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The early epaxial enhancer is essential for the initial expression of the skeletal muscle determination gene *Myf5* but not for subsequent, multiple phases of somitic myogenesis

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SUMMARY

Vertebrate myogenesis is controlled by four transcription factors known as the myogenic regulatory factors (MRFs): Myf5, Mrf4, myogenin and MyoD. During mouse development Myf5 is the first MRF to be expressed and it acts by integrating multiple developmental signals to initiate myogenesis. Numerous discrete regulatory elements are involved in the activation and maintenance of *Myf5* gene expression in the various muscle precursor populations, reflecting the diversity of the signals that control myogenesis. Here we focus on the enhancer that recapitulates the first phase of *Myf5* expression in the epaxial domain of the somite, in order to identify the subset of cells that first transcribes the gene and therefore gain insight into molecular, cellular and anatomical facets of early myogenesis. Deletion of this enhancer from a YAC reporter construct that recapitulates the *Myf5* expression pattern demonstrates that this regulatory element is necessary for expression in the early epaxial somite but in no other site of myogenesis. Importantly, *Myf5* is subsequently expressed in the epaxial myotome under the control of other elements located far upstream of the gene. Our data suggest that the inductive signals that control *Myf5* expression switch rapidly from those that impinge on the early epaxial enhancer to those that impinge on the other enhancers that act later in the epaxial somite, indicating that there are significant changes in either the signalling environment or the responsiveness of the cells along the rostrocaudal axis. We propose that the first phase of *Myf5* epaxial expression, driven by the early epaxial enhancer in the dermomyotome, is necessary for early myotome formation, while the subsequent phases are associated with cytodifferentiation within the myotome.

Key words: Epaxial, *Myf5*, Myogenesis, Somite, Dermomyotome, Myotome, Mouse

INTRODUCTION

Our aim is to understand how cells respond to the signals within the embryo that determine their identity, using, as a model, the commitment of paraxial mesoderm progenitors to the skeletal muscle fate. In the trunk, the segmental units, the somites, which are located on either side of the neural tube, form sequentially from the presomitic mesoderm such that the most cranial pair is the first to be born (Christ et al., 1992). While facial muscles derive from the unsegmented anterior paraxial mesoderm and the prechordal mesoderm, the somites produce the myoblasts of the trunk, limbs, diaphragm and tongue, in addition to other cell types such as dermal cells and chondroblasts. According to the classical model, muscle progenitor cells, which migrate into the prospective muscles dorsal to the transverse processes of the vertebrae, involute from the epaxial epithelial edge (lip) of the dermomyotome and accumulate on its inner surface to form the epaxial component of the myotome (Williams, 1910). Recent studies have expanded this view, suggesting several routes by which cells translocate from the dermomyotome to the myotome both directly from the dorsomedial lip (DML) and by migration from the DML to the rostral and caudal lips, and thence into the myotome (Denetclaw et al., 1997; Kahane et al., 1998; Cinnamon et al., 2001; Ordahl et al., 2001). The various models differ in the relative importance given to these routes

but they are not mutually exclusive, since the experiments supporting them were performed on somites from different levels along the rostrocaudal axis. The behaviour of hypaxial myoblasts also depends on their position along this axis (Summerbell et al., 2000; Carvajal et al., 2001).

Each of the groups of cells derived from the somites or the anterior and prechordal mesoderm that become myoblasts will differentiate into skeletal muscle but they follow different paths and respond to different environmental signals that govern their behaviour (reviewed by Buckingham, 2001). These signals induce the expression of a cascade of transcription factors that involves the four myogenic regulatory factors (MRFs): Myf5; myogenin; Mrf4 and MyoD, which are members of the basic helix-loop-helix (bHLH) superfamily of transcription factors (reviewed by Arnold and Braun, 2000). *Myf5* transcripts are first detected in the epaxial part of the somite at 8.0 dpc (days post coitum) and then in the epaxial myotome before they are seen in the hypaxial domain (Ott et al., 1991; Summerbell et al., 2000). The earliest epaxial *myogenin* (*myog* – Mouse Genome Informatics) and *Mrf4 (Myf6* – Mouse Genome Informatics), transcription follows within half a day and a day, respectively (Sassoon et al., 1989; Bober et al., 1991; Summerbell et al., 2002).

In *Myf5*$^{nlacZ/nlacZ}$ embryos myotome formation is delayed by 2.5-3 days (Tajbakhsh et al., 1996) but thereafter muscle development proceeds normally following *MyoD* (*myod1* - Mouse Genome Informatics) activation [(Braun et al., 1994; Tajbakhsh et al., 1996) and unpublished data]. Analysis of *Myf5*$^{-/-}$ mutant and other available *Mrf*-null models leads to a view of trunk skeletal muscle development whereby extracellular signals induce the expression of Myf5, which then activates the *myogenin* gene (reviewed by Arnold and Braun, 2000). Myf5 triggers early myotome formation, which may also depend on Mrf4 (Buckingham, 1994). However, *MyoD* can be activated independently of *Myf5* and plays a critical role in myogenesis in the limb (Kablar et al., 1997). Consistent with their role as upstream myogenic regulators both Myf5 and MyoD have been shown to act in the chromatin remodelling of loci involved in the myogenic programme (Gerber et al., 1997), while myogenin subsequently activates the genes encoding the terminal differentiation products. According to this model, Myf5 initiates myogenesis and occupies a key position in the myogenic cascade.

We have previously used reporter gene assays in transgenic mice to identify the elements that regulate *Myf5* expression. Our data show that the control mechanisms for *Myf5* expression, where enhancers are specific for particular precursor cell populations arising at distinct locations in the embryo (Summerbell et al., 2000), are dissimilar to those that operate for either *myogenin* or *MyoD* (Cheng et al., 1993; Yee and Rigby, 1993; Goldhammer et al., 1995; Kucharczuk et al., 1999). Our laboratories have shown that the components of the complicated and dynamic *Myf5* expression pattern are recapitulated in transgenic mice containing YAC- or BAC-based reporter constructs covering a 200 kb region (Hadchouel et al., 2000; Carvajal et al., 2001). Our data, together with those of others (Patapoutian et al., 1993; Zweigerdt et al., 1997), have begun to lead to the identification of the regulatory elements within this region. The transcriptional regulation of *Myf5* is further complicated by the interdigitation of *Myf5* control elements with those that regulate *Mrf4* and the possibility that some elements may act on both genes (Carvajal et al., 2001). *Mrf4* is located immediately upstream of *Myf5* and has a distinct but overlapping pattern of expression, under the control of several regulatory regions (Patapoutian et al., 1993; Pin et al., 1997; Carvajal et al., 2001).

Here, we focus on the first event of *Myf5* expression, which occurs in the epaxial part of the youngest somites at a location where *Mrf4* is not expressed. We have defined an enhancer, referred to as the Epaxial Element, which is sufficient to recapitulate this initial phase, and mapped it to a region immediately downstream of the *Mrf4* gene (Summerbell et al., 2000). This result has recently been confirmed by Gustafsson et al. (Gustafsson et al., 2002). This enhancer also drives ectopic expression in the dermomyotome and in the cephalic mesoderm (Summerbell et al., 2000). For reasons that will become apparent in this manuscript, we rename this regulatory element the Early Epaxial Enhancer (EEE).

To ascertain the role of this enhancer in the regulation of the *Myf5* locus, we deleted it from a reporter construct that can recapitulate the *Myf5* expression pattern (Hadchouel et al., 2000). We show that the EEE is necessary for directing the first phase of *Myf5* transcription, and therefore this first readout of myogenic signals during embryonic development. We also conclude that there are multiple, separable phases of epaxial somitic expression during primary myogenesis driven by different regulatory elements. The first event of epaxial somitic expression is activated prior to myotome formation and the appearance of the first skeletal muscle cells, while the other regulatory elements come into play during subsequent steps of myogenesis.

MATERIALS AND METHODS

All positions in the locus are indicated by their distance from the transcription start site of *Myf5*. Embryos were staged in days, taking 0.5 as the morning post coitum (dpc), and, more precisely, by somite counts. Somites were numbered using roman numerals, from the caudal end of the embryo to the rostral end, with the most newly formed somite designated as somite I (Ordahl, 1993).

Preparation of plasmid constructs

Plasmids were prepared using standard recombinant DNA techniques (Sambrook et al., 1989). The plasmids pE(paxial)EBZ and pE(paxial)XBZ contained a fragment, the 651 bp *Eco*RI-*Bam*HI and 450 bp *Xmn*I-*Bam*HI, respectively, cut out of construct #1 and a promoter-reporter insert equivalent to construct #9 (Summerbell et al., 2000). Full information on all cloning steps can be obtained on request.

YAC mutagenesis

y200-Myf5-nlacZ was modified following the protocol previously described (Hadchouel et al., 2000) using the split-marker vectors pUR and pRA (Fairhead et al., 1996). The early epaxial enhancer region, from the *Eco*RI site located at –6.3 kb to the *Bam*HI site at –5.7 kb was deleted from y200-Myf5-nlacZ. A 727 bp *Xba*I-*Eco*RI fragment, located between –7.0 and –6.3 kb, was inserted into pUR to create pUR-Epax. A 532 bp *Bam*HI-*Eco*RI fragment, located between –5.6 and –5.1 kb, was inserted into pRA to create pRA-Epax. These two vectors were used to create y200ΔE-Myf5-nlacZ.

Production of transgenic mice

Transgenic mice were produced by pronuclear injection of single-cell embryos from CBA × C57Bl/10 crosses as previously described (Yee and Rigby, 1993). Founder mice for pEEBZ, pEXBZ and y200ΔE-

Myf5-nlacZ were detected by PCR analysis of tail DNA as previously described (Hadchouel et al., 2000; Summerbell et al., 2000). Two pEEBZ lines, three transient pEXBZ transgenic embryos between 9.5 and 10.5 dpc plus two pEXBZ lines, and one transient y200ΔE-Myf5-nlacZ transgenic embryo plus five y200ΔE-Myf5-nlacZ lines expressed the transgene and were analysed. All embryos carrying a given construct showed the same pattern of expression except where noted in the results.

Whole-mount histochemistry for β-galactosidase activity and histology

Embryos were fixed in ice-cold Mirsky's fixative (National Diagnostics) overnight or in ice-cold 4% paraformaldehyde for 10 minutes, rinsed in three changes of PBS over 30 minutes and incubated in staining solution overnight, at 37°C for embryos ranging from 8.5 to 11.5 dpc, or at room temperature for older embryos. Staining solution was prepared in PBS and contained 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.01% Nonidet P-40 and 0.1% X-gal. Following histochemistry, embryos were washed in three changes of PBS over 30 minutes. Some of the whole-mount stained embryos were embedded in 2% agarose and 70 μm sections were cut using a vibratome (Leica VT1000 S).

In situ hybridisation

In situ hybridisation was performed as previously described (Summerbell et al., 2000) using an InsituPro robot (Intavis, Bergisch-Gladbach, Germany). Red-Phos (Research Organics, Cleveland, Ohio) was used instead of BCIP in some instances. Riboprobes were prepared using T7 RNA polymerase (Promega) on a 497 bp *Mlu*I-*Eco*RI subclone of *lacZ*.

Photomicroscopy

Microscopic images were captured by analog to digital conversion using a Scion Graphics card, at a resolution of 768 by 576 by 24 bits, a JVC 3CCD colour video camera and a Nikon SMZ1500 stereomicroscope under dark-field optics. Images of sections were captured using a Kodak DCS620X camera, at a resolution of 2048 by 1536 by 36 bits, and a Leica DMR microscope and differential interference contrast. Figures were assembled using Adobe Photoshop and Free-Hand.

Immunofluorescence

For cryostat sections, embryos were fixed in fresh 4% PFA at 4°C for 1 hour and rinsed in PBS before being transferred to 15% sucrose in PBS and then to 15% sucrose/7% gelatin in PBS for sectioning. 12-15 μm cryostat sections were fixed in 1% PFA in PBS for 2 minutes at room temperature, rinsed in PBS and then permeabilised in PBS containing 0.2% Triton X-100 for 10 minutes. After a 5 minute wash in PBS, sections were incubated with primary antibodies in PBS containing 1% BSA, 1% heat inactivated goat serum (Sigma) and 0.025% Tween-20 (Sigma). Monoclonal anti-desmin (Dako, 1:100 dilution) or anti-myosin heavy chain (MF20; Developmental Studies Hybridoma Bank; dilution 1:50), and polyclonal anti-β-galactosidase antibodies (Molecular Probes; dilution 1:100) were used for overnight incubation at 4°C in a humidified chamber. After several rinses in PBS, sections were incubated for 1 hour at room temperature with secondary antibodies (goat anti-mouse IgG Alexa Fluor 594 or anti-rabbit Alexa Fluor 488; Molecular Probes; dilution 1:200) and bisbenzimide (Hoechst, Sigma; dilution 1:1000). Sections were rinsed in PBS, mounted with Mowiol (Calbiochem) and observed with a fluorescence microscope (Zeiss Axiophot) equipped with an AxioVision system (Zeiss).

RESULTS

The early epaxial enhancer recapitulates the initial phase of *Myf5* expression

The activity of the regulatory element controlling the earliest phase of *Myf5* expression was originally assessed in a classical enhancer test employing a heterologous (β-globin) promoter [(construct #10 of Summerbell et al. (Summerbell et al., 2000)]. The β-galactosidase product of the transgene appeared first in the DML of each somite as it formed from the presomitic mesoderm [fig. 5A,D of Summerbell et al. (Summerbell et al., 2000)]. However, transverse sections revealed that subsequently β-galactosidase-positive cells accumulated not only in the dorsal myotome, but also throughout the dermomyotome [fig. 5D of Summerbell et al. (Summerbell et al., 2000)]. The former location is fully consistent with the earliest site of endogenous *Myf5* expression (Ott et al., 1991; Summerbell et al., 2000) but the latter is clearly ectopic. We have now re-characterised the EEE in more detail using the homologous context of the *Myf5* minimal promoter (for details, see Summerbell et al., 2000) driving an *nlacZ* reporter gene (construct pEEBZ, Fig. 1A). As with construct #10, in 8.5 and 9.5 dpc embryos pEEBZ drove reporter gene expression at the correct time and place (epaxial part of the somites, Fig. 1B,C) for the earliest *Myf5* expression (Ott et al., 1991; Summerbell et al., 2000). At 10.5 dpc (Fig. 1D), strong dermomyotomal expression could be

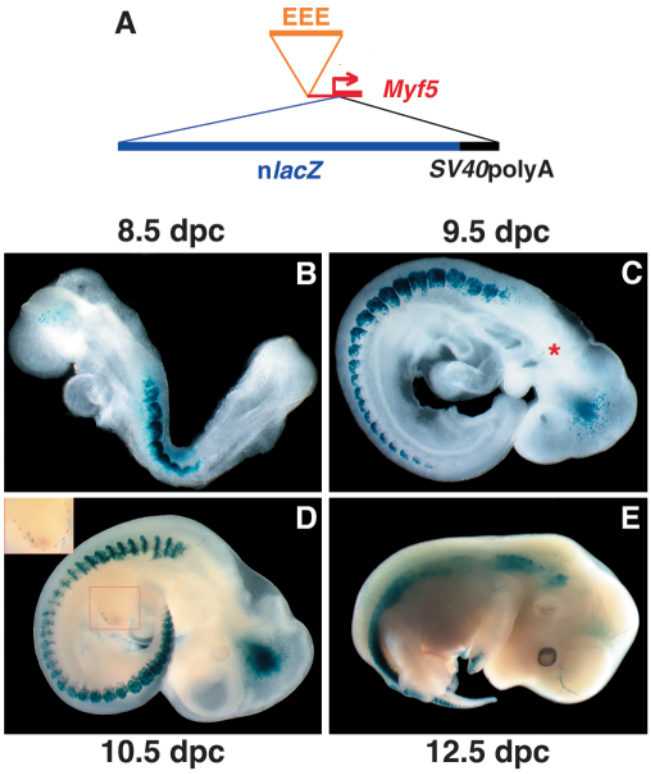

Fig. 1. Time-course of pEEBZ transgenic embryos stained for β-galactosidase. (A) pEEBZ construct map. (B) Transgene expression starts before 8.5 dpc. At 9.5 dpc (C), epaxial somitic expression is evident in all somites and expression is also seen in the branchial arches (star) and in the head. At 10.5 dpc (D), dermomyotomal expression can be seen in the youngest somites. Transgene expression can be observed in the myotome in older somites where the dermomyotomal structure is breaking down. Additional ectopic expression can be seen in limbs (higher magnification inset). At 12.5 dpc (E), continued epaxial somitic expression and ectopic limb and head expression are seen.

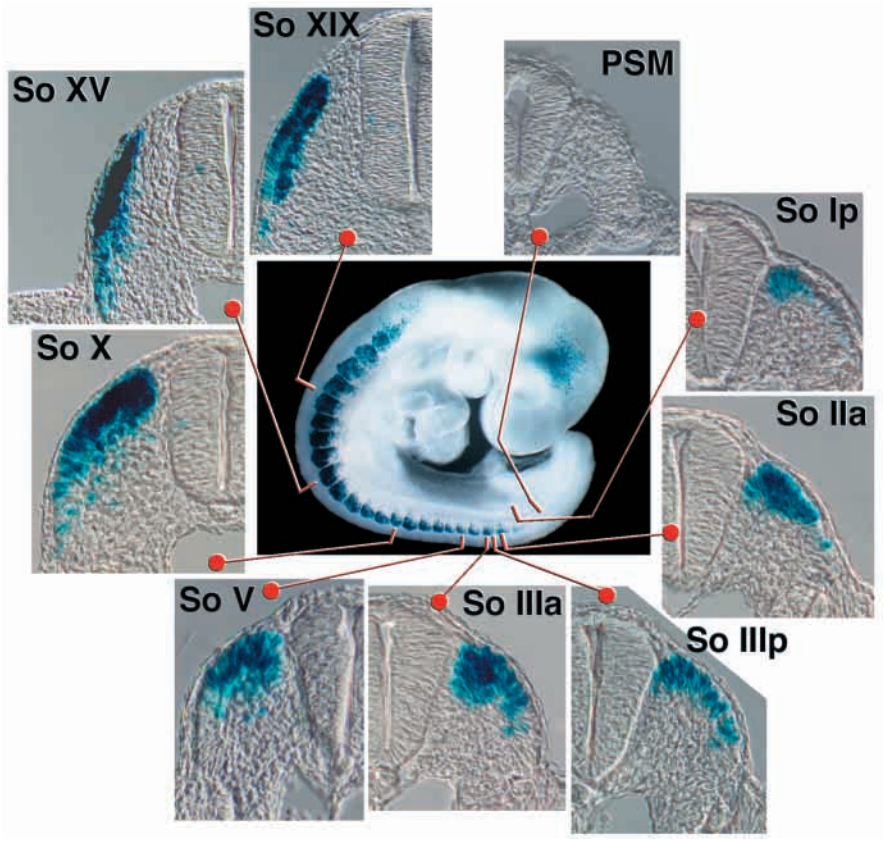

Fig. 2. Transverse sections of a 9.5 dpc (26 somite) pEEBZ transgenic embryo following X-gal staining. When using the criteria of Spörle and Schughart (Spörle and Schughart, 1997) this embryo is at the 27 somite stage. (PSM) No transgene expression can be seen in the presomitic mesoderm adjacent to the first somite. As soon as the somite is born (So Ip), β-galactosidase-positive cells are detected adjacent to the dorsal neural tube. (So IIa-IIIa) Stronger epaxial expression is seen in the dermomyotome of the subsequent, older somites. (So V-XIX) The expression of β-galactosidase is observed more hypaxially throughout the dermomyotome and the myotome as the somite matures (p, posterior; a, anterior).

seen in the youngest somites. Strong myotomal X-gal staining was observed in all mature somites, including the oldest where the dermomyotomal structure was disintegrating. Construct pEEBZ drove strong epaxial somitic expression at least until 13.5 dpc (Fig. 1E and data not shown). Moreover, the EEE also drove ectopic expression in the cephalic mesoderm, in the branchial arches (at a location where *Myf5* is not normally expressed: see star in Fig. 1C) and, from 10.5 dpc, although less consistently, in the posterior lateral edge of the limbs (Fig. 1D,E). Similar ectopic expression was observed with both the pEEBZ and pEXBZ constructs. Sporadic incidences of patches of ectopic expression at other locations were also occasionally observed.

Transverse sections of a 9.5 dpc (26 somite) embryo showed the location of the X-gal staining (Fig. 2). As soon as the somite (So) was born, β-galactosidase-positive cells were detected in the dorsal quadrant adjacent to both neural tube and ectoderm (Fig. 2, So Ip). Both the intensity of expression and the proportion of β-galatosidase-positive cells increased in the dermomyotome and expression extended further ventrally in slightly older somites (Fig. 2, So IIa-IIIa). β-galactosidase-positive cells also began to appear in the myotome (Fig. 2, So IIIa). As the somite matured, the X-gal staining broadened hypaxially in both the dermomyotome and the myotome (Fig. 2, So V-cervical). This contrasts with the X-gal staining pattern in *Myf5*$^{nlacZ/+}$ heterozygote embryos where dermomyotomal expression at a similar time and location is restricted to the DML [fig. 2C of Tajbakhsh et al. (Tajbakhsh et al., 1996)]. These data showed that the early epaxial enhancer directs the first phase of *Myf5* expression and that it does so with both

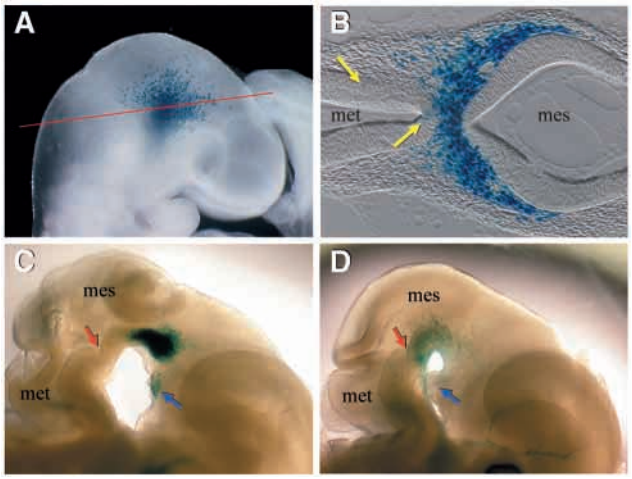

Fig. 3. Characterisation of the head expression in pEEBZ transgenic embryos. (A) Reporter gene expression is seen in the head at 9.5 dpc. (B) In transverse sections (plane of section shown in A) strong expression is detected in the cephalic mesoderm together with a few stained cells in the brain (arrows). (C) Dissection of the head of a 12.5 dpc y200-Myf5-nlacZ embryo shows reporter expression in prosomere p1 and in the mamillary body as previously described (Tajbakhsh and Buckingham, 1995; Daubas et al., 2000). (D) Dissection of the head of a 12.5 dpc pEEBZ embryo reveals that the reporter expression is scattered but centred on the posterior mesencephalon extending into the pons. β-galactosidase expression takes place predominantly outside of the previously described territories (red arrow indicates the boundary between the mesencephalon and the metencephalon; blue arrow indicates mamillary body; mes, mesencephalon; met, metencephalon).

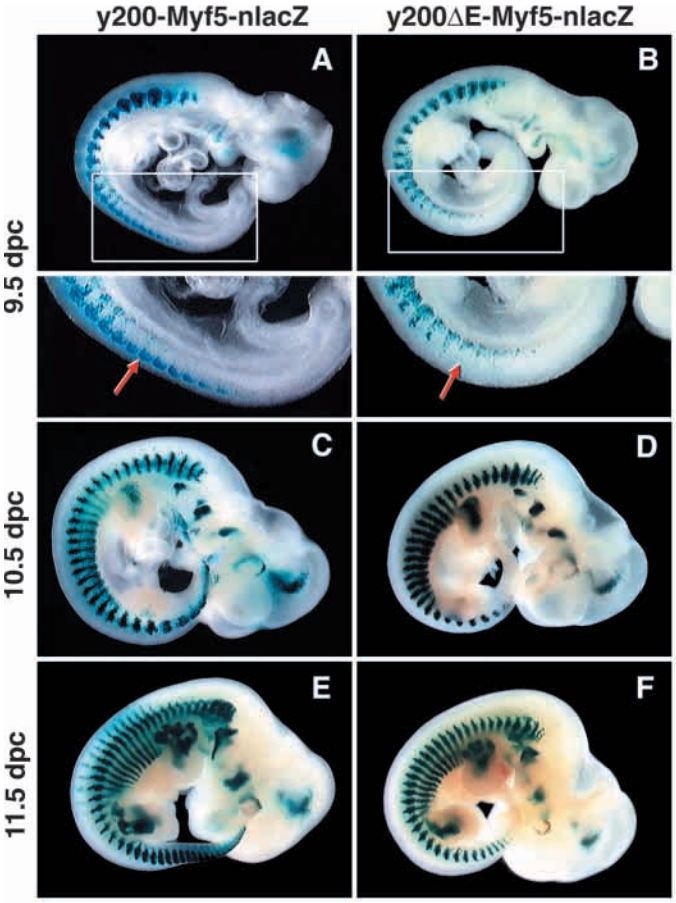

Fig. 4. Deletion analysis of the early epaxial enhancer in the y200-Myf5-nlacZ context. The expression patterns of 9.5 (A,B), 10.5 (C,D) and 11.5 (E,F) dpc embryos bearing y200-Myf5-nlacZ (A,C,E) and y200ΔE-Myf5-nlacZ (B,D,F) and stained for β-galactosidase. The deletion abolishes dermomyotomal transgene expression (arrows in A and B) although some epaxial myotomal expression remains. The only difference between the two series of embryos is that the early epaxial transgene expression is missing in the y200ΔE-Myf5-nlacZ line.

homologous and heterologous promoters but that it also drives extensive ectopic expression in the dermomyotome. The more extended dermomyotomal component of the pattern set by pEEBZ could be due to ectopic transcription or the perdurance of the β-galactosidase in cells that had moved within the dermomyotome and were no longer transcribing the gene, or both (but see below).

A second site of strong ectopic expression was the cephalic mesoderm. In 9.5 dpc pEEBZ whole-mount embryos (Fig. 3A), β-galactosidase-positive mesodermal cells masked the region of the brain in which we have previously observed *Myf5* expression [figs 1, 2 of Tajbakhsh and Buckingham (Tajbakhsh and Buckingham, 1995)]. Transverse sections of the head of the embryo shown in Fig. 3A revealed X-gal staining predominantly in the cephalic mesoderm (Fig. 3B). Although occasional stained cells could be seen in the brain (arrows), the only focus of this expression was at the junction of the telencephalon and mesencephalon, which was clearly ectopic. Dissection of the heads of 12.5 dpc y200-Myf5-nlacZ (Fig. 3C) and pEEBZ (Fig. 3D) embryos confirmed that the reporter expression in the former was in prosomere p1 and in the mamillary body, as previously described (Tajbakhsh and Buckingham, 1995; Daubas et al., 2000). In the pEEBZ embryo, expression in the brain was ectopic, centred on the posterior mesencephalon extending into the pons.

Deletion of the early epaxial enhancer in the context of y200-Myf5-nlacZ abolishes transgene expression in the epaxial region of early somites

We have shown that large constructs, such as y200-Myf5-nlacZ (Hadchouel et al., 2000) or BAC140Z (Carvajal et al., 2001), recapitulate the pattern of *Myf5* expression. At 9.5 dpc, control embryos of y200-Myf5-nlacZ lines (Fig. 4A) expressed the transgene in the epaxial dermomyotome (arrow) and the myotome of all somites as well as in the mandibular and hyoid arches. We deleted the region containing the EEE from the y200-Myf5-nlacZ construct producing y200ΔE-Myf5-nlacZ. We then made transgenic mice bearing this YAC construct and characterised the pattern of reporter gene expression between 9.5 and 14.5 dpc. In 9.5 dpc embryos stained for β-galactosidase activity, the onset of transgene expression in each epaxial somite was delayed by approximately 10 hours (arrows in Fig. 4A,B). This is equivalent to the time required to form 6 or 7 somites, so that the somitic

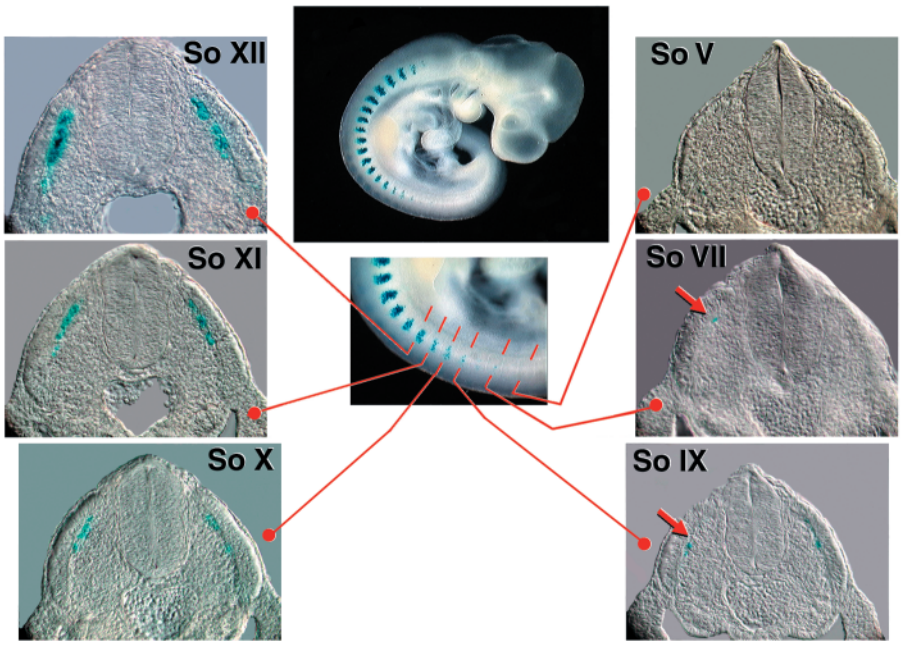

Fig. 5. Transverse sections of a 9.5 dpc (26 somite) y200ΔE-Myf5-nlacZ transgenic embryo stained for β-galactosidase. At this stage no transgene expression can be seen in the dermomyotome. Transgene expression starts in the epaxial half of the myotome of somite VII (arrow in So VII). The staining extends further epaxially and hypaxially in the consecutive somites until it occupies the entire length of the myotome (So IX-XII).

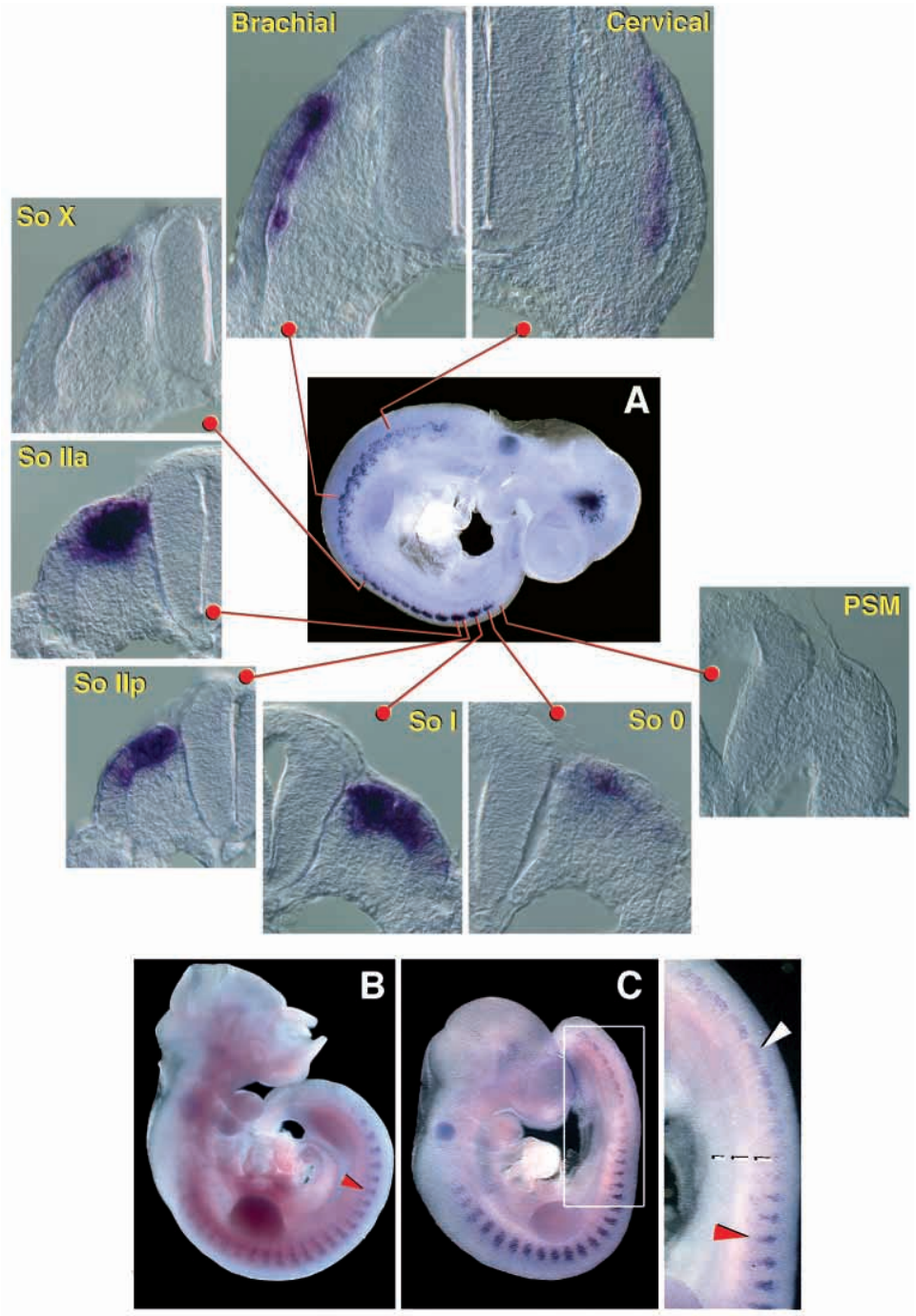

Fig. 6. Analysis of reporter gene transcription. In situ hybridisation was performed on 9.5 dpc embryos bearing (A) pEEBZ, (B) y200ΔE-Myf5-nlacZ and (C) y200-Myf5-nlacZ constructs. (A) Transverse sections of a 9.5 dpc (26 somite) pEEBZ transgenic embryo stained for β-galactosidase. When using the criteria of Spörle and Schughart (Spörle and Schughart, 1997), this is a is 27 somite stage embryo. (PSM) No transgene expression can be seen in the presomitic mesoderm adjacent to the first somite. (So O) As soon as the somite is born, *nlacZ* transcripts are detected in cells in the dorsal half of the somite. (So I-IIa) Stronger epaxial expression is seen in the dermomyotome of the subsequent older somites. (So X-Brachial) Intensity of dorsal dermomyotomal expression decreases thereafter. (Brachial-Cervical) The expression of *nlacZ* transcripts is observed more hypaxially through the myotome as the somite matures. (B) The y200ΔE-Myf5-nlacZ reporter transcripts are detected in the myotomes of all somites (red arrowhead) and in the hypaxial dermomyotome at the interlimb level. (C) The y200-Myf5-nlacZ transgene transcript pattern is the sum of those of pEEBZ and y200ΔE-Myf5-nlacZ. The higher magnification picture of the youngest somites illustrates the switch from the transcriptional output of the EEE (white arrowhead) to that of the enhancers that operate in the myotome (red arrowhead). Dashed line indicates the level at which this switch is seen.

transgene expression in each embryo first appeared more rostrally than normal. However, the hypaxial expression was identical to that seen with the non-deleted YAC (Fig. 4A,B and data not shown). Indeed, no difference could be seen between the expression patterns of y200-Myf5-nlacZ and y200ΔE-Myf5-nlacZ, in both skeletal muscle and in the brain, at all developmental stages analysed except, early, in the epaxial domain (Fig. 4C-F and data not shown).

Transverse sections of a 9.5 dpc (26 somite) embryo carrying the y200ΔE-Myf5-nlacZ transgene showed no expression in somites I to VI (Fig. 5, So V) and only occasional blue cells in somites VII to IX (Fig. 5, So VII to IX). Sections showed no epaxial dermomyotomal expression of the transgene in somites at any level along the rostrocaudal axis. The earliest expression was observed in the epaxial half of the myotome approximately 3 cell widths in from the edge (Fig. 5, arrows) and subsequently expanded through the myotome (So X-XII). These data showed that the EEE is required for the first event of *Myf5* expression and that at least one other element subsequently regulates *Myf5* expression in the myotome.

Analysis of reporter gene transcription and tracing of *nlacZ* expressing cells

We have previously illustrated the dramatic differences that can be seen between transcript and β-galactosidase activity patterns when using the *nlacZ* reporter gene (Hadchouel et al., 2000).

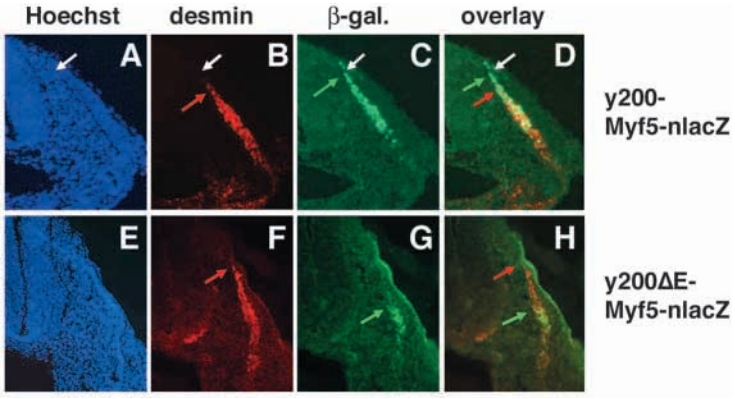

Fig. 7. Localisation of β-galactosidase-positive cells within the myotomes of y200-Myf5-nlacZ and y200ΔE-Myf5-nlacZ 9.5 dpc embryos. (A-D) Transverse sections of y200-Myf5-nlacZ, (E-H) transverse sections of y200ΔE-Myf5-nlacZ. A and E are stained with Hoechst 33258 to show the anatomy. Desmin (red), used as a marker of myogenic cytodifferentiation, is detected throughout the myotome except in cells immediately adjacent to the DML (dorsomedial lip) (B,F). β-galactosidase-positive cells (green) extend up to and within the epaxial DML in the y200-Myf5-nlacZ control sections (C) but are restricted to relatively ventral regions of the myotome in y200ΔE-Myf5-nlacZ (G). D and H show overlays (merged images) of B,C and F,G, respectively. White arrows mark the boundary of DML, red arrows mark dorsal desmin boundary, green arrows dorsal β-galactosidase boundary.

We therefore also assessed the patterns of transcript expression driven by pEEBZ, y200-Myf5-nlacZ and y200ΔE-Myf5-nlacZ. Fig. 6A shows a pEEBZ 9.5 dpc (26 somite) embryo hybridised with an antisense *nlacZ* probe. Compared to the β-galactosidase pattern (Fig. 1C, Fig. 2), the *nlacZ* transcripts in the somites were in general more dorsally restricted and at rostral levels of significantly lower intensity. Exceptions were the newly born somites where the in situ hybridisation pattern was both stronger and extended more ventrally.

Transverse sections of this embryo identified the cause of these differences. No transcripts were detected in the PSM adjacent to the first somite. The *nlacZ* transcripts were first observed in the dorsal half of the newly forming somite slightly earlier than the β-galactosidase activity and similar to the endogenous transcripts (Fig. 2A, PSM and So Ip). Stronger expression was seen in the dorsal dermomyotome of slightly older somites (Fig. 6A, So I-IIa), which extended quite ventrally. Expression intensity decreased thereafter and became progressively restricted to the DML (Fig. 6A, So X-brachial). In contrast, β-galactosidase steadily accumulated in the dorsal dermomyotome and increased both in intensity and in ventral extent (Fig. 2A, So I-XV). The contrast was even more striking in the myotome. Caudal somites contained very few, or no, cells clearly transcribing *nlacZ* in the myotome (Fig. 6A, So I-X), while β-galactosidase-expressing cells accumulated there (Fig. 2, So I-X). The pattern changed dramatically shortly after So X where *nlacZ* transcripts were detected in the myotome (Fig. 6A, So XV). At more rostral levels, expression continued in the myotome in an increasing number of cells throughout the cervical and occipital somites.

Our data showed that the enhancer also drives expression outside the DML. Similarly cells that had expressed the *nlacZ* transcripts while in the dermomyotome continued to display β-galactosidase activity as they accumulated in the myotome but very few cells were clearly transcribing *nlacZ* at the latter location (compare So V-X in Fig. 5 and Fig. 6A). This suggested that cells that initially switched on the transgene in the dermomyotome or in the DML switched it off again when they migrated into the myotome.

Importantly, the *nlacZ* transcript pattern was somewhat different from the endogenous pattern (Summerbell et al., 2000). The latter shows little or no expression in the dorsal dermomyotome outside the DML and stronger expression in the dorsalmost myotome. Indeed the isolated EEE drove both the recapitulation of the first phase of *Myf5* expression in the DML and ectopic expression in the dermomyotome. Moreover, the strong endogenous expression in the dorsalmost myotome was not recapitulated by the isolated enhancer.

Fig. 6B and C show a y200ΔE-Myf5-nlacZ and a y200-Myf5-nlacZ 9.5 dpc embryo, respectively, that were hybridised with the *nlacZ* antisense probe. The y200ΔE-Myf5-nlacZ reporter transcripts were detected in the myotomes of all somites and in the hypaxial dermomyotome at the interlimb level (Fig. 6B). The y200ΔE-Myf5-nlacZ transcript pattern was similar to the equivalent X-gal staining pattern (Fig. 4B). The y200-Myf5-nlacZ transgene transcript pattern was the sum of those of pEEBZ and y200ΔE-Myf5-nlacZ (Fig. 6C). The higher magnification view of the youngest somites allowed us to clearly distinguish the transcriptional output of the EEE (Fig. 6C, white arrowhead) from that of the enhancers that operate in the myotome (Fig. 6C, red arrowhead). It is noteworthy that this switch of expression occurred very rapidly as the somites matured.

Characterisation of the somitic cells marked by y200ΔE-Myf5-nlacZ

Deletion of the EEE and consequent loss of the earliest expression revealed at least one further phase of expression in the epaxial somite. Transverse sections at inter-limb level of 9.5 dpc y200-Myf5-nlacZ (Fig. 7A-D) and y200ΔE-Myf5-nlacZ (Fig. 7E-H) embryos were co-immunostained with anti-desmin, a marker of myogenic differentiation (Venuti et al., 1995), and anti-β-galactosidase antibodies. Hoechst 33258 staining (Fig. 7A,E) was employed to visualise the DML (white arrows). Desmin was detected throughout the myotome except in cells immediately adjacent to the DML (red arrow, Fig. 7B,F). The β-galactosidase staining in y200-Myf5-nlacZ embryo sections extended from the DML (green arrow) throughout the myotome (Fig. 7C) whereas in y200ΔE-Myf5-nlacZ embryo sections expressing cells were seen in a more restricted area (Fig. 7G). Fig. 7D and H confirm that the dorsal boundary of β-galactosidase expression (green arrows) had moved relative to the dorsal boundary of desmin expression (red arrows) revealing a desmin-positive domain dorsal to the β-galactosidase-expressing cells. This correlated well with the absence of β-galactosidase-positive cells, detected histochemically, in the same location in y200ΔE-Myf5-nlacZ transgenic embryos (Fig. 5). The desmin staining showed that cells had entered the myotome before the y200ΔE-Myf5-nlacZ transgene was expressed. Similar results were obtained in

sections taken from the brachial level and also when MHC expression was used to identify differentiated cells (data not shown). Therefore, the pool of cells marked by y200ΔE-Myf5-nlacZ defined a subset of differentiated myotomal cells again demonstrating that a separate element must be responsible for the expression of *Myf5* in the dorsalmost myotome.

DISCUSSION

The early epaxial enhancer is necessary for the initial expression of *Myf5*

We have previously shown that the regulation of *Myf5* expression is complex and involves a large number of elements which control both activation and maintenance (Hadchouel et al., 2000; Carvajal et al., 2001). Using the β-globin promoter in a plasmid-based reporter construct, we have demonstrated that one of these, which we then called the Epaxial Element, acts as an enhancer (Summerbell et al., 2000). We can now confirm, by the use of *Myf5* promoter based constructs, that it recapitulates the very first subset of *Myf5* expression in the epaxial dermomyotome early in somite development, and show that it does so with both the *Myf5* and β-globin promoters. By deleting this enhancer from a 200 kb YAC construct in which *nlacZ* is targeted to the *Myf5* locus we show that the EEE is essential for the earliest phase of *Myf5* expression in the context of the locus.

The deletion of the EEE shows that an entirely modular mechanism operates in the regulation of the epaxial expression of *Myf5*; the EEE acts for a short time and then other upstream enhancers take over. Furthermore, it shows that although a large number of regulatory elements lie in the locus, no functional redundancy can be seen for this earliest site of *Myf5* expression.

Ectopic expression and perdurance in the dermomyotome

We show that, as well as recapitulating the initial activation of *Myf5* transcription, the EEE drives expression at a number of ectopic sites, one of which is the dermomyotome. Gustafsson et al. (Gustafsson et al., 2002) have claimed that this same enhancer (which they called the early somite enhancer), when juxtaposed to the promoter of the herpes simplex virus thymidine kinase gene, directs transcription only in the DML. However, they showed no sections to support this assertion. Our *nlacZ* in situ hybridisation analysis (Fig. 6) demonstrates clearly that reporter gene transcripts are much more widely distributed in the dermomyotome than are the transcripts of the endogenous gene, which are confined to the DML in the epaxial domain (Summerbell et al., 2000). It must therefore be the case, as we have argued before (Summerbell et al., 2000), that in the context of the locus the activity of the EEE in the dermomyotome is constrained by some other element(s).

β-galactosidase activity driven by the EEE is much more widely distributed within the dermomyotome than are the corresponding transcripts (compare Figs 2 and 6). This indicates that cells originating in the epaxial dermomyotome move considerable distances into the hypaxial region of the dermomyotome. This labelling of hypaxial cells is presumably the result of the perdurance of the protein in cells that once expressed the transgene, or in their descendants. Indeed in some embryos we see labelled cells in the limb muscles (L. T., D. S. and P. W. J. R., unpublished data). Our observations suggest novel dorsoventral movements of cells within the dermomyotome and are in accord with models of cell migration during somite differentiation based on experiments in the chick (Kahane et al., 1998; Ordahl et al., 2001). It is noteworthy that such migration of cells labelled while in the epaxial dermomyotome cannot be seen in *Myf5*$^{nlacZ/+}$ heterozygote embryos, although they abundantly express the reporter (Tajbakhsh and Buckingham, 1994). This emphasises that the isolated EEE is active in locations where it is not active when in its normal context, and refutes the suggestion of Gustafsson et al. (Gustafsson et al., 2002) that no negative regulatory element is necessary for proper EEE expression.

Enhancer activity within the locus

The endogenous *Myf5* gene is expressed at specific locations in the brain although the protein is not detectable (Tajbakhsh and Buckingham, 1995; Daubas et al., 2000). We have shown that this brain expression is controlled by an upstream enhancer located between –58 and –48 kb (Hadchouel et al., 2000). Gustafsson et al. (Gustafsson et al., 2002) have claimed that correct brain expression is also controlled by the EEE; our data make it clear that the limited brain expression directed by the EEE is not in the proper location (Fig. 3C,D). Moreover, correct brain expression is driven by the YAC from which the EEE was deleted (Fig. 4). Furthermore, we have since shown by deleting the upstream region from an analogous BAC construct that it is required for the brain expression seen with the endogenous gene (J. H., J. Carvajal, P. D., D. Rocancourt, P. W. J. R. and M. B., unpublished data). The fact that the EEE, when isolated from the locus, directs strong ectopic expression in the cephalic mesoderm [this paper; (Summerbell et al., 2000)] may have confused interpretation of whole-mount staining patterns as used by Gustafsson et al., thus leading them to claim that the EEE directs brain expression.

Moreover, when isolated from its normal context, the enhancer also drives ectopic expression in the epaxial dermomyotome, the branchial arches and less frequently in the limb. This expression occurs with both the β-globin TATA box (Summerbell et al., 2000) and the homologous promoters, and is thus not due to the use of a heterologous promoter. Accurate EEE activity occurs only in the context of larger constructs, indicating that there must be other additional regulatory region(s) to correct the inappropriate activity. We have already described one case of such a limitation of enhancer activity by a negative regulatory fragment (Summerbell et al., 2000) in that the arch activity of the intragenic enhancer is down regulated by another element within the *Myf5* locus (Carvajal et al., 2001). The molecular basis of such co-operation between enhancers remains to be investigated. Furthermore, although the EEE lies closer to the *Mrf4* promoter than to the *Myf5* promoter, no *Mrf4* expression is found in domains in which the EEE is directing *Myf5* expression. The mechanism by which enhancers discriminate between different possible target genes will be the subject of further studies.

Multiple phases of somitic expression during primary myogenesis

We have previously shown (Summerbell et al., 2000), and

others have recently confirmed (Gustafsson et al., 2002), that the EEE is sufficient to drive the first (early epaxial) phase of *Myf5* expression in the somite. We now demonstrate that the element is necessary and that it appears to act only during this initial phase of expression. In situ hybridisation analyses of the reporter gene transcripts (Hadchouel et al., 2000), which monitor the transcriptional output of the isolated EEE, indicate that it is active for only a relatively short time (Fig. 6). When compared with those of the endogenous gene (Summerbell et al., 2000), these also show that a subsequent phase of *Myf5* expression in the dorsalmost myotome is dependent on another regulatory element. Deletion of the EEE from the YAC reporter construct shows that another element(s) activates expression in the myotome. By comparing the in situ hybridisation patterns of y200ΔE-Myf5-nlacZ, y23-Myf5-nlacZ and BAC59Z [Fig. 6B in this paper; fig. 6H in Hadchouel et al. (Hadchouel et al., 2000); data not shown], we can map this element to the –59/-23 kb interval. Furthermore, a regulatory element within y23-Myf5-nlacZ drives expression in only a sub-domain of the intercalated myotome (Spörle et al., 2001) at a later stage (Hadchouel et al., 2000), which may reflect a distinct origin of these cells. We have isolated from the locus enhancers that are able to recapitulate these phases of *Myf5* expression [(Hadchouel et al., 2000); T. Chang and M.B., unpublished].

We have previously shown that in the absence of Myf5, precursor cells leave the dermomyotome, undergoing an epithelial-mesenchymal transition and aberrantly accumulating along the edges of the dermomyotomal epithelium, but do not form a myotome (Tajbakhsh et al., 1996). In the wild-type embryo, as the desmin expression pattern shown in this paper illustrates (Fig. 7), differentiating myoblasts are laid out to form the myotome prior to the later phases of *Myf5* epaxial expression. This strongly suggests that the earliest phase of epaxial somitic expression is involved in the initial formation of the myotome. Deletion of the EEE in the context of the endogenous gene will indicate whether early expression in the dermomyotome is necessary for all of the precursors that enter the epaxial myotome.

Induction of early epaxial enhancer activity

Studies in the chick have predicted that several signals could be involved in specifying muscle along the rostrocaudal axis (Munsterberg and Lassar, 1995), and our data demonstrate multiple phases of epaxial expression of *Myf5* during the differentiation of the somite. It appears that the inductive signals that control *Myf5* expression switch rapidly from those that impinge on the early epaxial enhancer to those that impinge on the other enhancers acting later in the epaxial somite, indicating that there are significant changes in either the signalling environment or the responsiveness of the cells along the rostrocaudal axis.

The signalling molecules that turn on *Myf5* expression are beginning to be identified (reviewed by Buckingham, 2001). The Sonic hedgehog (Shh) pathway has been shown to be involved in the control of *Myf5* epaxial somitic expression (Tajbakhsh et al., 1998; Borycki et al., 1999; Gustafsson et al., 2002), although it has not been demonstrated to be sufficient for induction. Interestingly, three sites of ectopic expression driven by the EEE (posterior lateral edge of the limbs, non-muscle precursors in the branchial arches and cephalic mesoderm) correspond to regions where active Shh signalling has been shown (Riddle et al., 1993; Ahlgren and Bronner-Fraser, 1999; Schneider et al., 2001), raising the possibility that the response of the isolated enhancer to this signal is different from that in the context of the locus. Borycki et al. (Borycki et al., 1999) reported that *Myf5* early epaxial somitic expression is lost in 9.5 dpc $Shh^{-/-}$ mutants and proposed that *Myf5* is a target of Shh signalling. However, such a direct role was questioned because of reports of epaxial somitic *Myf5* expression in $Dsh^{-/-}$ (another Shh-null mutant) embryos (Kruger et al., 2001), in $Smo^{-/-}$ (a mouse mutant lacking a member of the Shh membrane receptor complex) and in the $Shh^{-/-}$ mutant itself (Zhang et al., 2001). This expression could result from the activity of other enhancers that operate in the epaxial myotome. A study that distinguishes between the activities of the various enhancers that regulate *Myf5* expression in the epaxial somite will be necessary to assess the exact role of Shh. Similarly, detailed mutational analysis of each currently defined regulatory element will show whether the various aspects of the pattern are controlled by one or several enhancers.

In conclusion we propose that the first phase of *Myf5* expression in the epaxial dermomyotome is necessary for early myotome formation, while the following phases are associated with cytodifferentiation within the myotome. It will be important to understand whether we have uncoupled distinct stages of the myogenic process, in which the same cells re-express *Myf5* under the control of different enhancers, or defined further heterogeneity amongst skeletal muscle precursors, or both.

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