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Integration of embryonic and fetal skeletal myogenic programs at the *myosin light chain 1f/3f* locus

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Abstract

The genetic control of skeletal muscle differentiation at the onset of myogenesis in the embryo is relatively well understood compared to the formation of muscle during the fetal period giving rise to the bulk of skeletal muscle fibers at birth. The *Mlc1f/3f* (*Myl1*) locus encodes two alkali myosin light chains, Mlc1f and Mlc3f, from two promoters that are differentially regulated during development. The *Mlc1f* promoter is active in embryonic, fetal and adult fast skeletal muscle whereas the *Mlc3f* promoter is upregulated during fetal development and remains on in adult fast skeletal muscle. Two enhancer elements have been identified at the mammalian *Mlc1f/3f* locus, a 3′ element active at all developmental stages and an intronic enhancer activated during fetal development. Here, using transgenesis, we demonstrate that these enhancers act combinatorially to confer the spatial, temporal and quantitative expression profile of the endogenous *Mlc3f* promoter. Using double reporter transgenes we demonstrate that each enhancer can activate both *Mlc1f* and *Mlc3f* promoters in vivo, revealing enhancer sharing rather than exclusive enhancer–promoter interactions. Finally, we demonstrate that the fetal activated enhancer contains critical E-box myogenic regulatory factor binding sites and that enhancer activation is impaired in vivo in the absence of myogenin but not in the absence of innervation. Together our observations provide insights into the regulation of fetal myogenesis and the mechanisms by which temporally distinct genetic programs are integrated at a single locus.

Keywords: Skeletal muscle development; Enhancer; Transgene; Fetal myogenesis; Gene regulation

Introduction

Skeletal muscle development in utero is biphasic: embryonic myogenesis takes place between embryonic day (E) 9.5 and E13.5 in the mouse to generate primary muscle fibers and is followed during fetal development by secondary myogenesis which gives rise to the bulk of skeletal muscle fibers present at birth (Kelly and Zacks, 1969). These successive waves of myogenesis are undertaken by embryonic and fetal myoblasts respectively, which have distinct growth factor responses and proliferative and differentiation capacities (Cossu and Molinaro, 1987; Harris et al., 1989; Condon et al., 1990; Barbieri et al., 1990; Ferrari et al., 1997, Pin et al., 2002). Fetal myogenesis is accompanied by selective upregulation or activation of a number of skeletal muscle genes. Evidence that different transcriptional programs operate in embryonic and fetal myoblasts supports a model by which they correspond to distinct genetic as well as temporal myogenic lineages (Kassar-Duchossoy et al., 2004; Biressi et al., 2007). Fetal myogenesis appears to depend on a Pax3/Pax7 positive progenitor cell population and activation of the myogenic regulatory factor (MRF) genes *Myf5* and *MyoD* by either Pax3 or Pax7 (Relaix et al., 2005, Kassar-Duchossoy et al., 2005). MyoD and Myf5, together with the remaining MRFs myogenin and Mrf4, are essential for the entry of a cell into the myogenic program and its consequent differentiation (Tapscott, 2005; Buckingham, 2006). While

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subsequent steps of the differentiation process and how this differs from that in the embryo are not well understood, recent evidence based on global and gene-specific analysis suggests that late myogenic gene expression is controlled by a MyoD-driven feed-forward mechanism involving myogenin, the MADS-box containing transcription factor Mef2D and the chromatin remodeling enzyme Brg1 (Penn et al., 2004; Ohkawa et al., 2006).

Analysis of the transcriptional regulation of the murine *Mlc3f* promoter provides a tractable system to examine the mechanisms controlling the embryonic and fetal myogenic programs. The *Mlc1f/3f* (*Myl1*) locus encodes two alkali myosin light chains, Mlc1f and Mlc3f, from differentially regulated promoters (Nabeshima et al., 1984; Robert et al., 1984; Periasamy et al., 1984). In contrast to the *Mlc1f* promoter, which is active in embryonic, fetal and adult skeletal muscle, the *Mlc3f* promoter is upregulated during fetal development and remains active in adult skeletal muscle (Barton et al., 1989; Cox and Buckingham, 1992; McGrew et al., 1996). Transcriptional regulation at the *Mlc1f/3f* locus is controlled by a series of regulatory modules, including elements in the *Mlc1f* and *Mlc3f* promoters and two differentially activated muscle-specific enhancers (reviewed in Kelly and Buckingham, 2000). An enhancer located 3′ to the gene (3′ enhancer) is active in embryonic, fetal and adult skeletal muscle (Donoghue et al., 1988; Rosenthal et al., 1989; Rao et al., 1996), while a second enhancer in the first intron (intronic enhancer) is active only in fetal and adult skeletal muscle (Kelly et al., 1997). Transgenic experiments have shown that these regulatory elements control muscle-specificity, temporal regulation, fiber-type specificity and patterned expression along the anterior–posterior axis (Grieshammer et al., 1992; Kelly et al., 1995, 1997; McGrew et al., 1996; Neville et al., 1996; Rao et al., 1996). However, how the activity of the 3′ and intronic enhancers is integrated at the *Mlc1f/3f* locus to regulate differential promoter activities remains unknown.

Transgenic analysis has revealed that different enhancers are generally responsible for expression in different cell types or sub-domains of a particular cell type or tissue, revealing a modular basis to the control of gene transcription (Arnone and Davidson, 1997). Distinct cis-acting modules activate a promoter in particular organs, cell-types or spatial domains in response to particular combinations of trans-acting regulatory factors, and together recapitulate the endogenous range of promoter activity. However, less is known about how different enhancers that function in the same cell type act together to regulate promoter activity. We therefore investigated the effect of both enhancers on the *Mlc3f* promoter in vivo and observed that they act combinatorially to confer the spatial, temporal and quantitative expression profile of the endogenous *Mlc3f* promoter on an *Mlc3f* transgene. Using a double reporter gene system in transgenic mice we observed that both the embryonic and fetal activated enhancers can act on early and late up-regulated promoters, ruling out a simple "one enhancer one promoter" model and revealing enhancer sharing at this locus. Finally, we focused on the molecular regulation of the fetal-activated enhancer and found that E-boxes binding the myogenic regulatory factors MyoD and myogenin are required for enhancer activity in skeletal muscle cells in culture. In vivo, myogenin was found to be required for correct regulation of fetal enhancer activity while innervation was not. Our results provide insight into how embryonic and fetal myogenic programs are integrated to temporally regulate gene expression.

Materials and methods

Mice

Transgenic lines were generated by pronuclear injection using standard protocols as reported previously (Kelly et al., 1997). The *3f-nlacZ-2, 3f-nlacZ-2E*, and *3f-nlacZ-9* transgenes have been previously described (Kelly et al., 1995, 1997). *Pma* mutant mice (Ashby et al., 1993a) were provided by John Harris (University of Otago, New Zealand), and *Myogenin* mutant mice (Hasty et al., 1993) by Eric Olson (University of Texas, Southwestern Medical Centre). Transgene copy numbers were calculated by quantification of Southern blot hybridization experiments with an *Mlc3f* 5′ UTR probe that detects both the endogenous and transgenic loci using a Phosphorimager. Mice were maintained under standard housing conditions and embryos dated using E0.5 day as the date of the vaginal plug. Mice were genotyped using PCR and Southern blot analysis as described in Kelly et al. (1997).

Construction of double reporter gene plasmids

Details of plasmid construction are available on request. Briefly, the human placental alkaline phosphatase reporter gene (*AP*) carrying a polyA sequence (kindly provided by James Sharpe, MRC, Edinburgh) was cloned into a *Hpa*I site in the *Mlc1f* 5′ UTR on a plasmid containing 1.6 kb upstream of the *Mlc1f* transcriptional start site, *Mlc1f* exon 1 and the *Mlc1f/3f* first intron up to a *Sal*I site 4.2 kb upstream of the *Mlc3f* transcriptional start site. A *Sac*II–*Sal*I fragment containing this entire plasmid insert was placed upstream of the same *Sal*I site in *Mlc3f-nlacZ-9* and *Mlc3f-nlacZ-9E* constructs (Kelly et al., 1997).

Transfections and reporter gene assays

Transfections were performed in C2/C7 myoblasts using calcium phosphate precipitation, myotubes harvested after 48 h of differentiation and β-galactosidase and luciferase assays carried out as described (Kelly et al., 1997). Briefly, β-galactosidase assays were performed on 0.2–7% of cell extract (from a 6-cm culture dish) as described in Sambrook and Russell (2001), and using a chemiluminescent reporter assay (Galactolight, Tropix, Bedford, MA) following the manufacturer's instructions. Luminescence was measured on a Berthold luminometer (Model LB-9501). Adult muscles and embryonic tissue were homogenized and quantified using Biorad protein quantification and the Tropix Galactolight kit as reported by Kelly et al. (1997).

Eletrophoretic mobility shift assays

Preparation of nuclear extracts and electrophoretic shift analysis were carried out as described in Catala et al. (1995). Antibodies used were mouse monoclonal anti-myogenin (clone F5D, Developmental Studies Hybridoma Bank), anti-MyoD1 (clone 5.8a, DakoCytomation) and rabbit polyclonal anti-MyoD (gift from Woody Wright).

β-Galactosidase and alkaline phosphatase histology

Embryos and fetuses were dissected and fixed in 4% paraformaldehyde for 20 min to 1 h, while cultured cells were only fixed for 5 min, before extensive rinses in PBS. To visualize β-galactosidase activity, tissues were incubated overnight at 37 °C in X-gal solution (4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$, 400 μg/ml X-gal and 0.02% NP40 in

PBS). Alkaline phosphatase (AP) activity was revealed as described by Cepko et al. (1995) and Sharpe et al. (1998). Briefly, tissues were first rinsed and then incubated in 2 mM $MgCl_2$/PBS for 1 h at 65 °C to inactivate endogenous phosphatases and equilibrated in AP buffer (100 mM Tris–HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween 20 and 2 mM levamisol) for 45 min before being transferred to BM purple (Roche) or 100 mg/ml BCIP 1 mg/ml NBT and incubated at room temperature in the dark. Double stained embryos and cells were incubated in X-gal solution prior to heat inactivation and AP revelation.

Northern blot analysis

Northern blot analysis was carried out according to standard procedures (Sambrook and Russell, 2001) using a probe from the 5′ UTR of the *Mlc3f* transcript.

In situ hybridization

In situ hybridization using ^{35}S labeled probes was carried out as described by Kelly et al. (1995); whole-mount in situ hybridization using digoxygenin labeled probes was carried out according to Kelly et al. (1998). *Mlc1f* exon1 and intronic probes and *Mlc3f* splice-specific probes are as described in Kelly et al. (1998).

Results

The 3′ and intronic enhancers act combinatorially during development

We previously identified a muscle-specific enhancer in the first intron of the *Mlc1f/3f* (*Myl1*) gene which is activated during fetal myogenesis and remains active in adult fast skeletal muscle fibers (Kelly et al., 1997). Intronic enhancer activity is first detected at E13.5 in particular muscles including the trapezius, and then increases rapidly, extending to the majority of skeletal muscles by E18.5 (Kelly et al., 1997). Fetal activation of transgenes carrying this enhancer, either in its genomic context (*3F-nlacZ-9*) or placed upstream of a *Mlc3f* promoter (*3F-nlacZ-2I*), contrasts with the early expression of the *3F-nlacZ-2E* transgene carrying the *Mlc1f/3f* 3′ enhancer element which is first expressed at E9 in the myotome, the time of activation of the *Mlc1f* promoter and 4 days before high-level transcription from the *Mlc3f* promoter (Kelly et al., 1995; McGrew et al., 1996). The intronic and 3′ enhancers therefore function as distinct modules activated in response to temporal transcriptional programs operative during fetal and embryonic myogenesis respectively.

In order to investigate how the activity of these two regulatory modules is integrated in vivo we examined the effects of the presence of both enhancers in a single transgene on transcription from the *Mlc3f* promoter. The 3′ enhancer was introduced into the *3f-nlacZ-9* construct and two transgenic lines containing both enhancers were generated (*3f-nlacZ-9E*; Table 1). Analysis of the spatiotemporal pattern of reporter gene activation in these lines revealed that reporter gene expression initiated in skeletal muscle from E9.5, as in the case of the *3f-nlacZ-2E* transgene (Fig. 1A). This result suggested that the endogenous *Mlc3f* promoter may also respond to the embryonic myogenic program. Re-evaluation of endogenous *Mlc3f* transcript distribution by whole-mount in situ hybridization revealed low level accumulation of *Mlc3f* transcripts in the myotome and embryonic muscle masses at E10.5 (Fig. 1B). The *Mlc3f* promoter is contained within the primary *Mlc1f* transcription unit raising the possibility of hybridization to unspliced *Mlc1f* transcripts. However, *Mlc3f* transcripts were detected using a riboprobe specific to spliced *Mlc3f* transcripts (Fig. 1B) and differed in distribution from *Mlc1f* transcripts (Fig. 1D) and, importantly, also from primary *Mlc1f* transcripts detected using a riboprobe from the first *Mlc1f/3f* intron, which gave a punctate nuclear signal (Fig. 1E). At E12.5 *3f-nlacZ-9E* transgene expression was observed throughout the embryonic skeletal musculature (Figs. 1C, F). Together these results reveal that activity of the 3′ enhancer is not repressed in the presence of the intronic enhancer, supporting an activation model for

Table 1
Mlc3f-nlacZ transgenic lines

	Intronic enhancer	3′ Enhancer	Transgenic line	Transgene copies	Embryonic expression	Fetal expression	Adult expression
3f-nlacZ-2[a]	−	−	5	8	−	−	−
			8	18	−	−	−
			10	50	−	−	−
3f-nlacZ-9[a]	+	−	1	2	−	+	+
			2	4	−	+	+
3f-nlacZ-2E[a]	−	+	1	7	+	+	+
			2	7	+	+	+
3f-nlacZ-9E	+	+	8	2	+	+	+
			13	1	+	+	+
1f-AP-3f-nlacZ-11.5	+	−	11a	30	−	+	+
			11b	20	−	+[b]	+
			13	3	−	+[c]	+[c]
1f-AP-3f-nlacZ-11.5E	+	+	1	3	+	+	+
			2	13	+	+	+
			4	6	+	+	+

[a] Described in Kelly et al. (1997).
[b] Transgene activated in the late fetal period.
[c] β-Galactosidase but no alkaline phosphatase activity observed at E13.5 and adult timepoints.

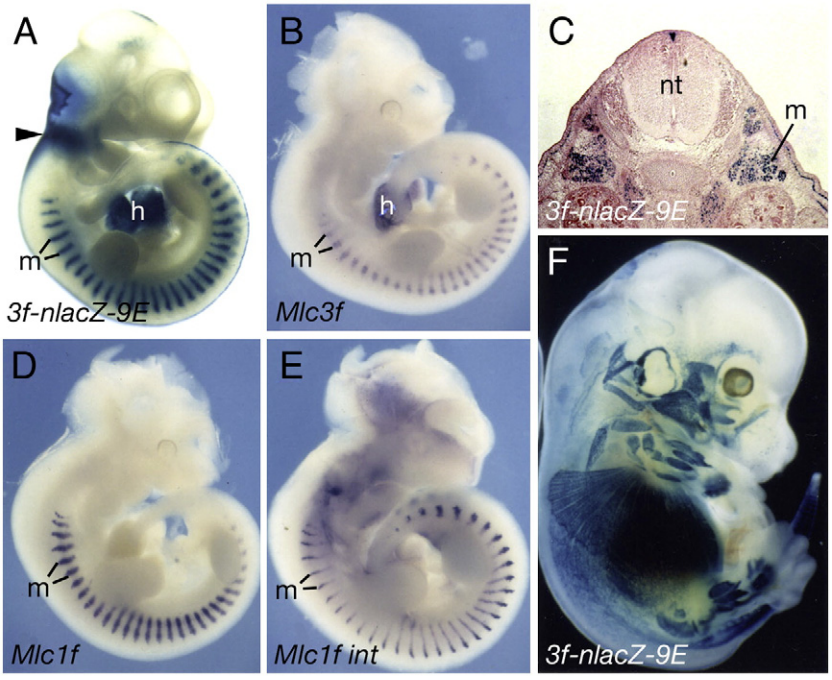

Fig. 1. *3f-nlacZ-9E* transgene and endogenous *Mlc1f/3f* expression in embryonic skeletal muscle. (A) β-Galactosidase activity in an E10.5 *3f-nlacZ-9E* transgenic embryo revealing reporter gene expression in the myotome (m) and heart (h) in addition to ectopic expression in the roof plate of the neural tube (arrowhead). (B) Whole-mount in situ hybridization of a non-transgenic embryo with a *Mlc3f*-specific riboprobe revealing low level *Mlc3f* transcription in the myotome (m) at E10.5; note also expression in the heart (h). (C) X-gal stained cryostat section through an E12.5 *3f-nlacZ-9E* embryo showing transgene expression in embryonic skeletal muscle (m) and the roof plate of the neural tube (nt). (D) In situ hybridization of a non-transgenic E10.5 embryo with an *Mlc1f*-specific riboprobe revealing robust expression in the myotome (m) but not in the heart. (E) An intronic probe specific for unspliced *Mlc1f* transcripts is also positive in the myotome (m—note the narrow myotomal expression domain due to the nuclear signal). (F) β-Galactosidase activity in an E13.5 *3f-nlacZ-9E* embryo showing transgene expression throughout the embryonic musculature.

intronic enhancer activity during fetal myogenesis and corresponding to a low level of endogenous *Mlc3f* transcription during embryonic myogenesis.

Integration of transcriptional programs during fetal myogenesis

The crural muscles of the hindlimb have been well studied as a site of the embryonic to fetal transition during skeletal muscle development (Ontell and Kozeka, 1984; Condon et al., 1990). The Tibialis Anterior (TA) and Extensor Digitorum Longus (EDL) are innervated before other crural muscles and express fetal activated muscle genes including *Mlc3f* and *muscle creatine kinase* (*Ckm*) prior to other crural muscles (Lyons et al., 1991; Ontell et al., 1993). The *3f-nlacZ-9* transgene switches on in the hindlimb at E16.5 (Fig. 2A): this transgene is first activated in the TA and EDL, whereas other crural muscles, including the gastrocnemius and soleus, remain β-galactosidase negative at this stage, and activate the transgene during late fetal and early post-natal development. In contrast, *3F-nlacZ-2E* transgene expression is equivalent in all hindlimb crural muscles at E16.5 (Fig. 2C) before being down-regulated after birth in the soleus muscle as the mature fiber-type distribution emerges (Kelly et al., 1995). Analysis of expression of the *3f-nlacZ-9E* transgene at E16.5 revealed a composite expression profile: expression was observed in all crural muscles with an elevated level of expression in the TA and EDL, suggesting that the reporter gene responds to both enhancer elements (Fig. 2E). This composite expression pattern resembles the endogenous *Mlc3f* expression profile, since *Mlc3f* transcripts are detectable by in situ hybridization at a low level throughout the crural muscles at E16.5, but are at a markedly higher level in the TA and EDL (Fig. 2B; Ontell et al., 1993). *Mlc1f* transcripts, in contrast, are found at an equivalent level in all crural muscles at this stage (Fig. 2D). This result demonstrates that the intronic and 3′ enhancers act in a combinatorial manner on the *Mlc3f* promoter.

The time of activation of the intronic enhancer coincides with innervation of the skeletal musculature during fetal development. For example, the TA and EDL receive innervation before the other developing crural muscles. In order to investigate whether motor innervation was required for intronic enhancer activation, we crossed the *3f-nlacZ-2I* transgene with mice carrying the *Peroneal muscular atrophy* (*Pma*) mutation. *Pma* mutant mice carry a recessive spontaneous mutation specifically affecting the peroneal branch of the sciatic nerve and so lack motor innervation of certain hindlimb muscles, in particular the TA and EDL (Ashby et al., 1993a). Analysis of homozygous *Pma* mutant embryos at E16.5 carrying the *3f-nlacZ-2I* transgene revealed that, despite the lack of motor innervation, activation of the transgene occurred normally in the TA and EDL (Fig. 2F) showing that motor innervation is not required for intronic enhancer activation in vivo.

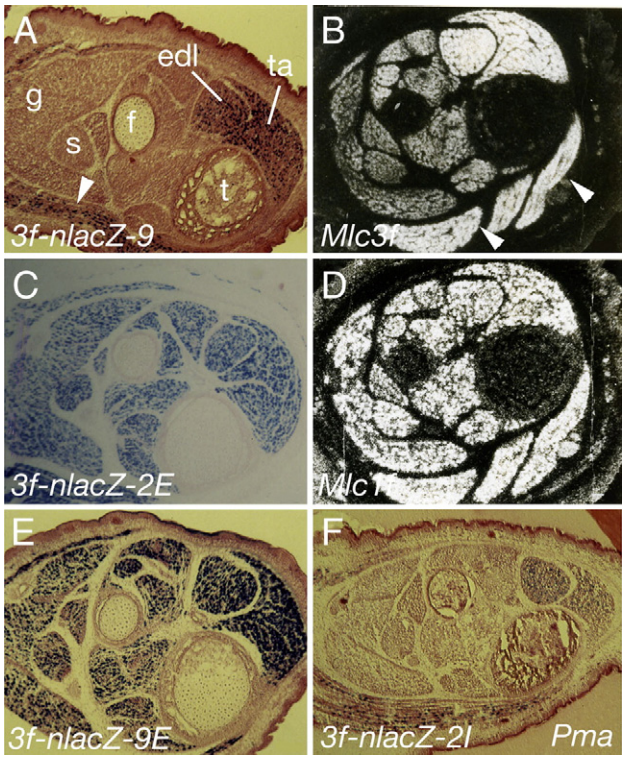

Fig. 2. *Mlc3f* transgene and endogenous *Mlc1f/3f* expression in crural hindlimb muscles. (A) Cryostat section through the lower hindlimb of an E16.5 *3f-nlacZ-9* transgenic embryo stained with X-gal and eosin. Transgene expression at this stage is detected in the Tibialis Anterior (ta) and Extensor Digitorum Longus (edl) muscles but not the gastrocnemius (g) or soleus (s) muscles: location of the fibula (f) and tibia (t) are indicated. Transgene expression is also observed in limb muscles above the knee (arrowhead). (B) In situ hybridization with an *Mlc3f*-specific riboprobe to non-transgenic muscles reveals that endogenous *Mlc3f* transcripts are present at a low level throughout the crus and at an elevated level in the ta and edl. High level *Mlc3f* expression is also observed in limb muscles above the knee (arrowheads). (C) Transgene expression is equivalent in different crural muscles of *3f-nlacZ-2E* mice, as is the distribution of endogenous *Mlc1f* transcripts (D) in non-transgenic muscles (note that this probe detects unspliced transcripts resulting in a punctate nuclear signal). (E) The *3f-nlacZ-9E* transgene is expressed throughout the crus, but at a higher level in the ta and edl, as seen for endogenous *Mlc3f* transcript distribution (B). (F) Section through the crural muscles of an E16.5 *Pma* mutant embryo carrying the *3f-nlacZ-2I* transgene showing normal transgene activation in the ta and edl.

The 3′ and intronic enhancers act synergistically to drive maximal transcription from the Mlc3f promoter in vivo

The observation that endogenous *Mlc3f* transcripts are up-regulated during the fetal period, and that in *3f-nlacZ-9E* transgenic mice, the two enhancers act combinatorially such that the fetal pattern is superimposed upon the embryonic pattern controlled by the 3′ enhancer, raised the question of whether the two enhancers interact to regulate the level of expression from the *Mlc3f* promoter. To investigate this we quantified β-galactosidase activity in adult skeletal muscle (Fig. 3A–D). *3f-nlacZ-9E* mice retained fast fiber-type specificity of transgene expression in adult muscle (Fig. 3A). Transgene activity in skeletal muscle of *3f-nlacZ-9E* mice was extremely high, equivalent to $1.5–2\times10^{-3}$ enzyme units (2–3 ng of β-galactosidase protein at 714 U/mg) per μg total extract (0.2–0.3% total protein). Expressed per transgene copy, β-galactosidase activity is at least 10-fold higher than in either *3f-nlacZ-2E* or *3f-nlacZ-9* mice, an effect not observed in extracts from cardiac muscle where the transgenes are also expressed (Figs. 3B, C). Indeed, levels of transgene mRNA in skeletal muscle in both *3f-nlacZ-9E* lines are equivalent to endogenous *Mlc3f* transcript levels, as detected by northern blot analysis using a *Mlc3f* 5′ UTR probe which hybridizes equivalently to the endogenous and transgenic transcripts (Fig. 3D). The intronic and 3′ enhancer elements therefore act synergistically in adult mice to drive maximal transcription from the *Mlc3f* promoter specifically in skeletal muscle.

In addition to quantifying transgene activity in adult skeletal muscle we quantified β-galactosidase activity in extracts from E12.5 embryos (Fig. 3E–F). At this developmental timepoint *3f-nlacZ-9E* transgene activity in limb muscle masses, expressed as β-galactosidase activity per transgene copy, was approximately 1.5 times that of *3f-nlacZ-2E* mice, while the transgene is not activated at this stage in skeletal muscle of *3f-nlacZ-9* mice (Fig. 3E). At E12.5, therefore, the two enhancers do not act synergistically in skeletal muscle; the level of transgene expression per transgene copy is approximately equivalent in *3f-nlacZ-2E* and *3f-nlacZ-9E* embryos (Fig. 3F). β-Galactosidase values per transgene copy in adult EDL are compared with embryonic hindlimb in Fig. 3G. The increase in *3f-nlacZ-2E* transgene expression between these timepoints is likely to reflect both up-regulation of transgene activity and the fact that only a sub-population of E12.5 hindlimb cells are differentiated skeletal muscle compared with the majority of cells in the adult EDL. There is, however, a 10-fold greater adult/embryonic differential, which is independent of transgene copy number, in *3f-nlacZ-9E* mice (approximate EDL/E12.5 HL ratio = 100) compared with *3f-nlacZ-2E* mice (approximate EDL/E12.5 HL ratio = 10), consistent with a 10-fold greater up-regulation of promoter activity between these timepoints.

Double reporter constructs reveal enhancer sharing rather than exclusive enhancer promoter interactions

The above results demonstrate that the 3′ and intronic enhancers at the *Mlc1f/3f* locus are activated during embryonic and fetal myogenesis respectively and act together to control spatiotemporal transcription from the *Mlc3f* promoter. However, given that there is high level transcription from the *Mlc1f* promoter in embryonic skeletal muscle it is possible that at the endogenous *Mlc1f/3f* locus enhancer–promoter interactions are largely exclusive, such that the 3′ enhancer interacts with the *Mlc1f* promoter and the intronic enhancer with the *Mlc3f* promoter. In order to investigate whether addition of the *Mlc1f* promoter modifies enhancer activity at the *Mlc3f* promoter and whether the intronic enhancer can interact with the *Mlc1f* promoter in vivo, we generated a DNA construct containing both promoters, both enhancers and two reporter genes: the *nlacZ* reporter gene under transcriptional control of the *Mlc3f* promoter and the human placental alkaline phosphatase (*AP*) reporter gene under transcriptional control of the *Mlc1f* promoter (containing 1.6 kb upstream of the *Mlc1f* transcriptional

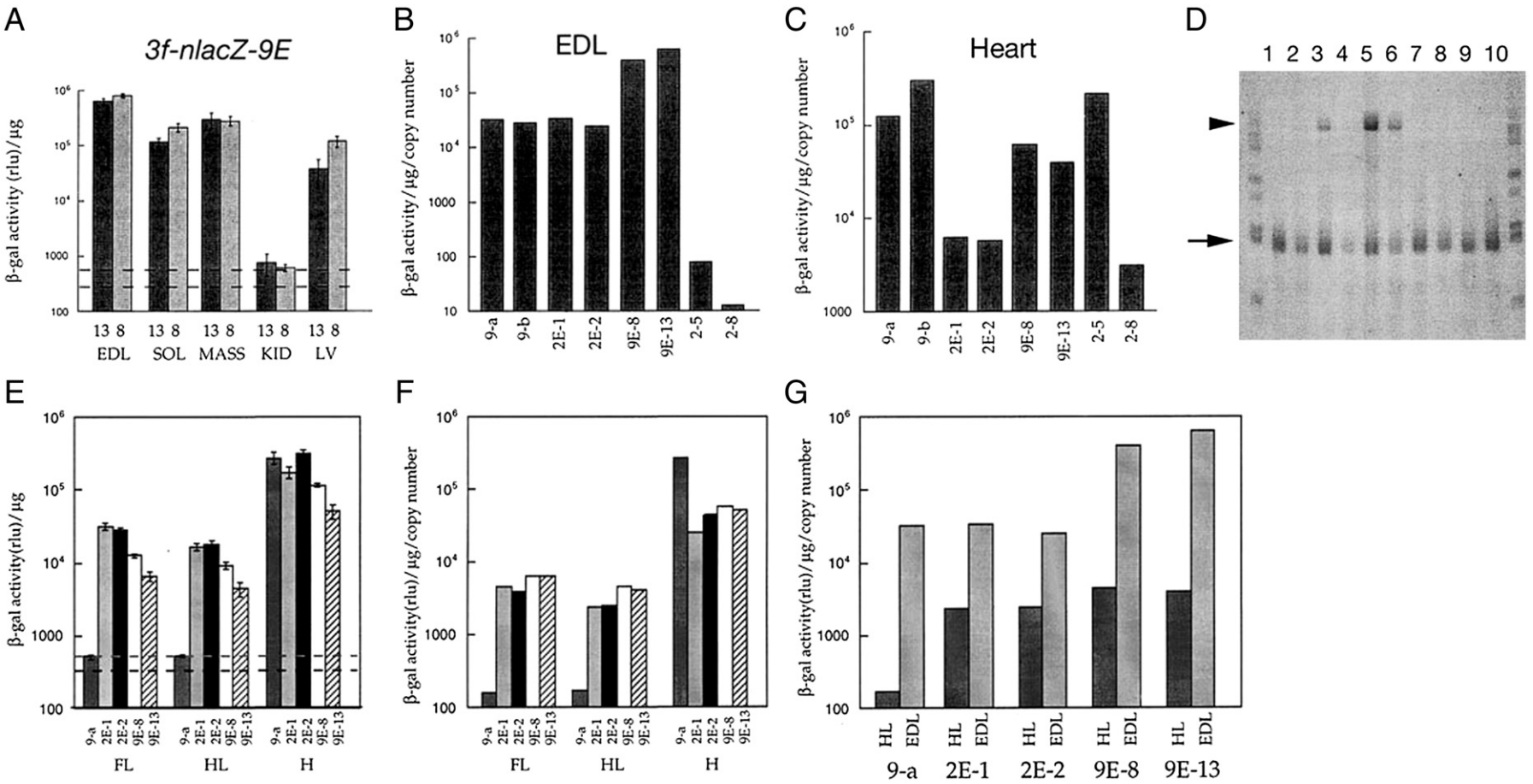

Fig. 3. Quantitative analysis of reporter gene expression in *Mlc3f* transgenic lines. (A) Mean β-galactosidase levels plus S.E.M. (expressed on a logarithmic scale as relative light units per μg protein) are shown for various adult tissues of the two transgenic lines (13 and 8) carrying construct *3f-nlacZ-9E*; EDL, extensor digitorum longus; SOL, soleus; MASS, masseter; KID, kidney; LV, left ventricle. Four adult (2–6 months) heterozygous mice were assayed per line; EDL and SOL values were determined in duplicate for each animal and represent the mean and S.E.M. of eight muscles. Background β-galactosidase activities in non-transgenic adult mice are indicated by broken lines and represent high and low extremes (KID and EDL respectively). (B, C) Comparison of mean β-galactosidase levels expressed per transgene copy number for various *Mlc3f-nlacZ* transgenic lines in skeletal muscle (EDL) and cardiac muscle (Heart). (D) Northern blot analysis of transgene (arrowhead) and endogenous (arrow) *Mlc3f* mRNA accumulation in EDL muscles dissected from various *Mlc3f-nlacZ* transgenic lines containing either, both or no *Mlc1f/3f* enhancers: 1, *3f-nlacZ-9* line 1; 2, *3f-nlacZ-9* line 2; 3, *3f-nlacZ-2E* line 2; 4, *3f-nlacZ-2E*, line 1; 5, *3f-nlacZ-9E* line 8; 6, *3f-nlacZ-9E* line 13; 7, *3f-nlacZ-2* line 5; 8, *3f-nlacZ-2* line 8; 9, *3f-nlacZ-2* line 10; 10, non-transgenic control. The 5′ UTR probe hybridizes equivalently to transgenic and endogenous transcripts. (E) Mean β-galactosidase levels plus S.E.M. in protein extracts from E12.5 forelimb (FL), hindlimb (HL) and heart (H) for various *Mlc3f-nlacZ* transgenic lines (mean values for at least five embryos). Background β-galactosidase activities obtained from non-transgenic embryos are indicated by broken lines and represent high and low extremes. (F) Expression levels per transgene copy, showing that the level of skeletal muscle activity per copy at E12.5 is approximately equivalent in *3f-nalcZ-2E* and *3f-nlacZ-9E* embryos. (G) Comparison of transgene activities in E12.5 hindlimbs (HL) and adult EDL extracts.

start site; construct *1f-AP-3f-nlacZ-11.5E*; Fig. 4A). The activity of both reporter genes can therefore be analyzed by whole-mount or histological analysis in the same embryo (Cepko et al., 1995; Sharpe et al., 1998). A second construct contained both promoters and both reporter genes but only the intronic enhancer (*1f-AP-3f-nlacZ-11.5*; Fig. 4A). Transient transfection of these reporter constructs into C2/C7 myotubes revealed efficient activity of both *nlacZ* and *AP* reporter genes which could be detected simultaneously: β-galactosidase under control of the *Mlc3f* promoter is localized to the nuclear membrane whereas AP, under control of the *Mlc1f* promoter, is localized on the cytoplasmic surface of the cell membrane (Fig. 4B).

Transgenic lines were generated carrying these reporter constructs and the spatiotemporal pattern of reporter gene activity scored (Fig. 4 and Table 1). In the case of the *1f-AP-3f-nlacZ-11.5E* transgene containing both promoters, reporter genes and enhancers (three lines) *nlacZ* activity was observed

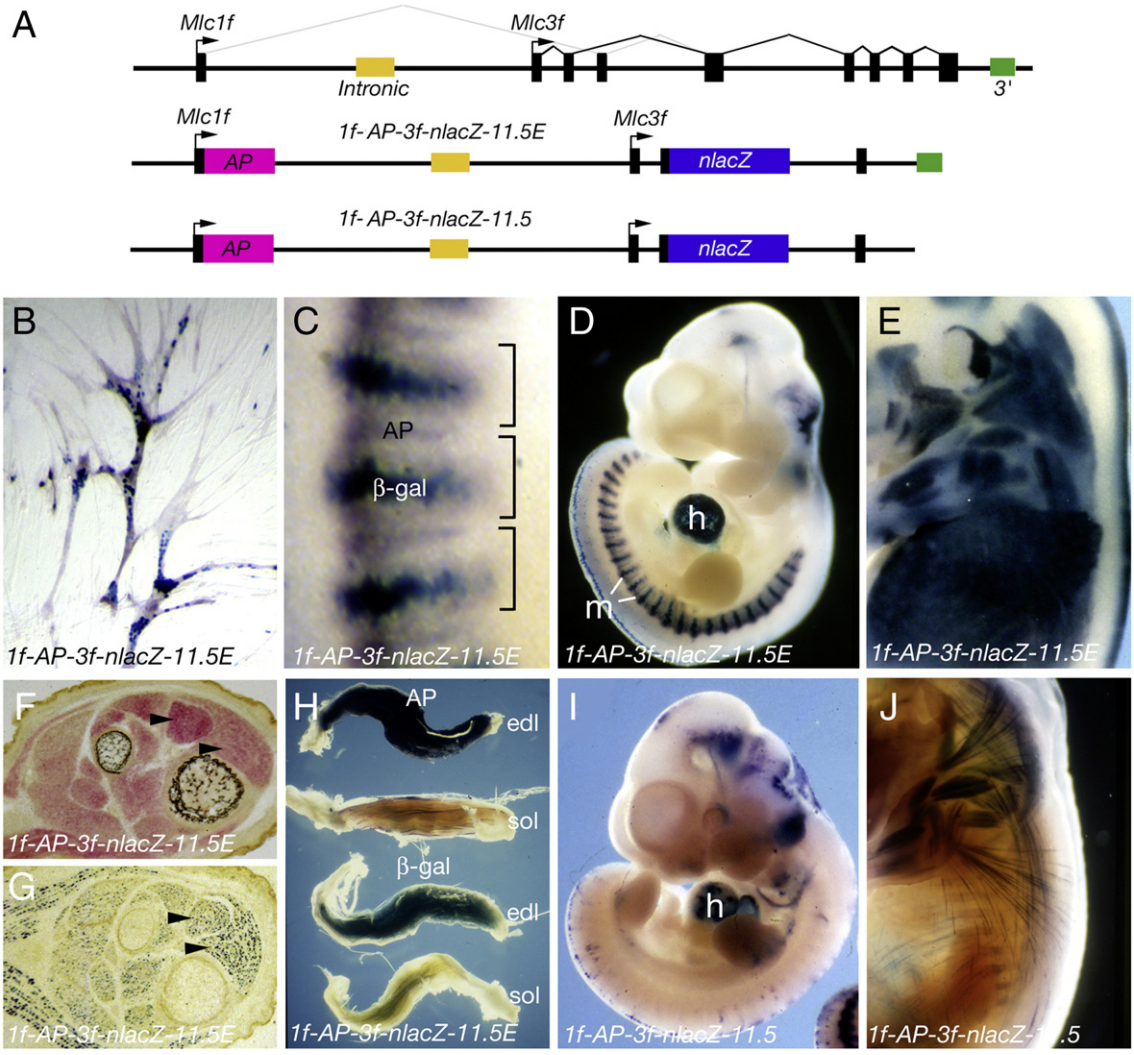

Fig. 4. Analysis of enhancer–promoter interactions at the *Mlc1f/3f* locus using double reporter transgenes. (A) Cartoon of the murine Mlc1f/3f locus showing the relative position of the intronic (yellow) and 3′ (green) enhancer elements and the structure of the *1f-AP-3f-nlacZ-11.5E* and *1f-AP-3f-nlacZ-11.5* transgenes. (B) C2/C7 myotubes transfected with construct *1f-AP-3f-nlacZ-11.5E* and stained for alkaline phosphatase and β-galactosidase activity showing nuclear β-galactosidase (intense blue) under control of the *Mlc3f* promoter and membrane bound alkaline phosphatase (purple) under control of the *Mlc1f* promoter. (C) *1f-AP-3f-nlacZ-11.5E* transgene expression in the myotomal compartment of the somites (brackets) of an E10.5 embryo stained for alkaline phosphatase and β-galactosidase activity. Both reporter genes are expressed: note the centrally aligned nuclei positive for β-galactosidase in contrast to the myotome spanning alkaline phosphatase signal. (D) *1f-AP-3f-nlacZ-11.5E* transgene expression in an E10.5 embryo stained for alkaline phosphatase and β-galactosidase activity. Both reporter genes are expressed in the myotome (m) but only β-galactosidase activity is observed in the heart (h). (E) At E13.5 both reporter genes are expressed throughout the embryonic musculature. (F) Cryostat section from a *1f-AP-3f-nlacZ-11.5E* E16.5 hindlimb stained for alkaline phosphatase activity: note the elevated expression in the TA and EDL (arrowheads); a similar distribution of reporter gene activity is observed for β-galactosidase (G). (H) *1f-AP-3f-nlacZ-11.5E* transgene expression in adult hindlimb muscles stained separately for alkaline phosphatase (top) and β-galactosidase (bottom). Note that both reporter genes are expressed at a higher level in the extensor digitorum longus (EDL) than the soleus (Sol), reflecting fast fiber specificity. (I) *1f-AP-3f-nlacZ-11.5* transgene expression in an E10.5 embryo stained for alkaline phosphatase and β-galactosidase activity. This transgene lacks the 3′ enhancer and neither reporter gene is expressed in the myotome; however β-galactosidase activity is observed in the heart (h). (J) At E14.5 activation of both reporter genes in *1f-AP-3f-nlacZ-11.5 line 11a* embryos has initiated in axial and proximal limb muscles, in a similar profile to that observed for the *3f-nlacZ-9* transgene (Kelly et al., 1997).

from E9.5, as in the case of the *3f-nlacZ-9E* transgene, revealing that the presence of the *Mlc1f* promoter did not deplete activity of the 3′ enhancer from the *Mlc3f* promoter in embryonic skeletal muscle (Figs. 4C, D). *nlacZ* activity was also observed in fetal and adult fast skeletal muscles fibers with this transgene (Figs. 4E, H). Similarly, *AP* activity was present from E9.5 and at all subsequent stages (Figs. 4C–H). Analysis of crural muscles at E16.5 revealed that the 3′ and intronic enhancers act combinatorially on both the *Mlc1f* and *Mlc3f* promoters: β-galactosidase and alkaline phosphatase activity were elevated in the TA and EDL relative to the gastrocnemius (Figs. 4F, G). Both reporter genes displayed a fast fiber-specific expression profile in adult skeletal muscle (Fig. 4H). Minor differences in the temporal activation of *AP* and *nlacZ* reporter genes were observed in second arch-derived craniofacial muscles, which activated *AP* under control of the *Mlc1f* promoter before *nlacZ* (Fig. 4E). In the case of the *1f-AP-3f-nlacZ-11.5* transgene containing both promoters, reporter genes and the intronic enhancer, but lacking the 3′ enhancer (three lines), neither promoter was active in embryonic skeletal muscle prior to E12.5 in any line (Fig. 4I). In two lines, both *AP* and *nlacZ* reporter genes were activated during the fetal period, consistent with the ability of the *Mlc1f* promoter to respond to the activity of the intronic enhancer suggested by the fetal upregulation observed in the *1f-AP-3f-nlacZ-11.5E* lines (Fig. 4J). Timing of onset varied between these lines though, with one active from E13.5 while the other was delayed to late fetal stages. In the third line the *nlacZ* but not *AP* reporter gene was activated from E13.5 (Table 1). Each enhancer can therefore interact with each promoter revealing the prevalence of enhancer sharing rather than exclusive enhancer–promoter interactions at the *Mlc1f/3f* locus.

E-boxes regulate activity of the intronic enhancer

We subsequently investigated the molecular mechanisms responsible for activation of the intronic enhancer using myogenic cells in culture and mutant mouse lines. Our previous work delimited the intronic enhancer to an 800 bp element between 4.2 and 5 kb upstream of the *Mlc3f* promoter (Kelly et al., 1997). This enhancer contains six E-box consensus binding sites (termed A–F), targets for MRF basic–helix–loop–helix transcriptional activators (Fig. 5A). Also present is an A/T-rich sequence containing a single mismatch with a Mef2 site in the 5′ enhancer of the *Ckm* gene (Gossett et al., 1989), located between E-boxes C and D (Fig. 5A). Since E-boxes and Mef2 sites are critically required for the regulation of many muscle-specific genes, including late-activated genes, we explored the requirement for these sites in enhancer function using deletion and mutational analysis assayed by transient transfection in C2/C7 myotubes.

Deletions removing regions containing E-boxes A and B from a construct containing 5 kb upstream of the *Mlc3f* transcription initiation site (*3f-nlacZ-5*) had only a marginal effect on transcriptional activity of the *Mlc3f* promoter. A bigger deletion however, also including a region containing E-boxes C and D and the A/T rich sequence, drastically reduced activity to that of the control 2 kb *Mlc3f* promoter alone (data not shown). Since the transcriptional activity of *3f-nlacZ-5* is indistinguishable from that of a construct containing the 800 bp intronic enhancer upstream of a 2 kb promoter, *3f-nlacZ-2I*, we used this to test the effects of single and combined mutations of E-boxes A–F and the A/T rich sequence (Fig. 5B). Only mutations of E-boxes C and F resulted in significantly reduced reporter gene activity. Mutation of either E-box C or F reduced activity of the *Mlc3f* promoter to approximately 60% of *3f-nlacZ-2I* control levels, while simultaneous mutations in both E-boxes C and F had an additive effect, resulting in activity of only ~45% of control levels (Fig. 5B). Mutation of the Mef2c site alone did not significantly reduce activity compared to control, and when the Mef2c site was mutated together with all E-boxes in the intronic enhancer, activity was not significantly different to when just both E-box C and F were mutated (Fig. 5B). These results demonstrate that intronic enhancer activity is primarily regulated by E-boxes.

MyoD and myogenin bind to the intronic enhancer

Having established that E-box mutations in the intronic enhancer significantly affected transcriptional activity of the *Mlc3f* promoter, we next investigated the DNA binding properties of E-boxes C, E and F using gel retardation analysis. Radioactively labeled oligonucleotides containing E-boxes C, E and F formed complexes after incubation with nuclear extract prepared from differentiated C2 myotubes; both E-boxes C and E bound the same large molecular weight complex, as shown by successful competition of E-box C with unlabelled E-box E and vice versa (Fig. 5C). E-box F however, failed to bind this complex. In addition, E-box C also bound lower molecular weight complexes, absent from E-box E, which were not competed by unlabelled E-box E (Fig. 5C, lane 5). Interestingly, E-box F also bound the same lower molecular weight complexes as E-box C, as shown by successful competition with unlabelled E-box C (Fig. 5C, lane 16) and vice versa (Fig. 5C, lane 9).

To characterize the proteins in these complexes, we used antibodies specific to MyoD, which caused a super-shift of a lower molecular weight complex formed with E-boxes C (Fig. 5C, lane 3) and F, but not of complexes formed by E-box E (Fig. 5C, lane 14). Similarly, incubation with antibodies specific to myogenin also caused a super-shift of lower molecular weight complexes in E-boxes C (Fig. 5C, lane 2) and F (Fig. 5C, lane 19), but not with E-box E (Fig. 5C, lane 13). Competition of E-box C with unlabelled E-box F and vice versa, but not E-box E, prevented a shift in the presence of MyoD (Fig. 5C, compare lanes 7 and 11) and myogenin antibodies (Fig. 5C, compare lanes 6 and 10), confirming that MyoD and myogenin bind to both E-box C and F. To confirm that the E-boxes, and not the flanking sequences, were responsible for binding complexes, oligonucleotides were prepared incorporating mutations to E-box C, E and F (Fig. 5A). These oligonucleotides failed to form complexes and were unable to compete the complexes formed by the wild type E-boxes (data not shown). MyoD and myogenin can therefore interact directly with the intronic enhancer element, binding to the two E-boxes which

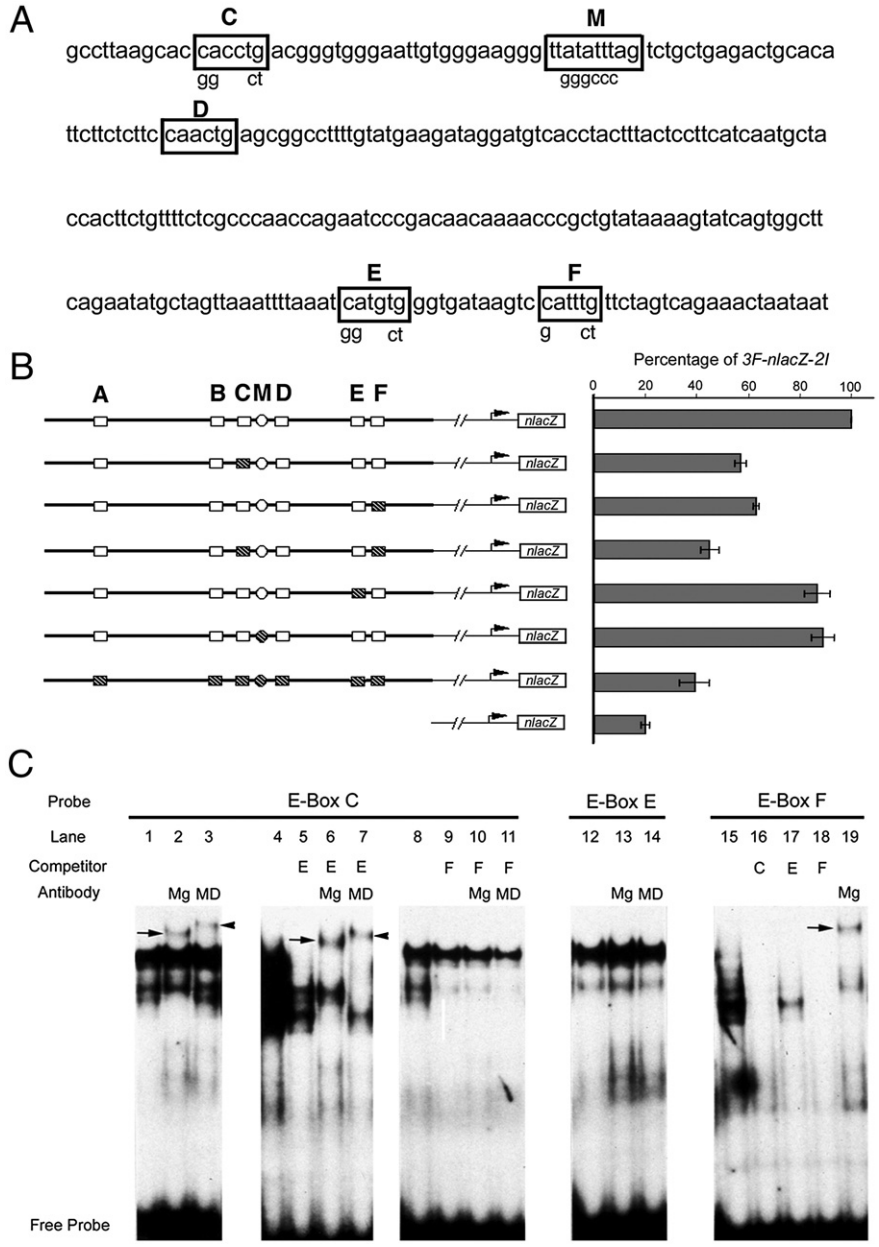

Fig. 5. Molecular analysis of the *Mlc1f/3f* intronic enhancer. (A) DNA sequence of the murine intronic enhancer showing the position of E-boxes C–F and the Mef2-related site (M). Introduced mutations are indicated under each site. (B) Mutation analysis of candidate transcription factor binding sites in the 800 bp intronic enhancer assayed in C2/C7 myotubes. The mutations introduced in E-boxes C and F (see panel A) significantly reduced enhancer activity, having an accumulative effect when both were mutated in *3f-nlacZ-2I*. Mutation of the Mef2-related site in conjugation with mutations in E-boxes A–F did not further reduce activity. Reporter gene expression is presented relative to *3f-nlacZ-2I* (100%) and represents the mean of at least six data points from three experiments. (C) Electrophoretic mobility shift assays using radiolabelled oligonucleotide probes containing the indicated E-box motifs after incubation with C2/C7 myotube nuclear extract. The complexes bound by E-box C (lane 1) and F (lane 15), but not E-box E (lane 12), contain myogenin and MyoD, as shown by a super-shift after pre-incubation of the nuclear extract with antibodies specific to myogenin (Mg—arrow, compare lanes 2, 6 and 19 with 13) and MyoD (MD—arrowhead, compare lanes 3 and 7 with 14). The presence of unlabelled competitor oligonucleotides of E-box F (lanes 10 and 11) prevented this shift with E-Box C, confirming that E-box F also binds MyoD and myogenin, whereas unlabelled E-box E (lanes 6 and 7) was unable to compete.

account for approximately 60% of enhancer activity in C2/C7 myotubes.

Myogenin controls the timing of intronic enhancer activation in vivo

The above in vitro analysis revealed that the activity of the intronic enhancer is controlled by E-boxes, two of which bind myogenin and MyoD. Of the different MRF mutations which have been analyzed, *Myogenin* mutant embryos have a phenotype specifically affecting fetal myogenesis: mutant mice have relatively normal primary myogenesis but perturbed secondary myogenesis, resulting in a failure of fetal myoblasts to differentiate and severe muscle hypoplasia at birth (Hasty et al., 1993; Nabeshima et al., 1993; Venuti et al., 1995). This phenotype is consistent with a feed-forward model of late

myogenic gene activation whereby myogenin activates loci previously bound by MyoD, together with Mef2D and the chromatin remodeling enzyme Brg1 (Penn et al., 2004; Ohkawa et al., 2007). Given that myogenin binds to functional E-box motifs in the fetal-activated *Mlc1f/3f* intronic enhancer we investigated whether myogenin contributes to the control of intronic enhancer activation in vivo. Transgenic mice containing the intronic (*3f-nlacZ-9*) or 3′ (*3f-nlacZ-2E*) *Mlc1f/3f* enhancers were crossed to mice heterozygous for a *Myogenin* null allele (Hasty et al., 1993). After crossing heterozygous mutant mice carrying either transgene, reporter gene expression was scored in myogenin null embryos and control littermates. This analysis revealed that the *3f-nlacZ-2E* transgene, containing the 3′ enhancer element, was normally activated during embryogenesis and remained expressed in the absence of myogenin at E13.5 (Figs. 6A and D). However, no activation of the intronic enhancer regulated transgene was observed at this stage (compare wt in Fig. 6B, with *Myog*$^{-/-}$ in 6E). Activation of the *3f-nlacZ-9* transgene was delayed by 3–4 days in the absence of myogenin and only low level transgene expression was observed in a small number of skeletal muscle fibers at E17.5 (compare wt in Fig. 6C with *Myog*$^{-/-}$ in 6F), in a similar distribution to the reported appearance of perinatal–myosin heavy chain expression and likely reflecting the severe deficiency in muscle at this stage (Venuti et al., 1995). The defined requirement for myogenin during fetal myogenesis thus contributes to control the time of activation of the *Mlc3f* intronic enhancer during normal development.

Discussion

The biphasic transcriptional regulation of the *Mlc1f/3f* locus provides a model of how gene expression is controlled during embryonic versus fetal myogenesis. Our results suggest that the 3′ and intronic enhancers act combinatorially to regulate spatiotemporal *Mlc3f* transcription and synergistically to confer maximal transcription rates in adult skeletal muscle. Together the spatiotemporal pattern and level of transgene expression in the presence of both enhancers approach that of the endogenous *Mlc3f* gene, indicating that the *3f-nlacZ-9E* construct may contain all the regulatory elements necessary for normal activation of this promoter. In the presence of both enhancers, β-galactosidase activity per transgene copy is an order of magnitude higher than in *3f-nlacZ-2E* or *3f-nlacZ-9* mice. This synergistic effect is specific to adult skeletal muscle and is not observed in cardiac muscle, another site of transgene expression. This combination of regulatory elements thus defines a regulatory cassette capable of driving extremely high levels of skeletal muscle expression of potential interest for gene therapy applications.

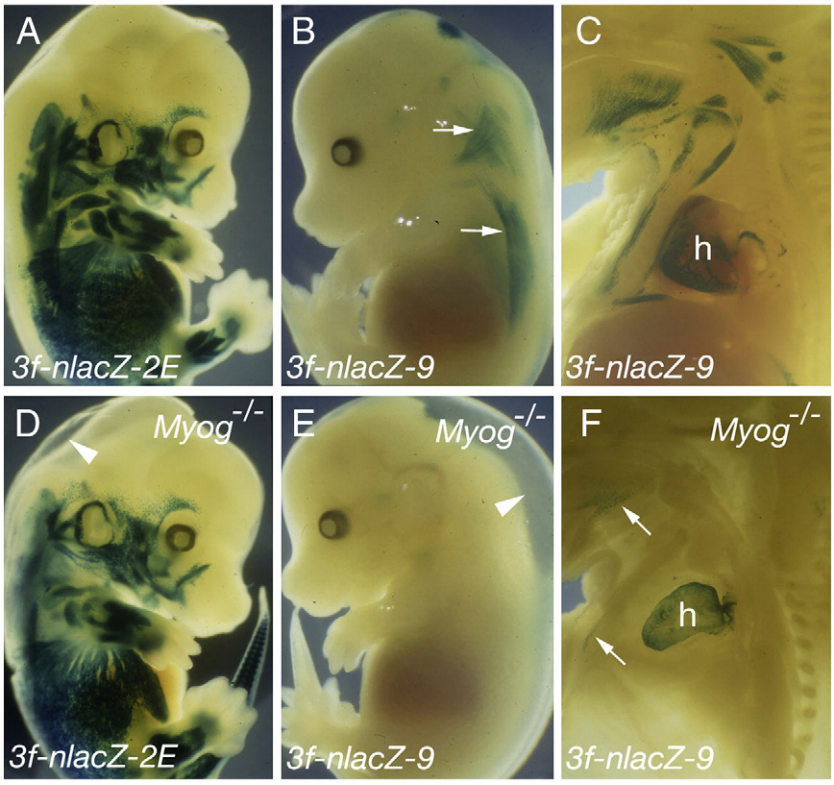

Fig. 6. Analysis of *Mlc3f* transgene expression in *Myogenin*$^{-/-}$ embryos. (A) X-gal stained E13.5 *Mlc3f-nlacZ-2E* embryo showing transgene expression throughout the embryonic musculature. (B) The *Mlc3f-nlacZ-9* transgene is activated at this stage in specific dorsal muscles including the cervical and thoracic regions of the forming trapezius muscles (arrows). (C) Bisected E17.5 *Mlc3f-nlacZ-9* embryo showing that at this stage, the transgene is widely expressed in skeletal muscle and the heart (h). (D) An approximately normal expression profile of the *3f-nlacZ-2E* transgene is observed in *Myogenin*$^{-/-}$ embryos; note the edema characteristic of mutant embryos (arrowhead). (E) In contrast, the *3f-nlacZ-9* transgene fails to be activated at E13.5 in the absence of myogenin. (F) At E17.5 a small number of *3f-nlacZ-9* transgene positive fibers are observed in *Myogenin*$^{-/-}$ embryos whereas normal transgene expression is observed in the heart.

In contrast to the situation in adult skeletal muscle, no synergistic effect in the presence of both enhancers is observed in embryonic skeletal muscle. The differential between embryonic and adult hindlimb β-galactosidase activity is therefore 10-fold greater when both enhancers are present than when only the 3′ enhancer is present in the transgene, a fold difference which is independent of transgene copy number. These data are consistent with the marked up-regulation of *Mlc3f* as opposed to *Mlc1f* transcription in fetal skeletal muscle (Cox and Buckingham, 1992; McGrew et al., 1996). Cox and Buckingham (1992) estimated by nuclear run-on analysis, using nuclei isolated from hindlimb skeletal muscle, that whereas *Mlc1f* transcription increases 2-fold between E14.5 and E18.5, *Mlc3f* transcription increases 14-fold in the same period to attain the level of activity of *Mlc1f*. Our data suggest that this substantial increase in *Mlc3f* transcription rate results from late activation of the intronic enhancer at E13.5, coupled with promoter-selective synergy (see below).

Other muscle-specific genes have been shown to contain more than one enhancer, each of which may regulate distinct subsets of transcriptional specificities (Johnson et al., 1989; Gremke et al., 1993; Zhu et al., 1995). A similar phenomenon of in vivo synergy in adult skeletal muscle has also been demonstrated in MCK-CAT transgenic mice, where a 5′ enhancer interacts with a sequence immediately upstream of the promoter to drive transcriptional activity in skeletal and cardiac muscle at a higher level than that observed with either cis-acting element alone (Donoviel et al., 1996). Functionally identical pairs of enhancers at the *Drosophila Troponin I*, *Troponin T* and *Paramyosin* loci have been shown to interact synergistically to control correct expression levels; in the case of *Troponin T,* synergism was higher in larval than adult muscles (Marin et al., 2004; Mas et al., 2004; Marco-Ferreres et al., 2005). Together with our results, these findings support the view that spatiotemporal specificity and maximal transcription levels in vivo depend on the concerted activities of distinct cis-acting regulatory sequences.

The *3f-nlacZ-9* construct lacks regulatory elements present at the endogenous locus, including the *Mlc1f* promoter and 3′ enhancer, whereas the presence of both enhancers in *3f-nlacZ-9E* mice may more closely approximate the endogenous situation. However, the *Mlc1f* promoter is also present at the endogenous locus, and since both enhancers can act on both promoters it was important to investigate the promoter specificities of the enhancer elements during development and in adult skeletal muscle. We addressed this question in transgenic mice containing *nlacZ* under control of the *Mlc3f* promoter and an alkaline phosphatase reporter gene under control of the *Mlc1f* promoter. A similar two reporter system has been used to analyze enhancer–promoter interactions at the *Hoxb* locus, revealing evidence for selectivity, sharing and competitive interactions which may contribute to the maintenance of *Hox* gene organization (Sharpe et al., 1998). Our results revealed no evidence for selectivity or competition in the regulation of the *Mlc1f* and *Mlc3f* promoters. Instead we observed that the 3′ enhancer continues to direct transcription from the *Mlc3f* promoter in embryonic skeletal muscle in the presence of the *Mlc1f* promoter, and that in the absence of the 3′ enhancer the intronic enhancer activates the *Mlc1f* promoter as well as the *Mlc3f* promoter during the fetal period. Rather than a "one enhancer one promoter" model of transcriptional control, enhancer sharing appears to dominate at the *Mlc1f/3f* locus. This may be achieved by a dynamic flip-flop mechanism, as proposed in the case of the *β-globin* gene cluster (Wijgerde et al., 1995) or by independent activation of the *Mlc1f* and *Mlc3f* promoters in different nuclei within syncitial myotubes. Mononuclear embryonic myocytes were observed to express both reporter genes, thus arguing in favor of a dynamic flip-flop mechanism. However, given that the *Mlc3f* promoter lies within the *Mlc1f* primary transcription domain, in contrast to the situation at adjacent promoters in the *β-globin* or *Hox* loci, a third possibility is allele-specific transcription of each promoter. Why the *Mlc3f* rather than *Mlc1f* promoter should be selectively upregulated during fetal myogenesis remains to be explained in the light of an enhancer sharing model. We favor the hypothesis that the synergistic effect of the intronic and 3′ enhancers on the level of reporter gene expression may be specific to the *Mlc3f* promoter. Quantification of *Mlc1f* promoter activity in the presence of each enhancer alone versus both enhancers may resolve this issue. Interestingly, synergy between enhancer elements has been reported at the *kappa immunoglobulin light chain* locus in B-cell lines (Blasquez et al., 1992; Fulton and Van Ness, 1993). High level transcription from a kappa V-region promoter is dependent on two enhancer elements which are activated at different stages of B-cell maturation, and which synergize to effect maximal transcription levels (Fulton and Van Ness, 1993). Like the *Mlc1f/3f* gene, the *kappa immunoglobulin light chain* gene uses two promoters only one of which supports synergistic enhancer activity. Promoter-selective enhancer synergy therefore provides a mechanism by which enhancer–promoter interactions may control the developmental modulation of gene expression. Our results point to the importance of such mechanisms in vivo, and complement models of enhancer action at other loci containing multiple promoters (Wijgerde et al., 1995; Sharpe et al., 1998; Spitz et al., 2005).

We have shown that transcriptional activity of the intronic enhancer in cultured myotubes is E-box driven, with both MyoD and myogenin binding specifically. The 3′ enhancer also contains multiple E-box motifs required for activity in muscle cells in culture and in transgenic mice (Wentworth et al., 1991; Rao et al., 1996). Our observation that the intronic enhancer fails to be activated at E13.5 in the absence of myogenin, whereas the activity of the 3′ enhancer is unaffected, suggests that a differential response of the enhancers to different MRFs may contribute to the temporal regulation of transcription at the *Mlc1f/3f* locus. The timing of MRF protein accumulation during development and the analysis of MRF null phenotypes suggests that myogenin is the principal MRF driving differentiation at the time of onset of intronic enhancer activation (see Tajbakhsh and Buckingham, 2000). The avian *Mlc1f/3f* gene has also been shown to have two enhancers, in this case both upstream of the *Mlc1f* promoter, one of which is MyoD responsive and one myogenin responsive (Asakura et al., 1993). Of the MRFs, MyoD was found to be most efficient at transactivating the

mammalian 3′ enhancer and Myogenin least so (Rosenthal et al., 1990). BC3H and L6E9 cells express *Mlc3f* but not *Mlc1f* transcripts and upregulate *Mlc1f* expression on *MyoD* transfection (Brennan et al., 1990; Muthuchamy et al., 1992). The intronic enhancer may therefore be selectively responsive to myogenin, which plays a critical role in generating muscle fiber mass during fetal myogenesis (Venuti et al., 1995). Indeed, a number of selective in vivo myogenin targets have recently been identified (Davie et al., 2007). Analysis of conditional mutant mice lacking myogenin in postnatal muscle suggests that myogenin does not play a role in transcriptional regulation of contractile protein encoding genes after birth (Knapp et al., 2006); once activated, the intronic and 3′ enhancer elements may be regulated by common mechanisms.

An alternative hypothesis is suggested by the recent finding that MyoD and myogenin have distinct regulatory roles at a similar set of target genes (Tapscott, 2005). Global and gene-specific analyses have shown that a feed-forward mechanism driven by MyoD underlies the temporal regulation of myogenic gene expression both in cell culture systems and during development. MyoD binding is sufficient to activate early myogenic regulatory genes whereas late activated genes are regulated by myogenin, Mef2D and the chromatin remodeling enzyme Brg1 (Penn et al., 2004; Cao et al., 2006; Ohkawa et al., 2006). During embryonic development MyoD protein accumulates after the onset of myogenesis suggesting a role for Mrf4 in myotome differentiation (see Tajbakhsh and Buckingham, 2000). Activation of late myogenic genes is dependent on prior MyoD-mediated regional histone modification and activation of the p38 mitogen activated protein kinase pathway (Penn et al., 2004; Ohkawa et al., 2007). Such a MyoD-driven feed-forward mechanism may operate at the *Mlc1f/3f* intronic enhancer; furthermore, our results suggest that sequential activation of early and late myogenic regulatory elements may take place at a single locus.

While E-box sites appear to be key regulatory elements controlling activity of the intronic and 3′ enhancers, additional transacting factors binding in the context of these two regulatory elements may also contribute to the different time of activation of these regulatory elements (Ferrari et al., 1997; Ernst et al., 1991; Gong et al., 1997; Ceccarelli et al., 1999; Penn et al., 2004; Ohkawa et al., 2007). Investigation of occupancy of the 3′ and intronic enhancers by chromatin immunoprecipitation with embryonic and fetal tissue will provide further insights into the molecular mechanisms controlling temporal gene expression at this locus.

In contrast to the importance of myogenin in fetal activation of the intronic enhancer, we demonstrate that activation is independent of innervation. Nerve–muscle contacts are established during the fetal period and innervation has been implicated in fetal upregulation of *Mlc3f* transcription. In avians, innervation was shown to be required for limb bud grafts to accumulate Mlc3f protein whereas aneural grafts accumulated only Mlc1f protein (Merrifield and Konigsberg, 1987). In utero injection of β-bungarotoxin in mice causing the destruction of peripheral nerves resulted in a major decrease in *Mlc3f* transcript accumulation (Barton et al., 1989); similarly, laser ablation of the lumbrosacral spinal cord at E14 prevented high level *Mlc3f* transcript accumulation in the hindlimb (Washabaugh et al., 1998). Despite this evidence for a role of neural influences on upregulation of *Mlc3f* expression we observed that the intronic enhancer is activated at the correct time in aneural hindlimb muscles of *Pma* mutant mice. Secondary myogenesis is known to occur relatively normally in aneural *Pma* hindlimb muscles, although this is perturbed by chronic paralysis induced by tetrodotoxin (Ashby et al., 1993b). Spontaneous contraction and passive stretch of aneural muscles in an otherwise innvervated limb, may therefore be sufficient for secondary myogenesis and possibly also for *Mlc3f* upregulation, indicating that direct nerve/muscle contact and electrical activity may not be necessary.

In conclusion, we have shown that the two regulatory modules characterized at the *Mlc1f/3f* locus act combinatorially to control spatiotemporal gene activation and synergistically to drive high level transcription; furthermore, the activities of these enhancers are non-exclusive but are shared by the *Mlc1f* and *Mlc3f* promoters. The fetal activation of one of these enhancers provides a direct demonstration of how myogenin orchestrates fetal myogenesis. These results provide insights into how biphasic myogenic programs impact on gene expression and how separate regulatory modules act together to control transcription from target promoters.

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