Analysis of a key regulatory region upstream of the Myf5 gene reveals multiple phases of myogenesis, orchestrated at each site by a combination of elements dispersed throughout the locus

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SUMMARY

Myf5 is the first myogenic regulatory factor to be expressed in the mouse embryo and it determines the entry of cells into the skeletal muscle programme. A region situated between −58 kb and −48 kb from the gene directs Myf5 transcription at sites where muscles will form. We now show that this region consists of a number of distinct regulatory elements that specifically target sites of myogenesis in the somite, limbs and hypoglossal cord, and also sites of Myf5 transcription in the central nervous system. Deletion of these sequences in the context of the locus shows that elements within the region are essential, and also reveals the combinatorial complexity of the transcriptional regulation of Myf5. Both within the −58 kb to −48 kb region and elsewhere in the locus, multiple sequences are present that direct transcription in subdomains of a single site during development, thus revealing distinct phases of myogenesis when subpopulations of progenitor cells enter the programme of skeletal muscle differentiation.

Key words: Myf5, Mouse embryo, Myogenesis, Transcriptional regulation, BACs, Myotome, Limb, CNS

INTRODUCTION

The emergence of different cell types during embryogenesis depends on signals, usually from adjacent tissues, leading to the expression of genes that determine the differentiation programme the cell will enter. Activation of a regulatory cascade culminates in the acquisition of a tissue-specific phenotype. Skeletal myogenesis conforms to this model. In the mouse embryo, Myf5 is the myogenic determination factor that is responsible for directing cells into the skeletal muscle programme (Braun et al., 1992; Tajbakhsh et al., 1996b). In keeping with this role, Myf5 is capable of remodelling the chromatin of muscle genes, rendering them accessible to transcriptional activation (Bergstrom and Tapscott, 2001; Gerber et al., 1997). At later stages of development, the expression of MyoD can also activate the myogenic cascade. The double null mutation of Myf5 and MyoD prevents the formation of skeletal muscle precursor cells (Rudnicki et al., 1993). However Myf5, together with Pax3, lies upstream of MyoD (Myod1 – Mouse Genome Informatics) in the genetic hierarchy that regulates myogenesis (Tajbakhsh et al., 1997). Mrf4 (Mycf6 – Mouse Genome Informatics) and myogenin, the other two members of the family of basic-helix-loop-helix transcription factors to which MyoD and Myf5 belong, are implicated in muscle cell differentiation in the embryo. Myogenin is directly involved in the transcriptional activation of muscle genes and in its absence many skeletal muscles are compromised (Venuti et al., 1995), whereas Mrf4 appears to play this role for the differentiation of the myotome, the earliest muscle mass to form in the embryo (Buckingham, 1994; Nabeshima et al., 1993). MyoD can also act as a differentiation factor, as evidenced by the phenotype of the MyoD/Mrf4 double mutant where myoblasts are present but fail to differentiate (Rawls et al., 1998).

During embryogenesis, the spatiotemporal expression of Myf5 marks skeletal muscle precursor cells and sites of myogenesis (see Tajbakhsh and Buckingham, 2000). In newly formed somites, it is expressed before the other myogenic factors, in the epaxial part of the dermomyotome, adjacent to the neural tube. This is the first source of muscle precursor cells, which involute and migrate from this epithelium to form the first differentiated skeletal muscle, the myotome. The hypaxial extremity of the dermomyotome and later somitic bud is also a source of muscle precursors, which express Myf5 and
form the hypaxial myotome, the origin of intercostal and body wall muscles (Christ et al., 1983). This part of the dermomyotome, in somites at the appropriate axial level, also gives rise to cells which migrate to other sites of myogenesis, to the limb buds or, via the hypoglossal chord (Mackenzie et al., 1998; Noden, 1983), to the pharynx, tongue and probably also diaphragm (Tremblay et al., 1998). Cells that migrate to the limb do not express Myf5 until they reach their destination (Tajbakhsh and Buckingham, 1994). Myf5-positive cells are already present in the hypoglossal cord where, in contrast to the limb progenitors, they do not migrate as separate mesenchymal cells, but rather as a coherent cell mass (Noden, 1983). Myf5 is also expressed in the branchial arches in cells derived from anterior paraxial mesoderm which will contribute to the formation of facial muscles. An unexpected site of Myf5 expression is in neurones, in prosomeres p1 and p4 of the brain (Tajbakhsh and Buckingham, 1995) and in a ventral domain of the neural tube (Tajbakhsh et al., 1994). The Myf5 protein does not accumulate in the central nervous system and there is no detectable neuronal perturbation in the Myf5 mutant embryos (Daubas et al., 2000).

The molecular mechanisms that lead to Myf5 activation at these multiple sites in the embryo are largely unknown. Signals from the axial structures, neural tube and notochord, and from surface ectoderm are required for the initiation of myogenesis in the somite in the mouse (Summerbell and Rigby, 2000; Tajbakhsh and Buckingham, 2000), as in the avian embryo (Christ and Ordahl, 1995). Sonic hedgehog from the notochord and floor plate of the neural tube, as well as Wnt proteins produced by the dorsal neural tube and surface ectoderm, have been implicated in Myf5 activation, which is particularly responsive to β-catenin-dependent Wnt1 signalling, as shown in mouse embryo explants of presomitic mesoderm or immature somites (Borycki et al., 1999; Tajbakhsh et al., 1998). Expression of Myf5 in explants from the region of the brain where the gene is transcribed also responds in this way to Wnt1, leading to the suggestion that this may reflect misfiring of a regulatory element that normally directs Myf5 transcription in the somite, in response to the Wnt1 signal (Daubas et al., 2000).

The multiple regulatory elements that direct the complete spatiotemporal expression pattern of Myf5 in the embryo have been mapped within 145 kb of genomic DNA by YAC (Hadchouel et al., 2000) and BAC (Carvajal et al., 2001a) transgenic analysis. Another gene, which encodes the myogenic regulatory factor Mrf4, lies 7 kb 5′ of the Myf5 gene (Braun et al., 1990; Miner and Wold, 1990). Within this intergenic region, just 3′ of the Mrf4 gene, an enhancer element has been identified that directs early expression of Myf5 in the epaxial dermomyotome (Summerbell et al., 2000; Tekolos et al., 2002). Gustafsson et al. have reported that this element responds to sonic hedgehog signalling, which is required for the expression of Myf5 specifically in this epaxial domain (Borycki et al., 1999; Gustafsson et al., 2002). Also in the intergenic region between Mrf4 and Myf5, further regulatory regions have been described, including one that directs expression to the branchial arches (Patapoutian et al., 1993; Summerbell et al., 2000) and another that leads to expression in the neural tube (Summerbell et al., 2000). Within the Myf5 gene itself, an enhancer that directs expression to the hypaxial domain of the somite has been identified (Summerbell et al., 2000). In the genomic DNA lying upstream of the Mrf4–Myf5 genes, there are a number of regulatory regions, including one responsible for maintenance of Myf5 expression (–88 kb to –81 kb) in some muscles of the trunk and head and others responsible for aspects of Myf5 expression in the hypaxial somite, arches and hypoglossal cord (Carvajal et al., 2001a; Carvajal et al., 2001b; Hadchouel et al., 2000). In particular, a sequence lying between –58 kb and –48 kb from the Myf5 gene (Hadchouel et al., 2000) directs expression of a transgene to sites of myogenesis in the somites, hypoglossal cord and limb buds, as suggested by deletions in this region (Carvajal et al., 2001a; Zweigerdt et al., 1997), and also to the brain. In this paper, we present the analysis of this region and show that it is necessary for transcription of Myf5 at these sites in the embryo. Different sites are targeted by distinct sequences and, furthermore, within a single site, regulatory subdomains emerge that are identified by dissection of the region or revealed after its deletion. Unexpectedly, a distinct sequence targets transcription of Myf5 to the central nervous system. At least three sequences are active at different times and to varying extents in fore- versus hindlimbs. In the somite, Myf5 expression is regulated by a minimum of six different sequences, with more than one regulatory module required even within an ostensibly uniform structure such as the myotome. The fine analysis of the region located between –58 kb and –48 kb from Myf5, and the consequences of its deletion add a further dimension to our appreciation of the complexity of the information that has to be integrated to ensure the spatiotemporal regulation of this myogenic determination gene. Furthermore, this analysis reveals subpopulations of cells that contribute to myogenesis as the embryo develops.

**MATERIALS AND METHODS**

**Plasmid transgene constructions** All plasmid constructs used in this study, except p-S8/-48tk-nlacZ and p8.8 (Summerbell et al., 2000), are derived from pphaMyf5-nlacZ, (Hadchouel et al., 2000), containing the n lacZ reporter gene with 2.6 kb of Myf5 5′ flanking sequence that includes the Myf5 branchial arch element located between –1.7 kb (Nhel site) and –561 bp (BsaI site), the neural tube element located between –561 bp and –141 bp (XbaI site) (Summerbell et al., 2000), and the Myf5 minimal promoter (–141 bp from the Cap site). We refer to this construct as pMyf5-nlacZ, p-S8/-48Myf5-nlacZ (Hadchouel et al., 2000) was totally digested by XhoI then partially digested by SnaHI and religated to itself to create a random 5′ deletion series of the –58/–48 fragment. Among the resulting constructs, p-56.4/-48Myf5-nlacZ and p-53.3/-48Myf5-nlacZ were selected for micro-injection. p-S8/-48Myf5-nlacZ was digested by Ncol and HindIII, and religated on itself to obtain p-S8/-54Myf5-nlacZ. A 1.4 kb Aval or a 2.9 kb Aval-AccI fragment was subcloned in pMyf5-nlacZ to create p-S8/-56.6Myf5-nlacZ or p-56.6/-53.7Myf5-nlacZ, respectively. A 5 kb ClaI-XhoI fragment located between –63 and –58 was subcloned in p-S8/-54Myf5-nlacZ to obtain p-S8-53.7Myf5-nlacZ. The fragments –58/-57, –57.5/-56.6 and –57.5/-57 were synthesized by PCR using the Advantage 2 Taq polymerase (Clontech), with XhoI and HindIII sites added to 5′ and 3′ primers, respectively. The forward and reverse primers used to generate the –58/-57, –57.5/-56.6 and –57.5/-57 fragments were, respectively: fwd (5′-GGG CTC GAG CAA TAT AAT GTC -3′), rev (5′-AAA AAG CTT TTT TCT TTA AAT GAG GCC TGG GGC ACC -3′); fwd (5′-AAA AAG CTT GAG GTA TGT TGT TGG GAA AGG CC -3′), rev (5′-AAA AAG CTT GGG ATT GTG ACC TAC AGA TGG G-3′); and fwd (5′-AAA CTC
GAG GTA TGT TTG TTG GAA AGG CC-3'), rev (5'-AAA AAG CTT TTT TCT TTA AGA GAG AGC TTG GGC ACC-3'). The PCR products were checked by sequencing (Genome Express) and cloned into pMyf5-ΔnlacZ, in front of the transgene.

p58-48tk-nlacZ was created by subcloning the 10 kb XhoI-HindIII fragment located between -58 and -48 kb into tk-nlacZ, which contains the nlacZ reporter gene under the control of the thymidine kinase (tk) promoter isolated from pBLCAT2 (Luckow and Schütz, 1987).

**BAC transgene constructions**

All BAC deletion constructs are based on BAC195APZ (Carvajal et al., 2001a). For BAC195APZ, homology arms were synthesized by PCR amplification of BAC195APZ with the following primer pairs: 5' homology arm (1220 bp), Δ54-49.5F (5'-TTA CTA TTG TCA GAA GAA TAG AAG AAA GGA-3') and Δ54-49.5R (5'-AAG GAT CCG ATC TTG AAG AAA TTT TGG TAA TTC C-3'); 3' homology arm (1209 bp), Δ54-49.3F (5'-GTI TTT TTG ATA GAG GAT GAA TAC TCA A-3') and Δ54-49.3R (5'-TCA TTT GAA TAG AGA CCT AAA GAT C-3'). The 5' homology arm fragment was cloned into pCRII (Invitrogen) to generate Δ54-49.5. The 3' homology arm fragment was digested with XhoI and cloned into a BamHI-digested pΔ54-49.5 to give pCasΔ54-49.

For BAC195APZ, homology arms were synthesized by PCR amplification of BAC195APZ with the following primer pairs: 5' homology arm (140 bp), Δ59-54.5F (5'-CTG CAT GAT CTT GGT CAT GGT-3') and Δ59-54.5R (5'-TGG ATC CTG AAA ACG TGA GGC ACC GGA GG-3'); 3' homology arm (140 bp), Δ59-54.3F (5'-CCCA TAG GAA TTA CCA AAA TTT TGG TAA TTC C-3') and Δ59-54.3R (5'-CGT AAA CCA TTA AGA TGG TGG-3'). To generate the deletion cassette, 5' and 3' homology arms were digested with BamHI and XhoII, respectively, ligated and re-amplified using Δ59-54.5F and Δ59-54.3R.

For BAC195APZ, homology arms were synthesized by PCR amplification of BAC195APZ with the following primer pairs: 5' homology arm (145 bp), Δ63-48.5F (5'-CTG CAT GAT CTT GGT CAT GGT-3') and Δ63-48.5R (5'-CAC ATA CAC AAC TAC ACA AAA GCT ATG CCA GTG TGC TAT CCC TCC-3'), including a 24mer tail homologous to the 5' -end of the 3' homology arm; 3' homology arm (144 bp), Δ63-48.3F (5'-AGC TTC TTT GGA GAT TGT GTG TAT GTG-3') and Δ63-48.3R (5'-GTC TGC ATG GAA CTA GTG TTA-3'). To generate the deletion cassette, 5' and 3' homology arm fragments were gel purified, mixed in a 1:1 ratio, denatured for 5 minutes at 95°C and left to re-annotate at 37°C for 30 minutes. Standard PCR mix, not including primers, was added and the reaction incubated at 72°C for 30 minutes. The extended products were then re-amplified by PCR using Δ63-48.5F and Δ63-48.3R.

For BAC195APZ, pCasΔ54-49 was digested with NolI to excise the deletion cassette. Fragments were isolated by gel electrophoresis and subcloned into a pSV-RecA vector (Yang et al., 1997), which had been modified by introducing a single NolI site replacing the Sall cloning site (D.C., J.J.C. and P.W.J.R., unpublished). Generation of the deletion BAC with this plasmid construct was carried out as previously described (Cox et al., 2002). For the generation of other BAC deletion constructs, we used our own modification of the linear recombination method (Lee et al., 2001; Swaminathan et al., 2001). BAC195APZ was generated by introducing the 5 kb deletion in BAC195APZ, while BAC195APZ was generated by deleting the entire region from BAC195APZ.

For BAC195APZ and BAC63-48, 25 ng of DNA from each BAC was used to transform electrocompetent E. coli DY380 cells. Single colonies growing under chloramphenicol (CAM) selection were analysed to check that BACs transferred to the new host were not rearranged. Single colonies were isolated and grown on LB-CAM media overnight at 32°C with constant agitation. This culture (220 μl) was used to seed 11 ml of LB-CAM. Cells were incubated at 32°C with shaking (250 rpm) until the OD600 was between 0.5 and 0.7. Cultures were then incubated at 42°C for 15 minutes, transferred to wet ice and left to cool down for at least 20 minutes. Cells were washed three times in ice-cold H2O and electroporated immediately after the last wash. Deletion cassettes were denatured by 10 minutes incubation in 300 mM NaOH, ethanol precipitated, resuspended in 20 μl of cold H2O and mixed with the electrocompetent DY380 cells carrying the BAC of interest. Electroporation conditions were as follows: 1.75 kV, 200 Ohms, 25 μF. After electroporation, cells were diluted and plated into a single 96-well plate at a density of 10-30 cells/well. After overnight growth at 32°C, colony pools were screened by PCR using primers outside the deletion cassette. Positive pools were diluted and plated on LB-agar CAM to obtain ~250 colonies/plate and incubated overnight at 32°C. Single colonies were picked onto 96 well plates, grown overnight at 32°C with constant agitation and PCR-screened with the same primer pair to identify positive clones. All clones were sequenced to confirm the deletion, and the integrity of the sequences corresponding to the homology arms. Furthermore, positive clones were digested with a panel of restriction enzymes to ensure no additional deletions, insertions or rearrangements had occurred.

**Generation of transgenic mice**

BAC DNA purification was carried out as described previously (Carvajal et al., 2001a) and plasmid fragments as described elsewhere (Kelly et al., 1995). Transgenic mice were generated by microinjection of purified BAC or plasmid DNA into fertilized (C57BL/6J×SJL) or (CBA×C57BL/6J) F2 eggs at a concentration of

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Numbers are given at each stage for embryos that showed Myf5-specific X-gal staining. The numbers in brackets indicate the total number of transgenic embryos examined.
~1-2 ng/μl using standard techniques (Hogan et al., 1994). Injected eggs were reimplanted the same day or the day after the injection into pseudopregnant (C57BL/6j × CBA) F1 foster mothers.

Identification of transgenic animals

DNA was prepared from mouse tails or, for transient transgenics, a region of the embryo, and analysed by PCR, using standard techniques.

Analysis of transgene expression

Heterozygous transgenic males were crossed with non-transgenic females ([C57BL/6j × SJL or C57BL/6j × CBA] F1). Embryos were dated, taking E0.5 as the day of the appearance of the vaginal plug. Transient transgenic embryos were dated taking the day of reimplantation into the pseudo-pregnant foster mothers as E0.5. Numbers of positive transient transgenics analysed at each time point are indicated in Table 1. The following transgenic lines, with numbers given in brackets, were also analysed: –58/–48Myf5-nlacZ (2), BAC195Z (6), BAC195APZ (5), BAC195A63-48 (3), BAC195A59-54 (3) and BAC195A54-49 (4).

X-gal staining: embryos were dissected in PBS, fixed in 4% paraformaldehyde for 5 to 60 minutes depending on the age of the embryo, or in Mirsky’s Fixative (National Diagnostics) for 1 hour to overnight. X-gal staining was performed for 1 to 2 hours at 37°C from 2 hours to overnight. Transgenic embryos were examined by whole-mount microscopy or cryostat sectioning, as described previously (Kelly et al., 1995).

Immunofluorescence: cryostat sectioning and immunofluorescence experiments were performed as described elsewhere (Teboul et al., 2000), with use of a monoclonal anti-myosin heavy chain antibody (MF20, dilution 1:50) from the Developmental Studies Hybridoma Bank.

RESULTS

In order to investigate the spatiotemporal regulation of Myf5 expression by the sequence that we had identified between –58 kb and –48 kb (Hadhchouel et al., 2000), we constructed transgenes containing fragments of this region as indicated in Fig. 1A. They were placed in front of a proximal Myf5 sequence (2.6 kb), which includes the promoter, and ventral neural tube and branchial arch elements (Summerbell et al., 2000), which serve as positive controls for expression of the n LacZ transgene. In Fig. 1B, we show the n LacZ expression at embryonic day 11.5 of a transgene where the reporter is regulated by the –58/–48 sequence with a heterologous thymidine kinase promoter. The expression pattern at this stage was similar to that previously described with the Myf5 promoter (Hadhchouel et al., 2000). The fact that the sequence acts with a heterologous promoter, independently of position relative to the promoter suggests that it has the properties of an enhancer.

The –53.3/–48 region directs expression mainly to the most anterior somites and to the hindlimb

The region between –53.3 kb and –48 kb placed in front of the proximal Myf5 sequence directed reporter gene expression to musculature in the cervical region and in the limb buds at E12 as shown in Fig. 2A. There was also labelling at this stage of some facial muscles derived from the branchial arches because of the branchial arch element. Hindlimb muscle masses were more strongly labelled than those in the forelimb, despite the anteroposterior developmental gradient (see Fig. 1B). With a –56.4/–48 Myf5-n LacZ transgene, the hindlimb bud was also more strongly labelled than the forelimb (Fig. 2B) at E11, β-galactosidase-positive cells were present in anterior somites, and the section shown in Fig. 2CD indicates that these represent a subpopulation of differentiated muscle cells in the myotome, which is marked by myosin heavy chain expression. The myotomal expression is also seen with the –53.3/–48 Myf5-n LacZ transgene (results not shown). In some embryos where transgene expression was particularly strong, labelled cells were also detected in more posterior myotomes. At E10.5, when Myf5 is normally expressed in the forelimb, no β-galactosidase positive cells were detectable with these transgenes in the limb buds. At this earlier stage, occasional embryos showed weak myotomal labelling, varying in its extent on the anteroposterior axis. At later stages (e.g. E13.5) shoulder and limb muscles continued to be positively labelled with this transgene (results not shown).

Fig. 1. The regulatory region at –58/–48 kb upstream of Myf5. (A) Schematic representation of the different fragments of the –58/–48 kb region and its upstream extension to –63 kb, which are subcloned in front of the Myf5-n LacZ cassette (2.6 kb, including a branchial arch element, neural tube element and the Myf5 promoter) (Hadhchouel et al., 2000; Summerbell et al., 2000). For each construct, the cloned region is indicated by a black line. Distances in kb are from the Myf5 Cap site. (B) The n LacZ expression pattern, revealed by X-gal staining, is shown for an embryo at E11.5 (~40-45 somites) with a –58/–48 tk-n LacZ transgene, where the region between –58 kb and –48 kb has been placed in front of a heterologous thymidine kinase promoter.
The –53.3/–48 region drives Myf5-nlacZ expression mainly in cervical somites and the hindlimb bud. X-gal staining at E12, with a –53.3/–48 Myf5-nlacZ transgene (A), and at E11.0, with a –56.4/–48 Myf5-nlacZ transgene (B). Black arrows indicate X-gal staining in fore- and hind-limbs. β-galactosidase-positive cells are detected in the brain in prosomeres p1 and p4 (white arrowheads). The embryo shown in B was sectioned at the level of the cervical somites (white line) and the corresponding transverse cryostat section is shown under phase contrast (C) or fluorescence (D), after immunoreaction with an antibody against muscle myosin heavy chain (red labelling) to show differentiated muscle cells in the myotome, the extent of which is indicated by white arrows. Black arrowhead in C indicates β-galactosidase-positive neurones within the ventral neural tube (NT). DRG, dorsal root ganglia.

The –56.6/–53.7 region directs expression in the central nervous system

In Fig. 2B, expression in the brain and ventral neural tube (Fig. 2C) was detected, suggesting that an element responsible for the transcription of Myf5 in the central nervous system (Tajbakhsh and Buckingham, 1995) is located between –56.4 kb and –53.3 kb (compare Fig. 2A with 2B). Fig. 3A shows that this is indeed the case. With a –56.6/–53.7 Myf5-nlacZ transgene, prosomeres p1 and p4 were labelled. A section through prosomere p1 (Fig. 3C) shows β-galactosidase-positive cells in this region of the diencephalon, where the endogenous gene is expressed, extending from the mantle layer into the subventricular zone where neuronal precursors are present. In contrast, the labelled cells in prosomere p4 in a section through the mesencephalon (Fig. 3D) were confined to the outer layer of the neuroepithelium where differentiated neurones are present, as seen with the endogenous gene (Daubas et al., 2000; Tajbakhsh and Buckingham, 1995). Expression of the transgene was also seen in cells in the ventral neural tube (Fig. 3B). As in the case of prosomere p1, expression was in the same region, but not identical to that seen with the endogenous gene, possibly because further regulatory elements which refine and restrict transcription are absent from the 2.9 kb fragment of the enhancer present in this transgene. As the 2.6 kb proximal region of Myf5, which is present in this and other transgenes, contains a neural tube element (Summerbell et al., 2000) and the complete –58/–48 region, when placed in front of a minimal tk promoter (Fig. 1B), also directs expression to the ventral neural tube, we conclude that two distinct elements are responsible for this site of expression, and probably cooperate to target the subset of neurones marked by transcription of the endogenous gene (Tajbakhsh et al., 1994).

The –58/–56.6 region contains elements which target limb buds, hypoglossal cord, and distinct regions of the somite

A transgene with the region between –58 and –54 kb directed Myf5-nlacZ expression in the central nervous system, as expected, and at additional sites in the embryo (Fig. 4). At E9.75 (Fig. 4A), there was no labelling in the most caudal immature somites, where myogenesis is initiated by the early epaxial enhancer that activates Myf5 transcription in the dermomyotome (Teboul et al., 2002). In more mature somites, β-galactosidase positive cells were present in the myotome, underlying the epithelial dermomyotome in a central position (Fig. 4B) where muscle differentiation first takes place (Spörle, 2001). This corresponds to the region of the myotome that is revealed when the earlier epaxial enhancer is deleted (Teboul et al., 2002). At E10.5 (Fig. 4C), labelled cells continued to be located in the central part of the myotome. By E11.5 the expression pattern in the somite has changed (Fig. 4D). Cells in the epaxial myotome were still β-galactosidase-positive in a region that is intercalated between epaxial most and hypaxial domains of the myotome (Fig. 4E). Labelling was now also detectable in more mature anterior somites in the epaxial lip of the dermomyotome and, in the interlimb region, the hypaxial somitic bud also contained β-galactosidase-positive cells (Fig. 4E). At this stage, the epithelial structure of the dermomyotome is no longer present except at the extremities of the somite. Some labelled cells could also be seen in the hypaxial myotome. In previous studies, the –58 kb end point was chosen arbitrarily. Given that sequences up to this point conferred muscle expression, we tested more upstream sequences for activity in transgenic assays. In Fig. 4F, an E11.5
embryo with a –63/–54 Myf5-nlacZ transgene is shown. The X-gal labelling is essentially similar to that with the –58/–54 sequence. This additional 5 kb of 5′ sequence therefore did not extend the expression pattern.

Fig. 5A shows that a –58/–56.6 Myf5-nlacZ transgene, which no longer contains the distal central nervous system element, directed expression at E11.5 to the sites of myogenesis shown in Fig. 4D. The –58/–57 part of this fragment (Fig. 5B) continued to direct expression in the hypoglossal cord and limb buds, whereas labelling in the somite was confined to the epaxial and hypaxial extremities (Fig. 5C), where β-galactosidase-positive cells were clearly seen in the epithelial structures of the epaxial lip (Fig. 5D) and hypaxial somitic bud (Fig. 5E). In contrast, a –57.5/–56.6 fragment directed expression to the intercalated domain of the epaxial myotome (Fig. 5F,G), showing that this site of Myf5 transcription in the somite is under distinct regulation. This transgene showed variable expression in the epaxial lip and somitic bud. Both the –58/–57 and –57.5/–56.6 fragments directed expression in the limb buds and hypoglossal cord (Fig. 5B,F). Consistent with this, the overlapping 500 bp fragment (–57.5/–57) also directed expression to these sites (Fig. 5H). This fragment also showed strong expression in the hypaxial somitic bud and in the epaxial lip of the dermomyotome.

Deletion of the –58/–48 region reveals its essential role in directing Myf5 expression in the embryo

The preceding observations demonstrate that the –58/–48 region directs transcription of Myf5 to the brain, limbs, hypoglossal cord and to specific domains of myogenesis in the somite. In order to establish whether this region is necessary for Myf5 expression at these sites in the context of the locus, it was deleted

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**Fig. 4.** The –58/–54 region drives Myf5-nlacZ expression to several sites of myogenesis in the somites, limb buds and hypoglossal cord. (A,C,D) X-gal staining of an embryo with a –58/–54 Myf5-nlacZ transgene at E9.75 (A, 26 somites), E10.5 (C, 30 somites) and E11.5 (D, 36 somites). At E9.75, β-galactosidase-positive cells are detected in the developing myotome in the central epaxial region, as shown in a transverse cryostat section (stained with Eosin) (B) of the same embryo shown in A (white line indicates level of sectioning). At E10.5 (C), Myf5-nlacZ expression is still restricted to the same area of the myotome. It is detected in the extremities of the more mature somites at E11.5 (D). (E) Phase contrast microscope view of a section in the interlimb region of the embryo shown in D (white line indicates level of sectioning) where both the epaxial lip of the epithelial dermomyotome and the hypaxial somitic bud contain cells with blue nuclei (black arrowheads). At this stage, there is strong X-gal staining in the forelimb buds and labelling is also detectable in the less mature hindlimb buds. Cells in the hypoglossal cord are also positive for β-galactosidase. (F) X-gal staining of an embryo at E11.5 (40 somites), with a –63/–54 Myf5-nlacZ transgene showing a pattern which is essentially similar to that shown in D. With these two constructs, Myf5-nlacZ activity is also detected in the brain and neural tube, owing to the central nervous system element (–56.6/–53.7), and in the branchial arches, owing to the control branchial arch sequence. There is also ectopic labelling of head mesenchyme, which is particularly notable in C. NT, neural tube; HC, hypoglossal cord.

**Fig. 5.** The –58/–56.6 region contains elements that direct transgene expression to distinct regions of the somite, as well as to the limb buds and hypoglossal cord. X-gal staining of embryos at E11.5 (38-40 somites) in which the Myf5-nlacZ transgene contains a –58/–56.6 kb fragment (A) or subfragments of it: –58/–57 (B), –57.5/–56.6 (F) or –57.5/–57 (H). In A, expression is no longer seen in the brain (compare with Fig. 4D), but in somites, limb buds and hypoglossal cord. In B, the –58/–57 subfragment directs expression to the limb buds and hypoglossal cord (black arrow) and to the epaxial and hypaxial extremities of the somite. The cryostat section in C (stained with Eosin), corresponds to a transverse section (white line in B) at the thoracic level of embryo (B). Higher magnifications (D,E) show β-galactosidase-positive cells in the epithelial structures of the epaxial lip (D) and hypaxial somitic bud (E). In F, somite expression is mainly seen in the epaxial intercalated region of the myotome, shown on a transverse cryostat section at the thoracic level (white line) in G. The outline of the myotome, which is marked by immune staining with a myosin heavy chain antibody (not shown) is presented as a broken line. In H, the 500 bp (–57.5/–57) fragment directs expression to the limb buds, hypoglossal cord, and epaxial and hypaxial extremities of the somite.
from BAC195APZ, which contains 195 kb of genomic DNA 5' to the Myf5 gene and 39.6 kb 3' to it. This BAC recapitulates the complete pattern of Myf5 expression, as monitored with an nlacZ reporter sequence, introduced at the ATG of the gene (Carvajal et al., 2001a). The deletion extends from –63 kb to –48 kb, in order to include any potential sequences that reinforce activity at the 5' end. As expected, comparison of the nlacZ expression profile in the deleted BAC195APZΔ63-48 and wild-type BAC195APZ constructs at different developmental stages showed no difference at E8.5 when β-galactosidase-positive cells, which are located in the epaxial dermomyotome, were present in both cases (results not shown). At this stage, Myf5 expression is directed by the early epaxial enhancer situated at –6.5 kb from the Myf5 gene (Teboul et al., 2002). At E9.0 (Fig. 6A,B), Myf5 expression in immature caudal somites was unaffected by the deletion. In more mature somites, the pattern of dermomyotomal expression was skewed towards the caudal edge of the somite (Fig. 6B), compared with that seen in the undeleted BAC (Fig. 6A). At E11.5, myotomal expression was perturbed in BAC195APZΔ63-48 with foreshortening of the epaxial and hypaxial extent (Fig. 6C-F), as shown on interlimb level sections (Fig. 6G,H), which also suggest loss of β-galactosidase-positive cells in the central epaxial domain. At this stage, it was clear that all brain and limb expression of Myf5 was abolished by the deletion (Fig. 6D). At E10.5, when expression of the transgene begins to be detectable in the forelimb bud, this is not seen with the deleted BAC (results not shown). Expression in the hypoglossal cord is also affected at this stage. These results demonstrate that the region deleted in the BAC is essential for Myf5 expression in the limb buds, in the hypoglossal cord and in the brain, and for aspects of (dermo)myotomal expression, notably those in the epaxial and hypaxial extremities of more mature somites. The epaxial intercalated expression in the myotome is more difficult to follow in the deleted BAC, probably because it tends to be obscured by more proximal elements that also target β-galactosidase in the myotome, and in particular by the perdurance of β-galactosidase as a result of the activity of the early epaxial enhancer (Teboul et al., 2002). This is certainly also the case for the element at –53.3/–48 that targets anterior somites.

Further deletions of the 5' (BAC195APZΔ59-54) and 3' (BAC195APZΔ54-49) parts of the region are shown in Fig. 7 and compared with p8.8, a plasmid that contains the intragenic hypaxial element (Summerbell et al., 2000) and the Mrf4/Myf5 intergenic region, which includes the early epaxial enhancer (Summerbell et al., 2000; Teboul et al., 2002). At E9.5, BAC195APZΔ63-48 (Fig. 7A) and BAC195APZΔ59-54 (Fig. 7B) showed the skewed dermomyotomal expression with a pronounced caudal somitic expression already noted in Fig. 6, which is due to this intragenic element (Fig. 7D). By contrast, epaxial dermomyotome expression in newly formed somites, directed by the early epaxial enhancer (Teboul et al., 2002), is maintained (Fig. 7A-C). Deletion of only the 3' half of the region in BAC195APZΔ54-49 (Fig. 7C) corrects this pattern, probably owing to the dermomyotomal suppressor element.
Fig. 7. Multiple deletions within the –63 kb to –48 kb interval reveal interactions between different regulatory regions. Comparison of Myf5-nlacZ expression profiles between BAC195ΔPZΔ63-48 (A,E), Δ59-54 (B,F), Δ54-49 (C,G) and p8.8 (D,H) constructs. At E9.5 (A-D), deletion of the entire –63 kb to –48 kb region (A) or the –59 kb to –54 kb region (B) results in a marked caudal somitic expression in the dermomyotome, reminiscent of the expression pattern obtained with plasmid p8.8 (D), and driven by the intragenic enhancer element (Summerbell et al., 2000). Deletion of the –54 kb to –49 kb region does not seem to affect the expression pattern at this stage, as it results in a similar pattern to the wild type. This suggests that the –59 kb to –54 kb interval contains the predicted suppressor of the dermomyotome element. At E11.5 (E-H), limb expression is missing in the –63 kb to –48 kb deletion (E), whereas expression in both limbs is seen with both the half-deletion constructs (F,G). In the –59 kb to –54 kb deletion (F), expression in the hindlimb is stronger, whereas in the –54 kb to –48 kb deletion, the relative intensity of expression between fore- and hindlimbs is reversed. This suggests that both regions are required for the full recapitulation of limb expression at this stage. (H) The intragenic region in p8.8 directs a pronounced band of labelling in the myotome of more mature somites.

Previously mapped between –59 kb and –8.8 kb (Carvajal et al., 2001a), and maintains the epaxial myotome expression directed by the –58/–48 region (Fig. 4A,B). At E11.5, BAC195ΔPZΔ54-49 (Fig. 7G) showed the full epaxial/hypaxial extent of Myf5 expression with labelling of the epaxial lip and notably the hypaxial somitic bud, which is missing in BAC195ΔPZΔ63-48 and BAC195ΔPZΔ59-54 (Fig. 7E,F). Notably in these BACs, expression in prosomeres p1 and p4 was missing (Fig. 7E,F, compare with 7G). At this stage the intragenic region in p8.8 still directed caudal somitic expression and a pronounced band of labelling in the myotome (Fig. 7H). At E11.5, the BAC deletions clearly confirmed observations on limb bud expression. In the absence of the 5′ half of the region, with BAC195ΔPZΔ59-54, this was weak, but more pronounced in the hindlimb, in keeping with our observations on the –53.3/–48 sequence (Fig. 2A), while the presence of the 5′ sequence in BAC195ΔPZΔ54-49 directed strong forelimb and weaker hindlimb expression, following the normal developmental gradient.

At E13.5, some trunk and limb musculature continued to be compromised with BAC195ΔPZΔ63-48; however, labelling of limb muscle masses, particularly in the forelimb was now detectable (Fig. 8B). Observations on fore- and hindlimbs from E12.5 to E14.5 are shown in Fig. 8, for deleted and wild-type BACs. In the forelimb at E12.5 (Fig. 8D,E), more proximal muscles were mainly labelled in the deleted BAC, but by E13.5 (Fig. 8F,G) most muscle masses appeared to be labelled. This was also true for the hindlimb (Fig. 8H-K) with a delay of 1 day. We therefore conclude that the –58/-48 region is essential for early expression of Myf5 in the limbs, but that other elements located elsewhere in the locus direct later expression of the gene in the limbs. This expression initially overlaps with that directed by the –58/–48 region, which is still active in the limbs at E13.5 (Fig. 8C).

DISCUSSION

The dissection of the region situated between –58 kb and –48 kb upstream of Myf5 demonstrates that distinct regulatory elements direct transcription of this myogenic determination gene to the limb buds and hypoglossal cord, to sites of myogenesis in the somite and to specific regions of the central nervous system, where the endogenous gene is transcribed, in the mouse embryo (Table 2). The complete region would appear to be a composite of elements which function out of the normal context and with a heterologous tk promoter (Fig. 1B and results not shown). Deletions of this region, in the context of a large BAC, which recapitulates the endogenous expression of Myf5, show that the regulatory elements are necessary for these important aspects of the spatiotemporal regulation of Myf5 transcription. Furthermore, this analysis shows that more than one element targets the same site, revealing multiple phases of myogenesis in the different domains where Myf5 regulatory elements orchestrate the initiation of skeletal muscle formation.

Myf5 transcription in the central nervous system

The region between –56.6 kb and –53.7 kb specifically targets prosomeres p1 and p4 in the brain where endogenous Myf5 transcripts are detected and deletion of this region shows that it is required for transcription of the gene at these sites. It also directs expression to the ventral neural tube. The fact that this site of transcription is not entirely coincident with that of the
endogenous gene (Tajbakhsh and Buckingham, 1995; Tajbakhsh et al., 1994) probably reflects the action of other sequences in the locus in restricting and refining the expression pattern. Indeed, the isolated fragment also tends to be transcribed more widely in prosomere p1. This effect is clearly distinguishable from ectopic expression in head mesenchyme, which is seen sporadically with many Myf5 transgenes. In the case of the neural tube, another element located within the Myf5 proximal region (Summerbell et al., 2000) also directs expression to this site. The upstream sequence, like this proximal sequence, can act independently, as seen in –58/–48 tk-nlacZ transgenic embryos. There is no apparent function associated with the activation of Myf5 in specific neurones of the central nervous system, and based on observations that canonical Wnt signalling is implicated in Myf5 transcription in both somites (Tajbakhsh et al., 1998) and the central nervous system (Daubas et al., 2000), we had speculated that the latter is caused by inappropriate triggering of a somite regulatory element. This is clearly not the case because the –56.6/–53.7 sequence only directs Myf5 transcription to the specific sites in the central nervous system. It is probable that the region between –58 kb and –48 kb was put together by evolutionary tinkering (‘bricolage moléculaire’) (Jacob, 1977), which may have led to the inclusion of an unwanted regulatory element. However, it is surprising that it should have conserved its function in the absence of apparent selective pressure. It is also surprising that a second neural tube element is present in the 5’ proximal region of Myf5. Although the Myf5 protein is not detectable and does not appear to play a role in the embryonic central nervous system (Daubas et al., 2000), a possible role in the postnatal neural tube and brain was not examined because of the lethality of the Myf5 mutation at birth.

These results lead to the conclusion that, in somites, the –57/–56.6 kb fragment directs expression in the intercalated myotome and suggest that in younger embryos this element targets the early epaxial myotome. Other regulatory elements that direct expression to somites, notably to the early epaxial dermomyotome (Teboul et al., 2002) and hypaxial myotome (Summerbell et al., 2000), and to later limb muscles, are revealed by deletion of this region, as discussed in the text. N.D., not done; (), weak.

### Table 2. Regulatory elements within the –58/–48 kb region: a summary of transgenic results

<table>
<thead>
<tr>
<th>Sites of expression</th>
<th>Sequence interval (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>–58/–54</td>
</tr>
<tr>
<td>Somites</td>
<td></td>
</tr>
<tr>
<td>Early epaxial and later intercalated epaxial myotome</td>
<td>+</td>
</tr>
<tr>
<td>Intercalated epaxial myotome</td>
<td>+</td>
</tr>
<tr>
<td>Subset of cells in central/hypaxial myotome (mainly anterior somites)</td>
<td>–</td>
</tr>
<tr>
<td>Later hypaxial somitic bud</td>
<td>+</td>
</tr>
<tr>
<td>Later epaxial lip</td>
<td>+</td>
</tr>
<tr>
<td>Limbs</td>
<td></td>
</tr>
<tr>
<td>Forelimb</td>
<td>+</td>
</tr>
<tr>
<td>Hindlim</td>
<td>+</td>
</tr>
<tr>
<td>Hypoglossal cord</td>
<td>+</td>
</tr>
<tr>
<td>Central nervous system</td>
<td></td>
</tr>
<tr>
<td>Brain prosomeres p1, p4</td>
<td>+</td>
</tr>
<tr>
<td>Ventral neural tube</td>
<td>+</td>
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</tbody>
</table>

Fig. 8. Deletion of the –63 kb to –48 kb region reveals an additional element(s) directing a second phase of limb expression. Transgenic embryos at E13.5 where nlacZ expression is directed by BAC195APZ (A) or by BAC195APZΔ63–48 (B). In C, an embryo with the –58/–48 Myf5–nlacZ transgene is shown. Comparison of the expression patterns of the limbs between embryos of BAC195APZ (D,F,H,J) and BAC195APZΔ63–48 (E,G,I,K) transgenic lines. (E) At E12.5, all pre-muscle masses show weak X-gal staining, not seen in the hindlimb at this stage (not shown). (F,G) By E13.5, the forelimb expression pattern driven by the two constructs is similar. (I) Very weak expression is detected in hindlimb pre-muscle masses. (J,K) By E14.5, the hindlimb expression pattern driven by the two BAC constructs is indistinguishable.
Myf5 expression in the limbs

Sequences within the −58/−48 kb region are essential for early expression of Myf5 in limb buds. An element located between −57.5 kb and −57 kb directs robust transcription of the nlacZ reporter to the fore- and hindlimb buds, following the anteroposterior developmental gradient, in the same spatiotemporal pattern as the endogenous gene. This 500 bp sequence, which is conserved between human and mouse, is therefore a potential target of signalling pathways/transcription factors responsible for the activation of this myogenic determination gene once the muscle progenitor cells have migrated from the somite to the limb bud. The same sequence also directs activation of Myf5 transcription in the hypoglossal cord. This is yet another example of multiple elements targeting the same site, because another element that also directs expression to the hypoglossal cord is present in the region between −81 kb and −63 kb (Carvajal et al., 2001b) and is necessary in the context of the locus. Cells of the hypoglossal cord also delaminate from the hypaxial dermomyotome of occipital somites, but move as a coherent mass (Noden, 1983), with activation of Myf5 before they attain their final location around the larynx or in the tongue, where they contribute to the formation of skeletal muscle (Mackenzie et al., 1998). It is therefore surprising that this different mode of myogenesis is activated by the same element, and it remains to be seen if the same regulation is involved. It is also striking that this element directs reporter gene expression to the hypaxial somitic bud in the interlimb region, at a stage when this is a remnant of the epithelial dermomyotome, which still harbours myogenic progenitor cells that activate Myf5. This is in contrast to cells of the hypoglossal cord and limb buds, which express Myf5 only when they have left the somite.

A second element in the region between −53.3 kb and −48 kb also contributes to Myf5 expression in the limb buds. This element is more active in the hindlimb, where reporter gene expression is clearly observed in the developing muscle masses at a time when β-galactosidase activity is barely detectable in the forelimb. Deletion of the region between −59 kb and −54 kb, where the other limb element is located, confirmed this result. Forelimb expression appears to be delayed and generally weaker, but seems to extend to most of the muscle masses. This distinction between fore- and hindlimbs is particularly interesting in view of the unexpected results of mutations in the Lbx1 and Mox2 homeobox genes. Despite the fact that they are present in myogenic cells in fore- and hindlimbs, the absence of Lbx1 seriously compromises the formation of many forelimb muscles (Brohmann et al., 2000; Gross et al., 2000; Schafer and Braun, 1999), whereas mutations in Mox2 mutants mainly affect hindlimb muscles (Mankoo et al., 1999). Recently, it has been shown that Tbx5 and Tbx4 genes are expressed in fore- and hindlimbs, respectively (Gibson-Brown et al., 1996) and required for their development (Rodriguez-Esteban et al., 1999). Ptx1 (Logan and Tabin, 1999) is another example of a gene that plays a role in hind- but not forelimb development. These mutant phenotypes point to regulatory differences in the limb environment that the muscle progenitor cells enter, in addition to potential intrinsic differences in fore- and hindlimb progenitors themselves. Further analysis of the Myf5 limb element at −53.3/−48 will provide more insight into the differences in the regulatory programme between fore- and hindlimbs.

Deletion of the −58/−48 region, although demonstrating that it is required for early expression of Myf5 in limb buds, also shows that by E12.5 other regulatory sequences begin to participate in Myf5 transcription in the muscle masses of the limbs. This is an autonomous function and therefore not simple maintenance, because it is initiated in the absence of the −63/−48 region. All muscles appear labelled eventually by the BAC transgene with this deletion, although initially this is more readily detectable in proximal muscles. It may be associated with the initiation of secondary myogenesis, which makes a major contribution to the growth of differentiated limb muscles from about E14 (Ontell and Kozeka, 1984).

Myf5 expression in the somite

Several sites of myogenesis in the somite are targeted by the region between −58 kb and −48 kb that, together with the previously identified early epaxial enhancer (Teboul et al., 2002), intragenic hypaxial enhancer (Summerbell et al., 2000), upstream hypaxial sequence (Carvajal et al., 2001a) and myotome sequence present in the −23 kb region (Hadchouel et al., 2000), constitute a set of regulatory modules that orchestrate Myf5 transcription in the somitic cells that form the myotome. Initially, the early epaxial enhancer (Teboul et al., 2002) activates Myf5 transcription in the epaxial dermomyotome, from which cells delaminate, and then, in the presence of Myf5, become correctly positioned to form the epaxial myotome (Tajbakhsh et al., 1996b). A second element contained in the −57.5/−56.6 region of the upstream enhancer then activates Myf5 transcription in the epaxial myotome, which, as the dermomyotome continues to grow in an epaxial direction (Denetclaw and Ordahl, 2000; Spörle, 2001) and to produce myogenic precursors, becomes positioned more centrally, intercalated between epaxial-most and hypaxial components of the myotome. It is here that transcripts of Mrf4 and those for differentiation markers, such as myosins (Lyons et al., 1990; Spörle, 2001), are first detected. Myf5 is required at this stage to activate Mrf4/myogenin transcription. The −57.5/−57 kb fragment does not direct epaxial myotome expression, indicating that the 400 bp at −57/−56.6 kb contains this transcriptional module. This region is conserved between human and mouse genomic DNA, in part because it also contains an exon of the Ptprq gene (Carvajal et al., 2001a). Another regulatory element contained within −23 kb of Myf5 also targets a subdomain of the epaxial myotome (Hadchouel et al., 2000).

The region between −53.3 kb and −48 kb also displays some somitic activity in a subset of cells in a more hypaxial part of the myotome. Labelling is seen at E11.5 in the most anterior somites and later in some muscles anterior to the forelimb. At earlier stages of development, somitic labelling is only occasionally seen. More extensive transgene expression in somites on the anteroposterior axis is also sometimes seen, indicating that the −53.3/−48 region can potentially direct such transcription. In the deleted BACs, it is difficult to distinguish the contribution of this region from that of the intragenic hypaxial enhancer. The hypaxial enhancer, located within the Myf5 gene (Summerbell et al., 2000) directs transcription in the early hypaxial myotome, and labels the caudal edge of the somite. This labelling, together with that due to the early epaxial enhancer, is evident when the −63/−48 region is deleted from BAC195APZ. A further hypaxial element is present in the 5¢ region upstream of −88 kb (Carvajal et al., 2001a). As the somite matures, and the dermomyotome disintegrates, epithelial structures, which probably continue to be
Myf5 regulation reveals multiple phases of myogenesis

Myf5 genes are distinct. Although in the same locus in all vertebrates examined (Braun et al., 1990; intimately related to that of the gene, which is linked to it), little or no regulatory capacity (Summerbell et al., 2000).

Somite, whereas the proximal promoter itself appears to have region, together with the early epaxial and hypaxial enhancers play a major role in the expression of Myf5 in the mouse somite, whereas the proximal promoter itself appears to have little or no regulatory capacity (Summerbell et al., 2000).

The way in which Myf5 regulation may have evolved is intimately related to that of the Mrf4 gene, which is linked to it in the same locus in all vertebrates examined (Braun et al., 1990; Miner and Wold, 1990). The expression profiles of these two myogenic factor genes are distinct. Although Mrf4 appears to be regulated, in part, by sequences immediately 5’ to it (Patapoutian et al., 1993; Pin et al., 1997), it is also dependent on sequences further upstream (Carvajal et al., 2001a); it will be interesting to establish to what extent the –58/-48 kb enhancer region interacts with the Mrf4 promoter, which lies 5’ to that of Myf5. Another myogenic factor gene, MyoD, which, like Myf5, plays a role in skeletal muscle determination (Rudnicki et al., 1993), has a distal enhancer that is located at –22 kb from the gene (Goldhamer et al., 1995). This directs early expression at sites of muscle formation in the embryo, whereas more proximal 5’ elements are necessary for transcription at later stages of skeletal muscle development. However, the MyoD enhancer, which is also active in limb buds, hypoglossal cord and myotome, can be reduced to a single 258 bp core element (Goldhamer et al., 1995; Kucharczuk et al., 1999), in contrast to Myf5, where the –58/-48 kb enhancer region breaks down into discrete elements that target different sites. Furthermore, as discussed here, crucial elements that direct Myf5 expression in the embryo, as well as during later stages of development, are present elsewhere in the locus. These differences probably reflect the fact that MyoD lies genetically downstream of Pax3 and Myf5, which govern the entry of cells into the myogenic programme. Myf5 is therefore the target of the signalling pathways that specify myogenic cell fate and that differ between sites of muscle formation in the embryo, acting through different Myf5 regulatory sequences. Although it was not surprising that limb versus dermomyotome/myotome expression of Myf5 should be subject to different controls, the analysis presented here reveals the extraordinary complexity of myogenic patterning. At least three different regulatory circuits govern Myf5 transcription in the limb, two in the hypoglossal cord, two in the epaxial and three in the hypaxial dermomyotome, and probably at least three more in the myotome. Other upstream regulatory genes, which have partially overlapping patterns of expression, may well display a similar complexity, such that small numbers of cells may read a unique code specifying muscle fate. Such codes, which would determine the myogenic body plan of a mouse, no doubt reflect the many ways in which further cell populations were co-opted into the muscle programme as vertebrates evolved and ever more sophisticated muscle functions were required. Identification of multiple Myf5 regulatory elements now makes it possible to dissect muscle formation at any one site, both in terms of molecular regulation and of unique cellular contributions.

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