed toward different developmental fates. Studies of skeletal muscle gene expression patterns during development suggest that different myogenic programs also exist *in vivo* (see Lyons and Buckingham, 1992). Our results confirm that distinct embryonic and fetal programs of myogenic differentiation operate during development. Analysis of the expression profiles of MLC3F transgenes containing either the 3′ or intronic enhancer demonstrates that these temporally distinct myogenic programs can act through separate regulatory elements at a single locus. Furthermore, since embryonic myoblasts derived from MLC3F transgenic mice containing the intronic enhancer do not activate the transgene on differentiation in culture, our results support the hypothesis that fetal and embryonic myogenic programs occur in myoblast populations which are intrinsically different.

3F-*nlacZ*-9 and 3F-*nlacZ*-2I mice provide the first examples of muscle-specific transgenes activated at E13.5. The *nlacZ* reporter gene allows transgene activity to be monitored in whole-mount embryos by X-gal staining, revealing a previously undocumented profile of muscle-specific gene expression. Initiating in axial muscles, transgene activity rapidly spreads laterally as development proceeds, and by E18.5 expression is approximately equivalent to that of the 3F-*nlacZ*-2E transgene. Endogenous MLC3F transcripts, expressed at a low level throughout fetal muscle, are more abundant in those muscles that first activate the 3F-*nlacZ*-9 and 3F-*nlacZ*-2I transgenes, supporting our conclusion that the 3′ and intronic enhancers mediate differential accumulation of MLC1F and MLC3F transcripts. The onset and subsequent profile of fetal activated MLC3F transgene activity in skeletal muscle provides an *in toto* image of the developmental events associated with fetal myogenesis, and it will be interesting to determine whether other late-upregulated muscle markers (such as MCK, Lyons *et al.*, 1991) follow the same expression profile. MRF4*lacZ* transgenic mice also activate reporter gene expression at fetal stages (although 2–3 days later than 3F-*nlacZ*-9 mice); in one line the spinotrapezius muscle is the only skeletal muscle expressing the transgene at E15.5 (Patapoutian *et al.*, 1993). In contrast to 3F-*nlacZ*-9 mice this remains the only expressing muscle at birth.

Endogenous MLC3F expression has been shown to be nerve dependent in avian and mammalian embryos, in contrast to MLC1F expression (Merrifield and Konigsberg, 1987; Barton *et al.*, 1989). Fetal activation of the 3F-*nlacZ*-9 and 3F-*nlacZ*-2I transgenes occurs concomitantly with innervation and the generation of secondary myotubes. In the hindlimb, anterior crural muscles are innervated and express these transgene markers prior to posterior muscles (Ontell and Kozeka, 1984; R.K., P.Z., and M.B., unpublished data); innervation may therefore contribute to fetal activation of the intronic enhancer. Although coincident with secondary myotube formation, expression of the 3F-*nlacZ*-9 transgene is not exclusive to secondary myotubes since β-galactosidase-positive nuclei are found in both primary and secondary fibers in the tibialis anterior at E17.5; other fetal activated muscle markers such as perinatal MHC protein are also expressed in both secondary (high level) and primary fibers (low level) at this stage of development (see Fig. 8; Condon *et al.*, 1990). The diversity of adult muscle fiber types originates during fetal development (see Kelly and Rubinstein, 1994); analysis of the MLC3F transgenes described here suggests that gene activation through either embryonic or fetal myogenic programs can result in a simi-

lar adult fast-fiber-type distribution. Comparison of the emergence of the adult fiber-type pattern in transgenic lines containing either the 3′ or intronic enhancer will reveal how this convergent distribution is achieved.

A consensus is emerging from transgenic studies that transcription of a gene expressed in spatially diverse muscle cell types is controlled by separable *cis*-acting elements which together generate a composite expression profile. The results presented here demonstrate that temporally distinct profiles of muscle gene expression can also be mediated by separate regulatory elements at a single locus. Transcription at the MLC1F/3F locus is complex and it remains to be shown how the two promoters respond to the 3′ and intronic enhancers in the context of the whole gene. The intronic enhancer, however, reproduces the fetal accumulation of MLC3F transcripts seen *in vivo* from the MLC3F promoter alone, demonstrating that this is a key element in the temporal regulation of MLC1F/3F transcription during development.

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