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Myf5 expression in somites and limb buds of mouse embryos is controlled by two distinct distal enhancer activities

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SUMMARY

The initiation of skeletal muscle development in the mouse embryo is strictly associated with the expression of the muscle-specific transcription factor Myf5, the first of four myogenic regulatory factors (MRFs) to be expressed in muscle progenitors, and ablation of the *Myf5* gene prevents myogenesis. The complex spatiotemporal expression pattern of Myf5 depends on many discrete regulatory elements that are dispersed over long distances throughout the gene locus. These multiple control modules act differently in the various muscle precursor populations, presumably in response to diverse signals that control myogenesis. A potent enhancer region regulating *Myf5* expression in limb muscles and somites has been identified previously at –58/–48 kb upstream of the transcriptional start site (Hadchouel et al., 2000). Here, we focus on the physical and functional dissection of this control region. We demonstrate that a conserved sequence of 270 bp located around –57 kb is required and sufficient to drive *Myf5* expression in limbs and to maintain it in somites. A second enhancer nearby is responsible for *Myf5* transcription in occipital/cranial somites. This enhancer activity also directs expression accurately to the myotome, preventing ectopic expression in the dermomyotome during the second phase of *Myf5* gene activation in somites.

Our data suggest that the enhancer identified here collaborates with other somitic enhancers to ensure correct myotomal *Myf5* expression. Moreover, it constitutes an important element that mediates somitic expression after the initial and transient Myf5 activation through a previously described sonic hedgehog-dependent early epaxial enhancer.

Key words: Myogenesis, Complex Myf5 regulation, Distal enhancers, Mouse

INTRODUCTION

In vertebrates, skeletal muscles of trunk, limbs, tongue and diaphragm are derived from somites, whereas facial muscles arise from paraxial head and prechordal mesoderm (Christ et al., 1992; Noden et al., 1999). Starting at about embryonic day 8 (E8.0) in the mouse embryo, somites are generated in rostrocaudal sequence on both sides of the neural tube by segmentation of the paraxial mesoderm into epithelialized tissue blocks. Shortly after their formation, somites give rise to the mesenchymal sclerotome on the ventral side and the epithelial dermomyotome on the dorsal side (Christ and Ordahl, 1995). Beginning in cranial somites at about E8.5, the first myotomal cells segregate from the dorsomedial lip (DML) of the dermomyotome adjacent to the neural tube and become positioned underneath the epithelium. This part of the myotome is frequently referred to as epaxial myotome, because it later gives rise to the epaxial musculature in the back. Between E9.5 and E10.0, dermomyotomal cells from the ventrolateral edge also involute and form the hypaxial myotome and the somitic bud, which contribute progenitors to the intercostal and ventral body wall muscles (Cinnamon et al., 1999; Denetclaw and Ordahl, 2000; Ordahl and Le Douarin, 1992). There is also evidence for a third (dermo)myotomal region between the epaxial and hypaxial domains, referred to as intercalated myotome (Sporle and Schughart, 1998; Tajbakhsh and Sporle, 1998). All myotomal regions eventually develop into a continuous sheet of cells with no apparent histological distinction (Kalcheim et al., 1999). At the limb level, muscle progenitor cells delaminate from the ventral (hypaxial) dermomyotome and migrate to the limb buds. Similarly, cervical/occipital somites also contain migratory muscle precursors that will form the hypoglossal cord and eventually give rise to tongue and pharyngeal muscles (Mackenzie et al., 1998; Noden, 1983).

Genetic evidence argues that formation of myoblasts from myogenic precursor cells in somites and prechordal mesoderm, and their successive differentiation into muscle cells involve the expression of four myogenic regulatory factors (MRFs): Myf5 (Braun et al., 1989), myogenin (Edmondson and Olson, 1989; Wright et al., 1989), Mrf4 (*Myf6* – Mouse Genome Informatics) (Braun et al., 1990; Miner and Wold, 1990; Rhodes and Konieczny, 1989) and MyoD (Myod1 – Mouse Genome Informatics) (Davis et al., 1987). These are members of the basic helix-loop-helix (bHLH) family of transcription factors (reviewed by Arnold and Braun, 2000). *Myf5* is the first

gene expressed in all muscle progenitors, beginning in the DML of the dermomyotome, which rapidly generates the epaxial myotome at E8.0 of mouse embryogenesis (Ott et al., 1991). Somewhat later, *Myf5* is also expressed in the hypaxial somitic domain (Tajbakhsh et al., 1997). Expression of myogenin and *Mrf4* in somites follows that of *Myf5* by 0.5 days and 1 day, respectively (Bober et al., 1991; Sassoon et al., 1989). *MyoD* is also expressed in the myotome ~2.5 days after the onset of *Myf5* expression (reviewed by Buckingham, 1992). Interestingly, the migratory muscle precursors destined to the limbs do not express *Myf5* until premuscle masses have accumulated in limb buds. Here and in head muscles, *Myf5* expression is immediately followed by *MyoD* transcription. In *Myf5*-deficient mouse embryos, myotome formation is delayed until *MyoD* transcription begins independently of *Myf5*, suggesting that Myf5 alone is essential for the formation of the early myotome and the onset of myogenesis (Braun et al., 1992; Braun et al., 1994), whereas MyoD appears to contribute to myogenesis in a separate, parallel pathway (Rudnicki et al., 1992). Consequently, mice lacking both Myf5 and MyoD are unable to form myotomes and muscle elsewhere (Rudnicki et al., 1993).

Activation of the myogenin gene apparently depends on the prior expression of *Myf5*, as deduced from transgenic studies and the temporal order of MRF expression in mouse embryos (Cheng et al., 1993; Yee and Rigby, 1993). These observations and those in other MRF-deficient mouse mutants have led to the model that *Myf5* and *MyoD* are myogenic determination genes that are essential for the specification of myogenic cell fate that act upstream of myogenin and possibly Mrf4, which both seem to be involved in the terminal differentiation program (Braun and Arnold, 1995; Venuti et al., 1995). Consistent with this view are observations that Myf5 and MyoD are capable of remodeling chromatin and opening gene loci that participate in further muscle differentiation (Bergstrom and Tapscott, 2001; Gerber et al., 1997). Taken together, the demonstrated functions of *Myf5* and its early expression prior to the other MRFs argue that, under normal circumstances, *Myf5* acts at the top of the myogenic cascade and initiates myogenesis in vertebrates.

Activation of the myogenic determination gene *Myf5* in the various muscle-forming regions undoubtedly depends on multiple signals that have to be integrated to regulate initiation and maintenance of transcription. Indeed, control elements that mediate *Myf5* gene regulation are beginning to emerge. Using yeast artificial chromosomes (YACs) in chimeric mouse embryos, we have shown previously that regulatory elements driving faithful *Myf5* expression in limb buds are located at least 45 kb upstream of the transcription start site (Zweigerdt et al., 1997). More recently, multiple proximal enhancer elements and large, distantly located control regions for particular progenitor cell populations in distinct myogenic locations have been identified in transgenic mouse embryos (Carvajal et al., 2001; Gustafsson et al., 2002; Hadchouel et al., 2000; Summerbell et al., 2000; Teboul et al., 2002). Deletion series of YACs and bacterial artificial chromosomes (BACs) carrying the *Myf5*/*Mrf4* gene locus demonstrated that most of the regulatory regions seem to function in a modular fashion, each affecting specific aspects of the spatiotemporal expression pattern of *Myf5*, emphasizing the enormous complexity of this regulation (Carvajal et al., 2001; Hadchouel et al., 2000).

Based on plasmid-derived transgenes in mouse embryos, three distinct enhancers have been found proximal to *Myf5* (Summerbell et al., 2000). The intragenic enhancer located within the transcribed sequence of *Myf5* functions in the hypaxial domain of somites but drives reporter gene expression incorrectly in the dermomyotome and in the posterior half of the somites. A second enhancer in the intergenic region between *Mrf4* and *Myf5* initiates transcription in muscle progenitors within branchial arches that subsequently give rise to facial muscles (Patapoutian et al., 1993; Summerbell et al., 2000). A third sequence, located –6.1 kb upstream of *Myf5* and close to *Mrf4*, directs early, transient expression in the epaxial dermomyotome as a direct target of long-range sonic hedgehog (Shh) signaling (Gustafsson et al., 2002; Teboul et al., 2002). None of these three regulatory elements, however, was capable of mediating *Myf5* activation in limb buds and maintaining it appropriately in the other muscle-forming regions. Moreover, these enhancers failed to restrict *Myf5* expression to the myotomal compartment in somites, suggesting that additional cis-acting elements were required to ensure accurate *Myf5* expression. In fact, sequences from –96 to –63 kb upstream of the transcriptional start site were shown to be necessary for later expression of *Myf5* in head muscles and in a subset of hypaxially derived trunk muscles (Hadchouel et al., 2000). This element did not behave like a classical enhancer, because it failed to function when linked directly to the Myf5 minimal promoter. Another element required for Myf5 expression in the ventral domain of tail somites and the most ventral component of thoracic somites was found to be located in the region –140 to –88.2 kb (Carvajal et al., 2001). Hadchouel et al. (Hadchouel et al., 2000) identified a 10 kb enhancer fragment –58/–48 kilobases (kb) upstream of the *Myf5* transcription start site that is required for expression in limb muscles and also in somites. In the present study, we particularly focused on further characterization of the –58/–48 kb region of *Myf5* and delineated key regulatory cis-acting elements. Here, we report that a 270 bp core enhancer located ~57 kb upstream of the *Myf5* transcription start site is necessary and sufficient to recapitulate the endogenous *Myf5* expression pattern in limbs and to maintain expression in somites. A second, closely spaced enhancer element is essential to direct transgene expression in cervical somites and to restrict transcription appropriately to the myotome. Thus, we have identified two enhancer activities that profoundly affect the complex regulation of *Myf5* during myogenesis in somites and limbs.

MATERIALS AND METHODS

Construction of *Myf5* transgenes

The *Myf5-lacZ* reporter gene together with 4.8 kb of the proximal promoter region was recuperated as *Spe*I fragment from the recombinant M5-YAC-95 construct (Zweigerdt et al., 1997). This reporter construct contains the *lacZ* gene fused in frame to the *Myf5* start codon and the rest of the *Myf5* gene including the intragenic enhancer described previously by Summerbell et al. (Summerbell et al., 2000). Subfragments of the –58/–48 kb enhancer (10 kb) were generated from M5-YAC-95 DNA by PCR with appropriate primer combinations and proofreading Pfu DNA polymerase (Promega). In order to clone individual subfragments into the pGEM-T easy vector (Promega) by standard recombinant DNA technology (Sambrook et al., 1989) they were briefly treated with Taq polymerase and dATP as

recommended by the manufacturer (Promega). The following primers were used: *Myf5-I*, forward primer GCCTGCCTTTAACGCAGTGTC, reverse primer GGGTGAACAAACTGGAAACCATG; *Myf5-II*, forward primer ACAGGCACATATACATACATACAC, reverse primer CCCACTCTCAAAATGTAAGAAGG; *Myf5-III*, forward primer GTGTCTGCCTTCAATAACGTCTG, reverse primer GGGTGAACAAACTGGAAACCATG; *Myf5-IV*, forward primer GCCTGCCTTTAACGCAGTGTC, reverse primer GGTAGATAGGAGCCTCAAAATAG; *Myf5-V*, forward primer CACACACCATACATCTAACGTGG, reverse primer ACTCGTCCTGTTTACAAAAGGAG; *Myf5-VI*, forward primer CACACACCATACATCTAACGTGG, reverse primer GCTAAAATACAGACATGCAGGCTT; *Myf5-VII*, forward primer GGTAACTGGAGAAATGCTTTCTCT, reverse primer ACTCGTCCTGTTTACAAAAGGAG, *Myf5-VIII*, forward primer GCTAACGAGGTTCTGTTTAATGC, reverse primer CAGACGTTATTGAAGGCAGACAC. Positions of fragments within the locus are indicated by their distance from the transcription start site of *Myf5*. All cloned PCR products were sequenced to ensure the correct nucleotide sequence. Resulting plasmids were linearized with *Spe*I in order to insert the *Myf5-lacZ* reporter fragment. For pronucleus injections, vector sequences were removed by digesting the plasmids with *Not*I and separating fragments by electrophoresis on agarose gels. The fragment carrying the transgene was isolated by electroelution, purified twice with phenol/chloroform and once with chloroform alone, and precipitated with ethanol. DNA was then dissolved in 10 mM Tris-HCl, pH 8.5, run over a ProbeQuant G-50 column (Amersham Biosciences), reprecipitated and washed several times with ethanol. Purified fragments were dissolved in 0.1× TE buffer made with sterile water specified for embryo transfer (Sigma).

Production of transgenic mice

Transgenic mice were generated by pronucleus injection of single-cell embryos from ICR crosses as described (Yee and Rigby, 1993). All constructs were analysed in multiple transient transgenic embryos to ascertain statistical significance of the observed expression patterns and filter out integration effects. Copy numbers of integrated transgenes were determined at least once for each construct and found to be in the range of two to ten, with no apparent correlation to *lacZ* expression levels. Most embryos carrying a given construct exhibited the same pattern and similar intensity of expression unless stated otherwise in Table 1.

Whole-mount staining for β-galactosidase activity and histological sections

Embryos were collected at various developmental stages, counting the day of transfer as E0.5. Isolated embryos were fixed in 4% glutaraldehyde dissolved in buffer B (PBS containing 5 mM EGTA and 2 mM $MgCl_2$) for 10-30 minutes at room temperature. Fixed embryos were then washed in three changes of buffer C (buffer B plus 0.01% sodium desoxycholate and 0.02% Nonidet P-40) for 30 minutes each and incubated in staining solution (2 mM $MgCl_2$, 5 mM EGTA, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.01% Nonidet P-40 and 0.1% X-gal dissolved in PBS) overnight at room temperature. For sections embryos were embedded in 2% agarose and cut at 70 μm using a vibratome (Leica VT 1000S). Pictures of whole-mount embryos were taken under a Leica MZ 12 stereomicroscope using a Polaroid 3CCD color camera and Polaroid DMC2 software. Sections were photographed on a Leica DM-RBE microscope equipped with camera. Figures were assembled with Adobe Photoshop.

RESULTS

Construction of *Myf5* transgenes containing fragments of the –58/–48 enhancer region

It has been shown previously that the distal 10 kb sequence located between –58 and –48 upstream of the *Myf5* transcription start site contains important regulatory elements for *Myf5* expression in limb buds and somites (Carvajal et al., 2001; Hadchouel et al., 2000). In order to dissect this region further and to locate the putative enhancer elements more precisely, subfragments were generated and linked to 4.8 kb of the *Myf5* proximal promoter, followed by the *Myf5* gene body in which the *lacZ* reporter gene was inserted in frame at the *Myf5* start codon (Fig. 1). The *Myf5* promoter sequence used in all of our constructs contains the previously described branchial arch enhancer (Patapoutian et al., 1993; Summerbell et al., 2000),

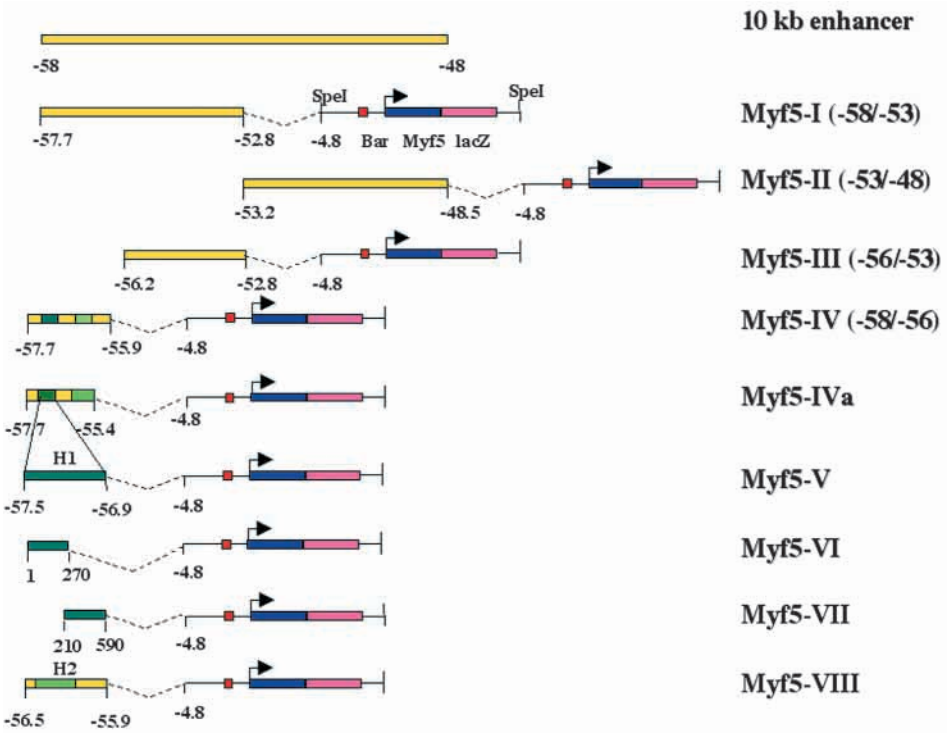

Fig. 1. Schematic representation of *Myf5* transgenes. The –58/–48 distal enhancer of the *Myf5* gene (Hadchouel et al., 2000) was divided into subfragments indicated by yellow bars and the corresponding coordinates. Each fragment was linked to a *Spe*I fragment containing 4.8 kb of the proximal *Myf5* promoter including the branchial arch enhancer (red), the *Myf5* gene body (blue) and the *lacZ* reporter gene (purple) fused in frame. The reporter fragment was obtained from M5-YAC-95 described previously (Zweigerdt et al., 1997). Dark and light green boxes indicate homology elements H1 and H2, respectively, which are highly conserved in sequence between mouse and human. The various constructs are referred to by roman numerals.

Table 1. Summary of the expession profile for all transgenic embryos

Constructs	Transient transgenics (*n*)			Expression of transgenes					
	E9.5-E11.5	E12.5-13.5	Total	Myotome	Dermomyotome	Cervical somites	Limbs	Maintenance* E 13.5	Ectopic
Myf5-I	4	6	10	10	–	10	6/6	3/3	a, b, c
Myf5-II	5	4	9	–	9	–	0/4	0/4	b, c
Myf5-III	5	3	8	–	8	–	0/3	0/3	b, c
Myf5-IV	4	3	7	7	–	7	3/3	3/3	a, b, c
Myf5-V	4	4	8	–	8	–	4/4	4/4	b
Myf5-VI	5	3	8	–	8	–	7/8	2/3	b
Myf5-VII	3	3	6	–	6	–	0/3	0/3	b, c
Myf5-VIII	4	4	8	8†	§	8	0/4	0/4	a

*Perdurance in somites.
†Expression shifted to myotome in older somites.
§Initial expression in young somites.
a, head mesenchyme; b, notochord; c, neuronal tissues.

which served as internal positive control for transgene expression. The transgene constructs also contain the intragenic enhancer that was shown to drive somitic expression in hypaxial dermomyotome (Summerbell et al., 2000). The transgenes *Myf5-I* and *Myf5-II* contain the distal and proximal halves of the 10 kb enhancer fragment (–58/–48), respectively, with some sequence overlap in the middle. The distal 5 kb (–58/–53) were further subdivided to yield a 3 kb fragment encompassing the sequence from –56 to –53, represented by the transgene *Myf5-III*. The remaining 2 kb region (–58/–56) was used to generate the transgene *Myf5-IV*. This fragment contains two sequence segments that are highly conserved between human and mouse *Myf5* genes, referred to as the homology elements 1 and 2 (H1 and H2). H1 (587 bp) was used to generate the transgene *Myf5-V* and H2 was part of the construct *Myf5-VIII*. The H1 enhancer was further subdivided into a 270 bp fragment, representing the 5′ end of H1, and a fragment encompassing nucleotides 210-590, representing the 3′ end of H1. Both fragments were fused to the reporter gene to create the transgenes *Myf5-VI* and *Myf5-VII*, respectively. For pronucleus injections in fertilized mouse eggs vector sequences were removed from all *Myf5* transgene constructs and transient expression was analysed in the appropriate number of embryos at the indicated developmental stages (Table 1). Relative expression levels of the various transgenes in somites and limb buds were estimated by comparison to the expression in branchial arches (internal control).

Expression of *Myf5-I* and *Myf5-II*

Transgene *Myf5-I*, containing the sequence from –58 to –53, was expressed in all somites following the typical rostrocaudal gradient of myogenesis in the transgenic embryos (Fig. 2). At E9.5, transgene expression in somites was confined to the dorsal (epaxial) myotome and extended more ventrally into the hypaxial domain of older somites in E12.5 and E13.5 embryos, similar to the endogenous *Myf5* gene activity (Fig. 2A-C) (Tajbakhsh et al., 1996a,b). Significantly, however, early expression in the epaxial dermomyotome was not seen and the onset of transgene expression was considerably delayed, because only the VIth or VIIth somites began to show β-galactosidase activity (Fig. 3A). This is in contrast to endogenous *Myf5* gene transcription, which normally starts in the DML of the most recently formed somite (Tajbakhsh et al., 1996b; Teboul et al., 2002). The delay is consistent with the absence of the early epaxial enhancer from our constructs that was previously shown to be required for the early onset of *Myf5* expression (Gustafsson et al., 2002; Summerbell et al., 2000; Teboul et al., 2002). Transverse sections through transgenic embryos of various developmental stages confirmed that the expression of *Myf5-I* in somites was restricted to the myotome

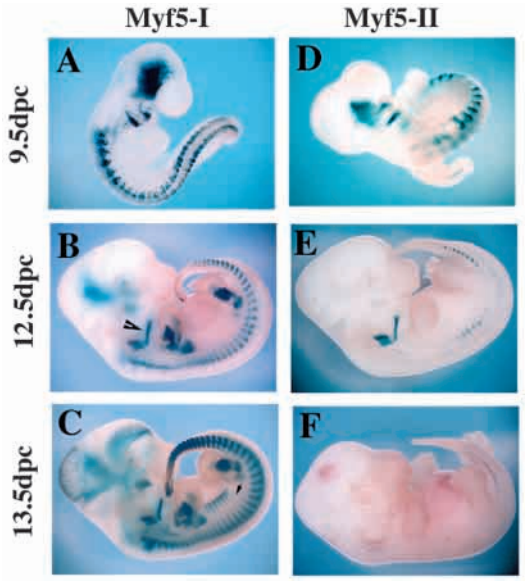

Fig. 2. Expression patterns of transgenic mouse embryos containing constructs *Myf5-I* (A-C) and *Myf5-II* (D-F). *Myf5-I* drives strong expression in the dorsal domain of somites at E9.5 (A), which extends more ventrally during later stages of developmental (B,C). Notice the expression in the hypaxial domain at the thoracic level, whereas the intermediate myotome appears to be free of *lacZ* staining (arrow in C). *Myf5-I* is also strongly expressed in limb muscles of E12.5 and E13.5 embryos (B,C), in the mandibular and hyoid arches (A), and later in hypoglossal cord (arrowhead in B). Ectopic expression is seen in head mesenchyme and in the notochord of the tail. The *Myf5-II* transgene is active in somites at E9.5, except for the cervical region (D), but somitic expression is not properly maintained at later stages (E,F). Also notice that β-galactosidase staining is inappropriately confined to the posterior margin of somites (D). *Myf5-II* is never activated in limb buds.

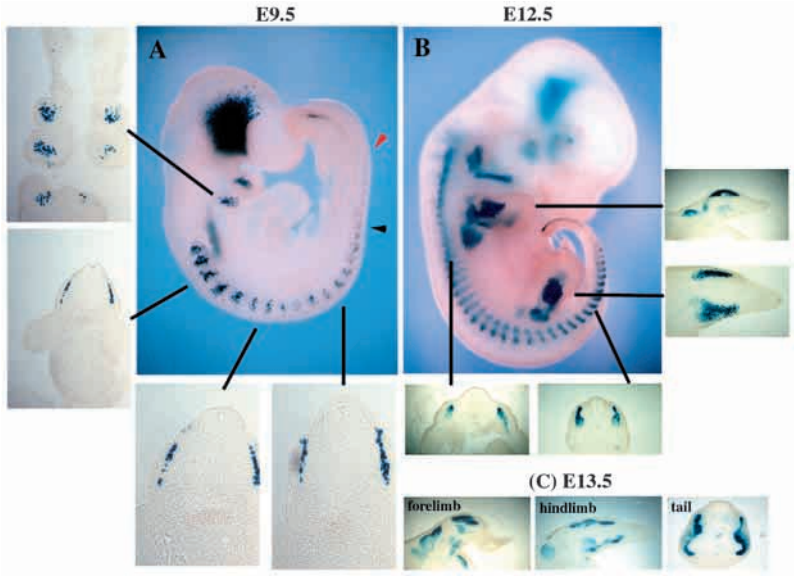

Fig. 3. Whole-mount β-galactosidase staining of *Myf5-I* transgenic mouse embryos and serial transverse sections. (A) An E9.5 embryo (26 somites) illustrating that activation of the transgene occurs correctly in the epaxial domain of somites but is delayed by 5-6 somites. The red arrowhead indicates the most recently formed somite, and the black arrowhead points to the first somite expressing the transgene. Sections confirm that *Myf5-I* expression is properly restricted to the epaxial myotome along the entire axis but does not reach into the hypaxial domain during early stages. Expression in branchial arches occurs in the correct cells. (B) Myotomal expression of *Myf5-I* is maintained in an E12.5 embryo and, in addition, the transgene is now also expressed in the muscle anlagen of fore- and hindlimbs. This expression persists in all limb muscles at E13.5 (C). At this stage, the ventral myotome of tail somites shows robust transgene activity.

and did not extend into the dermomyotome (Fig. 3A,B). At E13.5, both epaxial and hypaxial myotomes were positive for transgene expression, as demonstrated in the precursors of intercostal muscles at the thoracic level (Fig. 2C) and in ventral myotomes of tail somites (Fig. 3C). However, in the medial portion of the myotome, which might be part of the intercalated myotome, the transgene was expressed either at very low level or not at all. In E12.5 embryos, when myoblasts have reached the muscle-forming regions in both fore- and hindlimbs, the transgene was robustly activated in all prospective dorsal and ventral muscle masses (Figs 2, 3). This expression continued accurately in extensor and flexor muscles of later-stage embryos (see sections in Fig. 3C). As expected from the presence of the proximal branchial arch enhancer in the *Myf5-I* construct, all embryos showed expression in mandibular and hyoid arches, and later in the hypoglossal cord. Variable ectopic transgene activity was also observed in head mesenchyme and occasionally in neural tube and dorsal root ganglia. We have not examined expression of the transgene in brain that has been documented for the endogenous *Myf5* gene (Tajbakhsh and Buckingham, 1995; Tajbakhsh et al., 1994).

In contrast to the *Myf5-I* construct, the *Myf5-II* transgene, containing the –53 to –48 kb region of the 10 kb enhancer, completely failed to support expression in limb buds but showed limited activity in somites of E9.5 embryos (Fig. 2D-F). This expression, however, was located to the posterior aspect of somites and almost completely disappeared by E13.5. By E9.5, *Myf5-II* activity was already noticeably weaker or absent in cervical somites compared with thoracic and tail somites. Also the transgene seemed to be less active in the lumbar region, particularly at later developmental stages (Fig. 2E). Significantly, *Myf5-II* was prominently expressed in the dermomyotome and was not restrained to the myotome (data not shown). Similar somitic expression to that observed here has been described for the intragenic *Myf5* enhancer [construct #5 of Summerbell et al. (Summerbell et al., 2000)], which is present in our transgene constructs. These observations suggested that the *Myf5-II* transgene pattern in somites was predominantly due to the previously identified intragenic enhancer and not to the region between –53 and –48. Significantly, the –53/–48 fragment also failed to support expression in limb muscles. Comparison of *Myf5-I* and *Myf5-II* transgene activities clearly indicated that regulatory cis-acting elements within the sequence interval –58 to –53 were responsible for the spatially correct activation and maintenance of *Myf5* transcription in epaxial myotomes, expression in cervical somites and transgene activity in limb buds.

Myf5-III fails to direct correct expression in myotomes and limb buds, whereas *Myf5-IV* promotes it in both muscle-forming regions

To further dissect the –58/–53 region with respect to the observed enhancer activities, the fragment was subdivided into a proximal 3 kb (–56/–53) and a distal 2 kb (–58/–56) segment represented by transgenes *Myf5-III* and *Myf5-IV*, respectively. In addition to the expected activity in branchial arches, transgenic animals obtained with *Myf5-III* exhibited strong ectopic expression in the hypaxial dermomyotome with few positive cells in myotomes between E9.5 and E13.5 (Fig. 4A-D). Cervical somites never expressed *Myf5-III* and the initial activity in lumbar somites rapidly disappeared during embryonic development, similar to the pattern obtained with *Myf5-II* (Fig. 4B). *Myf5-III* also entirely failed to direct *Myf5* expression in limbs. Occasionally, ectopic transgene activity was observed in head mesenchyme and neural tube. These results strongly argued that the observed pattern was due largely to the previously described branchial arch and intragenic *Myf5* enhancers (Patapoutian et al., 1993; Summerbell et al., 2000). Apparently, the 3 kb fragment located –56 to –53 was unable to mediate normal *Myf5* expression in somites and limbs. However, in older embryos (E12.5/13.5), we consistently observed weak transgene activity in proximal muscles at the bases of the fore- and hindlimbs, probably in parts of the shoulder and hip musculature (Fig. 4C,E). This then suggested that the –56/–53 sequence might exert limited enhancer activity in a subset of proximal muscles in hip and shoulder girdles.

By contrast, *Myf5-IV* transgenic embryos (carrying the most-

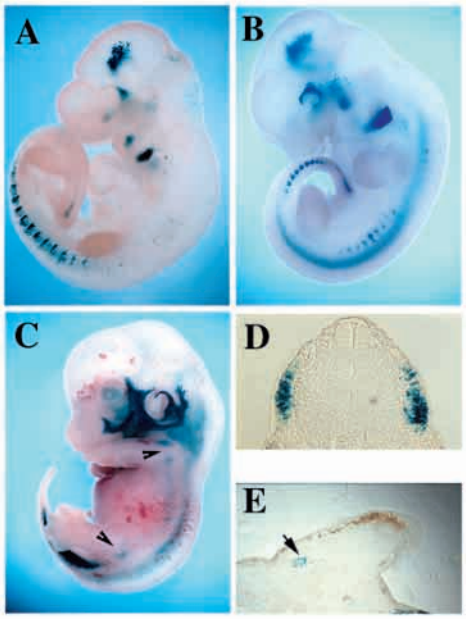

Fig. 4. Myf5-III fails to support correct expression in myotomes and limb buds. β-Galactosidase staining of transgenic embryos at E9.5 (A), E11.5 (B) and E13.5 (C) reveals expression in caudal but not in rostral somites at all stages of development. Transverse section of the embryo in (A) illustrates that the somitic expression is mostly limited to the ventral dermomyotome, with very few positive myotomal cells (D). At E13.5, distinct muscles at the base of the limbs weakly express the transgene in whole-mount (arrowhead, C) and in a section (arrow, E) of the hindlimb. *Myf5-III* is ectopically expressed in head mesenchyme and in tailbud notochord. Expression in branchial arches and later in head muscles reflects the activity of the proximal branchial arch enhancer present in *Myf5-III*. The incorrect activity in somites is probably due to the enhancer located within the *Myf5* gene body, as demonstrated previously (Summerbell et al., 2000).

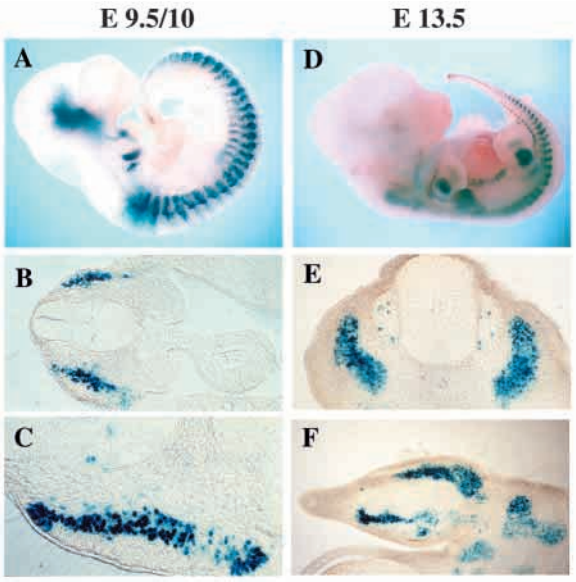

Fig. 5. The transgene *Myf5-IV* is correctly expressed in the myotomal compartment of somites and in limb muscles. Whole-mount β-galactosidase histochemistry and sections of transgenic mouse embryos show myotomal expression in all somites including the occipital/cervical somites at E9.5/10.0 (A-C). Somitic expression is maintained at E13.5 in the dorsal and ventral parts but not in the intermediate region of the myotomes at thoracic and lumbar levels (D,E). The transgene is also expressed normally in limb muscles (F). Few cells in neural tube (B) and dorsal root ganglia (E) exhibit ectopic expression.

distal 2 kb fragment of the 10 kb enhancer region) essentially reiterated the expression patterns of the *Myf5-I* construct and that reported for the entire –58/–48 kb region (Hadchouel et al., 2000) in both somites and limbs (Fig. 5). Activation of the *Myf5-IV* transgene started accurately in the epaxial myotome of all somites including occipital/cervical somites. In older somites, the transgene was also active in the hypaxial domain. Moreover, *Myf5-IV* supported correct spatiotemporal expression in the limb musculature during the appropriate developmental stages. The same result was obtained with *Myf5-IVa*, a truncated version lacking ~500 bp at the 3′ end (data not shown). Taken together, these data suggested that most, if not all, of the important cis-acting control elements of the 10 kb distal enhancer were actually located within the 2 kb upstream sequence (–58/–56). Interestingly, this region contains two closely spaced sequences of approximately 500 and 350 bp that are 87% and 93% identical in mouse and human *Myf5* genes. We refer to these homologous sequences as the H1 and H2 elements, respectively (Fig. 6). Intriguingly, both elements are located within the putative tyrosine phosphatase RQ locus but only partially overlap with annotated exons of the *ptprq* gene (Carvajal et al., 2001). H1 encompasses exon 36, which is 27 nucleotides long, and H2 contains exon 37, which is 82 nucleotides long. Thus, most of the homologous sequence of H1 and H2 is situated within the intron and therefore suggests conserved regulatory functions.

H1 element directs *Myf5* expression in fore- and hindlimb muscles

To examine the particular role of H1, transient transgenic embryos carrying the *Myf5-V* construct were analysed at E9.5, E10.5 and E13.5. Transgene expression in caudal (youngest) somites was initiated dorsally at about the right time (considering that the early epaxial enhancer is not present in the construct) and spread ventrally with further maturation of somites (Fig. 7A,B). The most rostral (occipital/cervical) somites, however, failed to express the transgene, indicating that an important control element for this axial level was absent from the H1 region. In all other somites along the rostrocaudal axis, transgene expression was properly maintained, suggesting that the temporal pattern of *Myf5-V* activity in somites was fairly normal. However, closer inspection of the embryos revealed that the spatial expression of *Myf5-V* was not correct, because β-galactosidase-positive cells were inaccurately located along the posterior rather than the anterior margin of somites (Fig. 7A,B,b). Moreover, transverse and coronal sections showed that the transgene was predominantly expressed in the dermomytome (Fig. 7a,b) and only more-mature somites showed some (limited) activation in myotomal cells, initially in the epaxial region, and later also hypaxially (Fig. 7a′,b′). Significantly, the correct *Myf5* expression pattern in muscles of fore- and hindlimbs was obtained in older

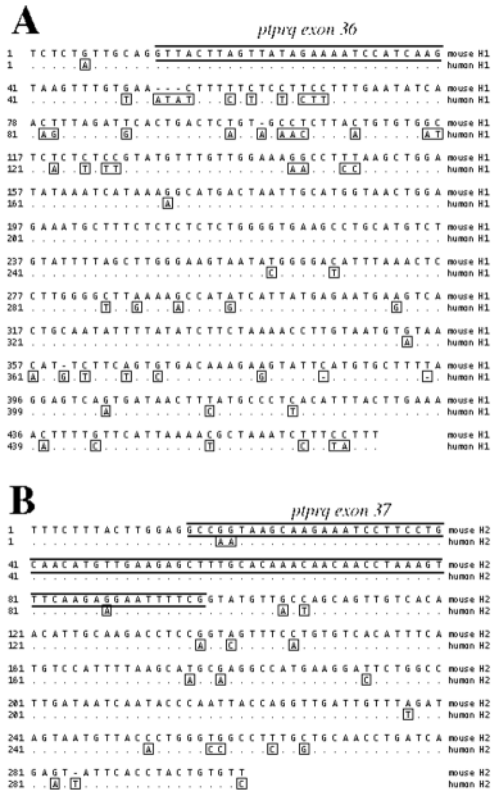

Fig. 6. Sequence comparison of H1 (A) and H2 (B) elements in mouse and human *Myf5* enhancer. The alignment was performed by the Clustal method. Nucleotide residues in the human sequence that are identical to the mouse sequence are indicated by dots. Different nucleotides are shown in boxes. Sequence gaps are marked by dashes. Exon sequences of the overlapping *ptprq* gene are highlighted between lines.

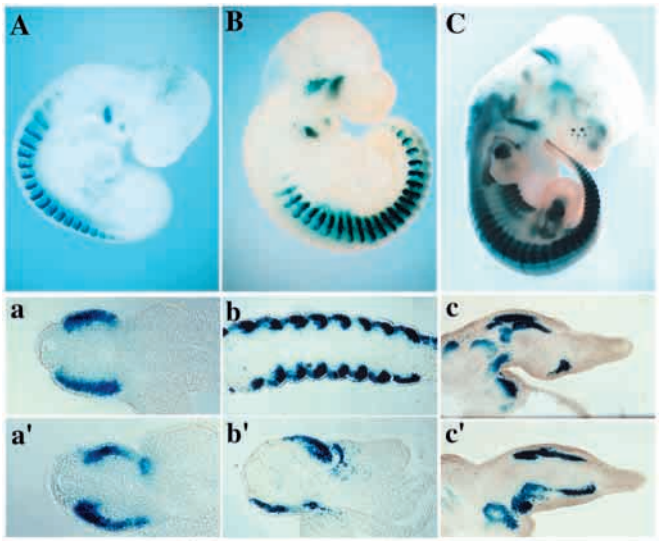

Fig. 7. The H1 element in the transgene *Myf5-V* drives correct expression in limb muscles but not in somites. Whole-mount *lacZ* stainings of E9.5 (A), E10.5 (B) and E13,5(C) embryos illustrate transgene activation in posterior margins of somites but no expression in cervical somites. Transverse sections at different axial levels indicate the expression in the dermomyotome at early stages (a,a′). At later stages, some expression is also seen in myotomal cells (b′). A sagittal section of the embryo in (B) shows prominent expression in the posterior part of somites (b, rostral to the left). Sections through fore- and hindlimbs of the E13.5 embryo demonstrate that all limb muscles express the transgene (c,c′).

transgenic embryos that carried the *Myf5-V* construct (Fig. 7C,c,c′). These results indicate that the H1 element contains the sequences required for *Myf5* activation in limbs but lacks essential control elements necessary to suppress ectopic expression in the dermomyotome and to drive expression in the myotome.

To dissect H1 further and to locate the limb enhancer more precisely, the first 270 nucleotides of H1 were used to generate transgene construct *Myf5-VI*, and the remaining sequence with some overlap (210/590) was used to construct *Myf5-VII*. Embryos containing either transgene at E9.5 exhibited very similar somitic expression that was falsely located to the dermomyotome and the posterior half of somites, and largely absent from cervical somites, confirming the results obtained with H1 (Fig. 8A,C,A′,C′). At E10.5, E11.5 and E13.5, however, *Myf5-VI* was correctly expressed in limb muscles, whereas *Myf5-VII* was not (Fig. 8B,D). Furthermore, somitic expression of *Myf5-VI* was maintained until E13.5, whereas *Myf5-VII* expression in somites was downregulated prematurely, although not quite to the same extent that was observed with other constructs (e.g. *Myf5-II*). Thus, the limb enhancer of the *Myf5* gene is delimited by 270 bp located ~57 kb upstream of the transcriptional start site. The same sequence also seems to mediate a maintenance function in somites. No evidence for a major role of the second half of the H1 element (*Myf5-VII*), at least in isolation, has been obtained, with the possible exception of a weak contribution to late expression in somites.

Enhancer directing *Myf5* expression to the myotome and in cervical somites is composed of H1 and H2

Our results argue that the 2 kb fragment (*Myf5-IV*) located 5′ within the 10 kb distal enhancer region but not the H1 element alone mimics the complete expression pattern that had been obtained with the entire enhancer (Hadchouel et al., 2000). This suggested to us that the regulatory element for correct myotomal expression might lie downstream of the H1 element but still within the *Myf5-IV* construct. To test this hypothesis, *Myf5-VIII* was generated encompassing the last 660 nucleotides of the 2 kb fragment, including the H2 element. Transgenic embryos were analysed at E9.5/10.0 and E13.5. All but one transgenic embryos exhibited expression in somites along the anteroposterior axis, including cervical somites at E9.5/10.5, suggesting that the element for the most rostral somites was contained within H2 (Fig. 9A). However, expression was not maintained until E13.5 (Fig. 9B) and, moreover, expression was initially localized to the dermomyotome in young somites but gradually shifted to the myotome in more anterior somites concomitant with their maturation (Fig. 9C-F). These results suggested that the H2 element and its flanking sequences can drive expression in the myotome but are not sufficient entirely to suppress the dermomyotomal activity. Based on the comparison of transgenic embryos expressing *Myf5-IV* and *Myf5-VIII*, we suspect that sequences within H1 and H2 function in

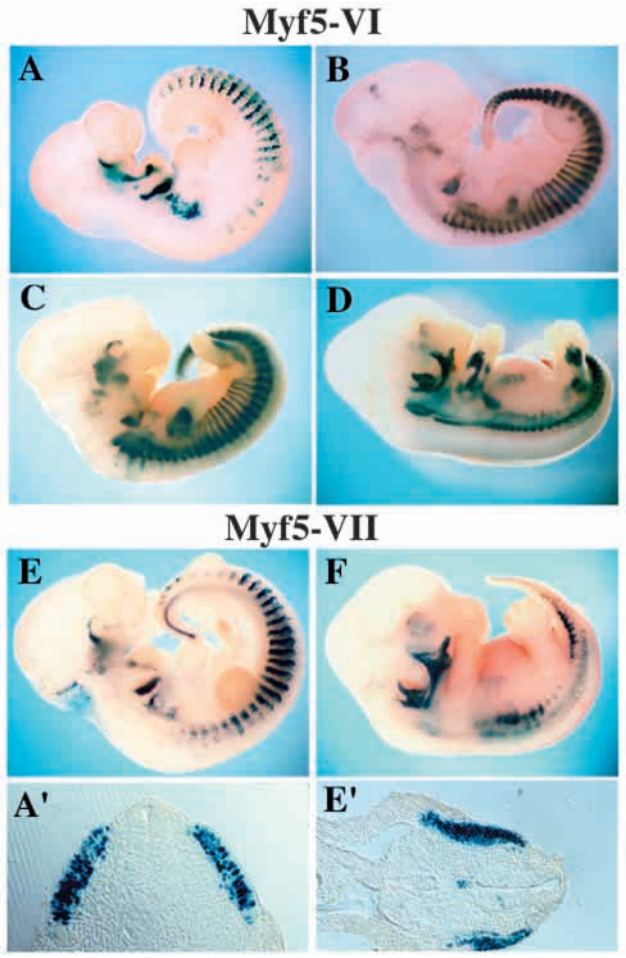

Fig. 8. The limb-specific enhancer is located within the first 270 bp of the H1 element (*Myf5-VI*), whereas the rest (*Myf5-VII*) fails to support expression in limbs. *lacZ* staining of whole-mount embryos carrying *Myf5-VI* (A-D) revealed somitic expression at E9.5 (A) that is maintained until E13.5 (D). Notice, however, that expression in cervical somites is lacking at E9.5 but becomes activated at later stages (B-D). *Myf5-VI* is accurately activated in limb buds from E11 (B) onwards and continues in limb muscles of E12 (C) and E13.5 (D) embryos. By contrast, *Myf5-VII* is not expressed in limbs, and cervical somites also fail to express the transgene (E,F). Moreover, expression of *Myf5-VII* is not correctly maintained in most trunk somites at E13.5 (F). Both transgenes are ectopically expressed in the dermomyotome as shown on transversal sections (A′,E′).

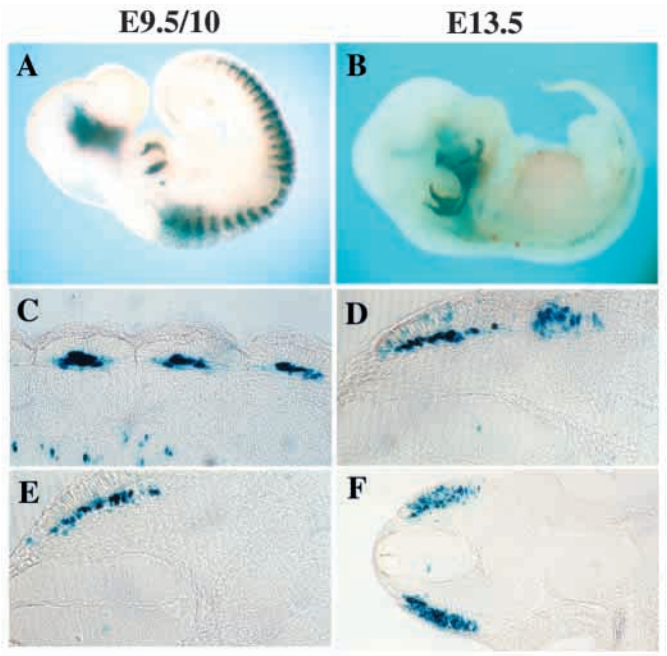

Fig. 9. Expression pattern of the transgene *Myf5-VIII* in somites of mouse embryos. *lacZ* staining on a whole-mount embryo at E9.5/10 indicates transgene activity in all somites including cervical somites (A). Transgene activity in the more caudal (younger) somites (C,E) is preferentially associated with the posterior half, but this is not the case in older somites (D,F). Serial transverse sections of E9.5/10 embryo show dermomyotomal expression in caudal somites (F) that gradually shifts to the myotome in rostral somites that are more mature (E,D). A sagittal section of the cranial somites confirms the expression in myotomes (C). Transgene activity in somites of E13.5 embryos is downregulated (B). Weak expression is seen in proximal muscles of fore- and hindlimbs at the dorsal side.

conjunction to exert the complete enhancer activity in somites. Sequence overlap with the limb enhancer cannot be ruled out. It is clear, however, that the H2 element does not drive expression in limb muscles because it was never seen with *Myf5-VIII*, except for weak activity in dorsal muscles at the limb base (Fig. 9B).

DISCUSSION

Myf5 expression is controlled by a complex set of modular enhancers

Previous studies have shown that the spatiotemporal expression pattern of *Myf5* during mouse embryogenesis depends on multiple distinct regulatory elements that are dispersed over 140 kb of the *MRF4*/*Myf5* gene locus (Carvajal et al., 2001; Hadchouel et al., 2000). Here, we report the identification of two discrete enhancers within the previously described –58/–48 control region (Hadchouel et al., 2000). The first enhancer, composed of a core sequence of 270 bp, is required and sufficient to drive correct *Myf5* expression in fore- and hindlimbs, and to maintain somitic expression during late embryonic stages. The second enhancer mediates accurate expression in the myotomal compartment of somites, subsequent to the initial activation in the DML, which is dependent on the early epaxial enhancer (Gustafsson et al., 2002; Summerbell et al., 2000; Teboul et al., 2002). The second enhancer also appears to be necessary to drive *Myf5* transcription in occipital and cervical somites.

To delineate these enhancer elements, we followed a gain-of-function strategy by adding subfragments of the 10 kb distal region (–58/–48) to the *Myf5*-promoter-driven *lacZ* reporter gene. The *Myf5* promoter including the branchial arch enhancer and the additional intragenic somite enhancer were used to maintain the natural gene context as closely as possible, and to facilitate comparison of our data with those published previously using a similar promoter fragment (Hadchouel et al., 2000). In good agreement with these earlier reports, all of our transgenes that did not receive enhancer activity by the

added subfragments consistently exerted expression in branchial arches and in the hypaxial dermomyotome that was poorly maintained (Summerbell et al., 2000). However, some of the transgenes tested here exhibited clearly different expression patterns, suggesting that the transcription profile of the basal reporter construct was modified by enhancers presented by the additional subfragments. In fact, most of the regulatory aspects of *Myf5* transcription during mouse embryogenesis appeared to be recapitulated by the enhancer modules tested in our transgenes, with the notable exception of the early epaxial expression and rather weak expression in the hypaxial domain and the intercalated myotome. These observations are in line with previous studies in which specific control regions for both the early epaxial and the most-ventral somitic domains were identified in different regions of the gene locus (Carvajal et al., 2001; Gustafsson et al., 2002; Hadchouel et al., 2000; Teboul et al., 2002). It is interesting that the enhancers identified here contain two conspicuous sequence elements that are highly conserved between mouse and human, suggesting that at least some of the molecular mechanisms regulating the murine *Myf5* gene might also apply to the human gene.

Another consideration relates to the fact that the *Mrf4* and *Myf5* genes in birds (Saitoh et al., 1993), mouse (Patapoutian et al., 1993) and humans (Braun et al., 1990) have been conserved in close linkage but exhibit different, albeit partially overlapping, spatiotemporal expression patterns. Regulatory elements controlling both genes are interspersed throughout the common gene locus (Carvajal et al., 2001), posing the problem of how enhancer elements distinguish between the two promoters. We have not actually tested the effects of the enhancers identified here on the *Mrf4* promoter but, clearly, the limb enhancer in its natural context does not lead to *Mrf4* expression in early limbs, despite the fact that it lies closer to the *Mrf4* than to the *Myf5* gene. Presumably, insulator elements are required to shield the *Mrf4* promoter from the influence of this enhancer. Whether somitic expression of *Mrf4* is affected by the distal *Myf5* enhancer cannot be evaluated by our experiments because the expression of both genes overlaps in this compartment (Bober et al., 1991; Hinterberger et al., 1991). Preliminary observations, however, suggest that *Mrf4* expression in the myotome is controlled by elements that lie much closer to its own promoter (M. Fomin and H.-H.A., unpublished). Further investigations will be needed to elucidate the mechanisms underlying the selectivity with which the various enhancers in the *Mrf4*/*Myf5* locus ensure specific regulation of both promoters.

Sequence of 270 bp specifically drives *Myf5* expression in developing limb muscles

Our data clearly demonstrate that 270 bp of the H1 element, located around 57 kb upstream of the *Myf5* transcriptional start site, are sufficient to initiate robust transcription in the post-migratory muscle precursors in fore- and hindlimbs, and presumably to maintain it in all developing flexor and extensor muscles at least until E13.5. In addition, the H1 element also contributes significantly to maintenance of expression in somites but lacks the element to restrain dermomyotomal expression to the myotome and to ensure accurate transcription in cervical somites. Although the limb-specific core enhancer of *Myf5* can be ascribed precisely to the first 270 bp of the H1 sequence, the maintenance function has not been delineated so clearly. It certainly overlaps with the limb enhancer but might extend into the second half of H1, as suggested by the weak maintenance effect that we observed with the transgene containing this sequence. Although published information and the results presented here support the notion that the limb enhancer is unique and solely responsible for *Myf5* expression in limb muscles, a redundant enhancer module cannot be ruled out completely. In fact, we consistently observed weak expression in a subset of proximal limb muscles with transgenes *Myf5-III* (–56.2/–52.8 kb) and *Myf5-VIII*. This might point to proximodistal heterogeneity of muscles in the extremities or, alternatively, might be the artefactual result of the ectopic expression in the dermomyotome, which harbors migratory cells. Ectopic activity caused by integration effects is unlikely because we observed this phenomenon in all transient *Myf5-III* and *Myf5-VIII* embryos. Even if the limb enhancer is not be unique within the *Myf5* locus, it is certainly sufficient to drive accurate expression in muscles of the fore- and hindlimbs.

Little is known about specific signaling molecules that might induce transcriptional activation of *Myf5* in myoblasts that have entered the limb buds, nor about how *Myf5* expression is suppressed in the migrating muscle progenitors. Clearly, Wnts, BMPs, FGFs and Shh are present in limbs and might affect *Myf5* expression. Moreover, calcineurin- and NFAT-dependent signals were recently implicated in regulating *Myf5* gene expression in skeletal muscle reserve cells (Friday and Pavlath, 2001). The identification of the limb core enhancer will facilitate investigation of which, if any, of the signals are actually directly involved in the transcriptional regulation of *Myf5*. Algorithms searching for binding sites of transcription factors within the 270 bp core enhancer predicted many potential consensus sequences including those for Lef1/TCF, Xvent1 and NFAT binding, suggesting that Wnt, BMP and calcineurin-dependent signaling pathways might actually play a direct role (H.-H.A. et al., unpublished). It is worth mentioning that all transgenes containing the enhancer exhibited ectopic expression in the notochord, a site of high Shh activity, which might suggest that an element responsive to this signal is present. Mutational analysis of potential binding sites is under way in order to clarify their roles and to identify cognate transcription factors.

Combinatorial control of *Myf5* expression in somites by multiple enhancer modules

Comparing the expression patterns of *Myf5-IV* and *Myf5-VIII* indicated that the enhancer activity for somitic expression within the distal 10 kb region (–58/-48) depended on the H1 element, at least partly, and the H2 element, because separation of both conserved sequences resulted in suboptimal enhancer activity. Thus, we have not separated this enhancer physically from the limb enhancer and both might indeed overlap. Timing and regionalization of *Myf5* expression in somites has turned out to be much more complex than anticipated. The distal enhancer identified here contributes to this complexity in several ways. First, it activates and maintains transgene expression in myotomes of occipital/cervical somites, indicating a previously unrealized additional control level of *Myf5* expression along the anteroposterior axis. None of the other regulatory regions examined in our transgenes promoted

expression in rostral somites, suggesting that the activity is unique to this enhancer. The early epaxial enhancer also drives expression in cervical somites but it does so only transiently in the DML and the early epaxial domain and not within the myotome during the second phase of myogenesis (Teboul et al., 2002). Second, the distal enhancer directs expression of *Myf5* to the myotome and represses expression in the dermomyotome of all somites along the anteroposterior axis. The ability to correct the ectopic dermomyotomal expression associated with our transgene constructs, presumably through the intragenic somite enhancer, is remarkable because it suggests that a transcriptional silencer function in the dermomyotome is associated with this region, in addition to the activation of transcription in myotomal cells. Moreover, it argues for some kind of co-operativity between the different regulatory modules controlling *Myf5* expression in somites. It seems likely, although it has not been proved experimentally, that the distal enhancer described here is also responsible for the correction of the early epaxial enhancer that drives ectopic dermomyotomal expression when tested in isolation but not in the context of the entire locus (Teboul et al., 2002). Taken together, our results thus provide evidence that the distal enhancer is required and interacts with other somite enhancers (possibly the intragenic, early epaxial and far-upstream hypaxial enhancers) to ensure correct *Myf5* expression in the myotome. Precise assessment of specific and redundant roles of these enhancers in myogenesis must await their individual deletion from the endogenous gene. This type of experiment will also provide information about whether or not the distal myotomal enhancer affects the somitic expression of *Mrf4* as it does *Myf5*.

Specification of myogenic cell fate during skeletal myogenesis is the result of signals from surrounding tissues (Cossu et al., 1996). Expression of *Myf5* (and, later, *MyoD*) is required for the acquisition of myogenic identity and might therefore be the first readout of signaling pathways. Multiple candidates of signaling molecules for myogenic cell specification and transcriptional activation of *Myf5* have been described, and control elements upon which signaling pathways might impinge are beginning to emerge (for reviews, see Buckingham, 2001; Cossu and Borello, 1999; Tajbakhsh and Buckingham, 2000). Wnts (which emanate from dorsal neural tube and surface ectoderm) and Shh (from the notochord and floorplate) have been identified as positive signals for myogenesis in mouse and chicken (Munsterberg et al., 1995; Stern et al., 1995; Tajbakhsh et al., 1998), whereas BMPs are thought to affect myogenesis and the expression of myogenic determination genes negatively, and this activity is counteracted by BMP antagonists like noggin or chordin produced in the dorsal midline, possibly in response to Wnt signals (Pourquie et al., 1996). In *Xenopus*, *Xmyf-5* expression in mesoderm can also be activated by Wnt signals (Marom et al., 1999). More recently, a regulatory mosaic of repression and activation involving Wnt and activin-like signals has been described to define the *Myf5* expression profile in the frog gastrula (Yang et al., 2002). A requirement has been shown in mouse null mutants for Shh signaling during initial *Myf5* expression in the early epaxial somite (Borycki et al., 1999), and the corresponding enhancer containing a Gli-binding-site has recently been identified (Gustafsson et al., 2002). However, direct induction of *Myf5* transcription by Shh has been disputed by others (Kruger et al., 2001). Clearly, regulation of *Myf5* must accommodate the complex input of various signaling circuits, which might explain the complexity of cis-acting control regions within this locus. The identification of the enhancers described previously and in this report and their delimitation to manageable size will now allow us to investigate the molecular mechanisms underlying the recognition and integration of signals that determine the complicated expression pattern of *Myf5* in myotomes and limb muscles.

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