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Identification of Epha4 enhancer required for segmental expression and the regulation by Mesp2

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Somites provide the basic body plan for metameric axial structures in vertebrates, and establish the segmental features through the sequential gene expression in the presomitic mesoderm (PSM). A crucial protein for segment border formation is the bHLH transcription factor Mesp2, the expression of which is restricted to the anterior PSM. A gene candidate that is activated by Mesp2 is *Epha4*, as its expression pattern resembles *Mesp2* and is absent in Mesp2-null embryos. We have analyzed the enhancer region of *Epha4*, which is responsible for its expression in the anterior PSM, and identified an E-box containing region. Subsequent transgenic and transient luciferase analyses successfully determined that the presence of repeated E-box sequences is a minimum essential requirement for the expression in the anterior PSM. We also show that Mesp2 directly binds to the enhancer sequence of *Epha4*. Furthermore, the forced expression of Mesp2 in somitic cells results in the activation of *Epha4* and repression of the caudal gene *Uncx4.1*, which may trigger the events leading to the formation of abnormal somites and rostralized vertebra. In addition, ectopic *Mesp2* expression induces abnormally epithelialized structures, which support to the idea that Mesp2 induces the formation of segmental borders by activating genes that play roles in cellular epithelialization.

KEY WORDS: Mesp2, Epha4, Somitogenesis, Segmental border, Mox1, mouse

INTRODUCTION

Somites are basic structures that underlie the segmental body architecture in vertebrates. The mechanisms involved in the generation of serially segmental units are a fascinating model system that has been used by many developmental biologists to further our understanding of the temporal and spatial control of gene expression. Somite precursors are derived as paraxial mesoderm from the primitive streak or tailbud region and these cells then come under the control of the segmentation clock, in which Notch signal oscillation generates the periodicity (for reviews, see Aulehla and Herrmann, 2004; Bessho and Kageyama, 2003; Maroto and Pourquié, 2001; Pourquié, 2003; Rida et al., 2004; Saga and Takeda, 2001). Notch signaling is suppressed in the anterior PSM by a bHLH protein, Mesp2, and the anterior limits of the Mesp2 expression domain demarcate the next segmental border (Morimoto et al., 2005). Mesp2 is a key transcription factor for both segment border formation and for the generation of rostrocaudal patterning within somites (Saga et al., 1997; Takahashi et al., 2000). The expression of many genes is affected in the Mesp2-null embryo, in which the genes required for rostral property are suppressed but those required for the development of caudal properties are enhanced. Among these genes, only lunatic fringe (*Lfng*) has so far been shown to be a direct target of Mesp2 (Morimoto et al., 2005).

Since the *Mesp2* expression domain is very similar to that of *Epha4*, and this gene is also suppressed in the Mesp2-null embryo (Nomura-Kitabayashi et al., 2002), it was probable that Mesp2 directly activated *Epha4* in the rostral compartment of the somites. Furthermore, Epha4 is implicated in segmental border formation in zebrafish (Cooke et al., 2005; Barrios et al., 2003; Durbin et al., 1998), although gene knockout studies indicate that Epha4 is not the sole protein required for segmental border formation in the mouse, as no somitic phenotype has been reported (Dottori et al., 1998; Kullander et al., 2001) (M. Asano, personal communication). The identification of target genes for a transcription factor is necessary to understand fully the genetic networks involved in a particular biological system. However, it is very difficult to achieve these using straightforward methods such as SELEX or immunoprecipitation, particularly in embryonic tissues. As an alternative method, we attempted to first identify the *Epha4* enhancer and then test whether Mesp2 directly binds to this region; if it does not bind, we can search the binding protein that might be a direct target of Mesp2. Fortunately, the *Epha4* enhancer elements identified showed direct binding to Mesp2, together with E47 (Tcfe2a – Mouse Genome Informatics) in vitro. Moreover, the forced expression of Mesp2 resulted in the reverse phenotype of the Mesp2-null embryo, whereby *Epha4* is activated, *Uncx4.1* is suppressed and ectopic epithelialization could be observed in the transgenic embryos.

MATERIALS AND METHODS

Construction of lacZ reporter constructs

An 8.8 kb fragment (*Nhe*I-*Xba*I) was isolated from an *Epha4*-containing bacterial artificial chromosome clone (415B3) and subcloned into the pBluescript vector (Stratagene). A series of deletion constructs were then generated using the appropriate restriction enzymes (Fig. 1A). These genomic fragments were inserted into a *lacZ* reporter vector, upstream of the hsp promoter (Kothary et al., 1989). E-box deletion and mutant constructs were subsequently generated via PCR using the 630 bp enhancer region (*Hin*dIII cut) as a template (Imai et al., 1991).

Formation of E-box multimer constructs

Synthetic oligonucleotides were designed to generate two repeats of 20 bp containing an E-box when annealed (Fig. 2C). These E-box-containing sequences were flanked by *Bgl*II and *Bam*HI sites. The complementary oligonucleotides were annealed and phosphorylated with T4 polynucleotide kinase prior to ligation. Ligated DNA were digested with *Bam*HI and *Bgl*II, and separated on 2% agarose gels. Multimerized bands were excised and subcloned into the pBluescript vector.

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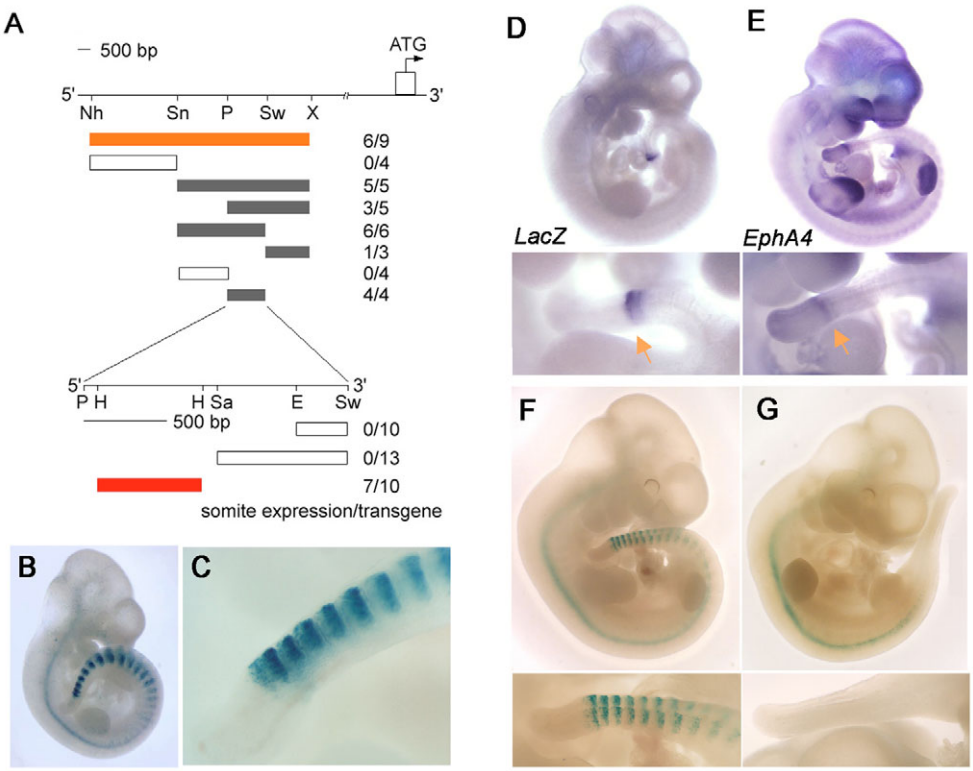

Fig. 1. Identification of a somite-specific *Epha4* enhancer. (**A**) The *lacZ* transgene constructs used to identify the cis-acting somite enhancer within the *Epha4* genomic region. The numbers of transgenic mouse embryos that expressed β-gal in the somites, among the transgene-positive embryos, are indicated on the right. E, *Eco*RI; H, *Hin*dIII; N, *Nhe*I; Sa, *Sac*I; Sw, *Swa*I; P, *Pma*CI; X, *Xba*I. (**B**,**C**) Lateral view of β-gal activity driven by the 630 bp (*Hin*dIII) *Epha4* enhancer fragment in a 10.5 dpc embryo. A magnified image in the somitic region of B is shown in C. (**D**,**E**) Comparison by in situ hybridization at 10.5 dpc of the transgene expression (*lacZ*) in a transgenic embryo (D) with endogenous *Epha4* expression in the wild-type embryo (E). In situ signals in the anterior PSM are indicated by arrows in the lower panels showing magnified images. (**F**,**G**) β-Gal activity driven by the 630 bp enhancer in 10.5 dpc wild-type (F) and Mesp2$^{L/L}$ embryos (G).

Generation of transgenic mice

All constructs were digested with restriction enzymes to remove vector sequences and then gel purified. Transgenic mice were generated by microinjection of fertilized eggs. Microinjected eggs were transferred into the oviducts of pseudopregnant foster females. The genotypes of the embryos were identified by PCR using DNA prepared from the yolk sac.

Luciferase assay

An *Epha4* somite enhancer insert (630 bp *Hin*dIII-*Hin*dIII fragment) and E-box multimers were cloned into the pGL3-Promoter vector (Promega). NIH3T3 cells were grown at 80% confluency in 24-multiwell plates and transfected with luciferase gene constructs using Lipofectamine Plus (Invitrogen). Cells were harvested 36 hours after transfection and luciferase activities were measured using a Dual Luciferase Assay Kit (Promega). The transfection efficiency was normalized by co-transfection of the Renilla luciferase expression vector pRL-TK (Promega), and the relative luciferase activity was determined as recommended by the manufacturer.

Electrophoretic mobility shift assay (EMSA)

For protein preparation, NIH3T3 cells were grown at 80% confluency in 10 cm dishes and transfected with expression vectors containing either 3×FLAG-tagged Mesp2 or Myc-tagged E47. Nuclear extracts were prepared using Nuclear Extract Kit (Active Motif). The protein concentrations were measured by the Bradford assay (Pierce). EMSA was performed using a DIG gelshift and detection kit (Roche). Binding reactions were carried out by mixing DIG-labeled and unlabeled (for competition experiments) probes with nuclear extracts from NIH3T3 cells. In experiments using antibodies, the nuclear extracts were preincubated with the antibody for 1 hour on ice.

Generation of *CAG-CAT-Mesp2* transgenic and *Mox1-cre* knock-in mice

A targeting vector was designed to introduce the *Cre* gene near to the translational initiation site of the *Mox1* gene (see Fig. S1 in the supplementary material) and used to establish the *Mox1-cre* mouse line, in which Cre recombinase is expressed instead of Mox1 and the gene activity is examined by crossing with a reporter line, R26R (Zambrowicz et al., 1997). To achieve the ectopic expression of Mesp2, a *CAG-floxed-CAT-Mesp2* transgene was constructed (Yamauchi et al., 1999), in which *CAT* gene can be excised by Cre recombinase and thus the *Mesp2* gene comes under the control of the *CAG* promoter (see Fig. S1 in the supplementary material). Transgenic mouse lines were established by microinjection of *CAG-floxed CAT-Mesp2* DNA as described above.

Analyses of embryos by LacZ staining, in situ hybridization, skeletal and the histological methods

Embryos were fixed and stained in X-gal solution for the detection of β-gal activity, as described previously (Saga et al., 1999). For histology analyses, samples stained by X-gal were postfixed with 4% paraformaldehyde, dehydrated in an ethanol series, embedded in paraffin and sectioned at 6 μm. Whole-mount in situ hybridization was performed using InsituPro robot (Intavis). The transcripts were visualized using anti-digoxigenin (DIG) antibodies conjugated to alkaline phosphatase. Color reactions were performed using BM Purple (Roche). Methods employed for section in situ hybridization and for the immunohistological detection of Mesp2 have been previously described (Morimoto et al., 2005). Skeletal preparations by Alcian Blue/Alizarin Red staining have also been described previously (Saga et al., 1997; Takahashi et al., 2000). Probes used for the in situ hybridization detection of *Uncx4.1* and *Sox9* were kindly provided by Dr Peter Gruss and Dr Veronique Lefebvre, respectively. For the detection of actin filaments, frozen sections were stained with AlexaFluor 488-conjugated phalloidin (Molecular Probes) according to the manufacturer's protocol.

RESULTS

Enhancer analysis of *Epha4*

Previous enhancer studies have indicated that a somite specific enhancer is not contained within the 7.5 kb region upstream of the *Epha4* transcriptional start site, in which only rhombomere-specific enhancer activity has been identified (Theil et al., 1998). To identify the somite specific enhancer region of the *Epha4* gene, we focused on the more upstream region of the gene. We subsequently found the enhancer within an 8.8 kb fragment, beginning 8 kb upstream of the *Epha4* transcriptional start site (Fig. 1A). β-Gal activity was observed in the rostral compartment of the segmented somites (Fig.

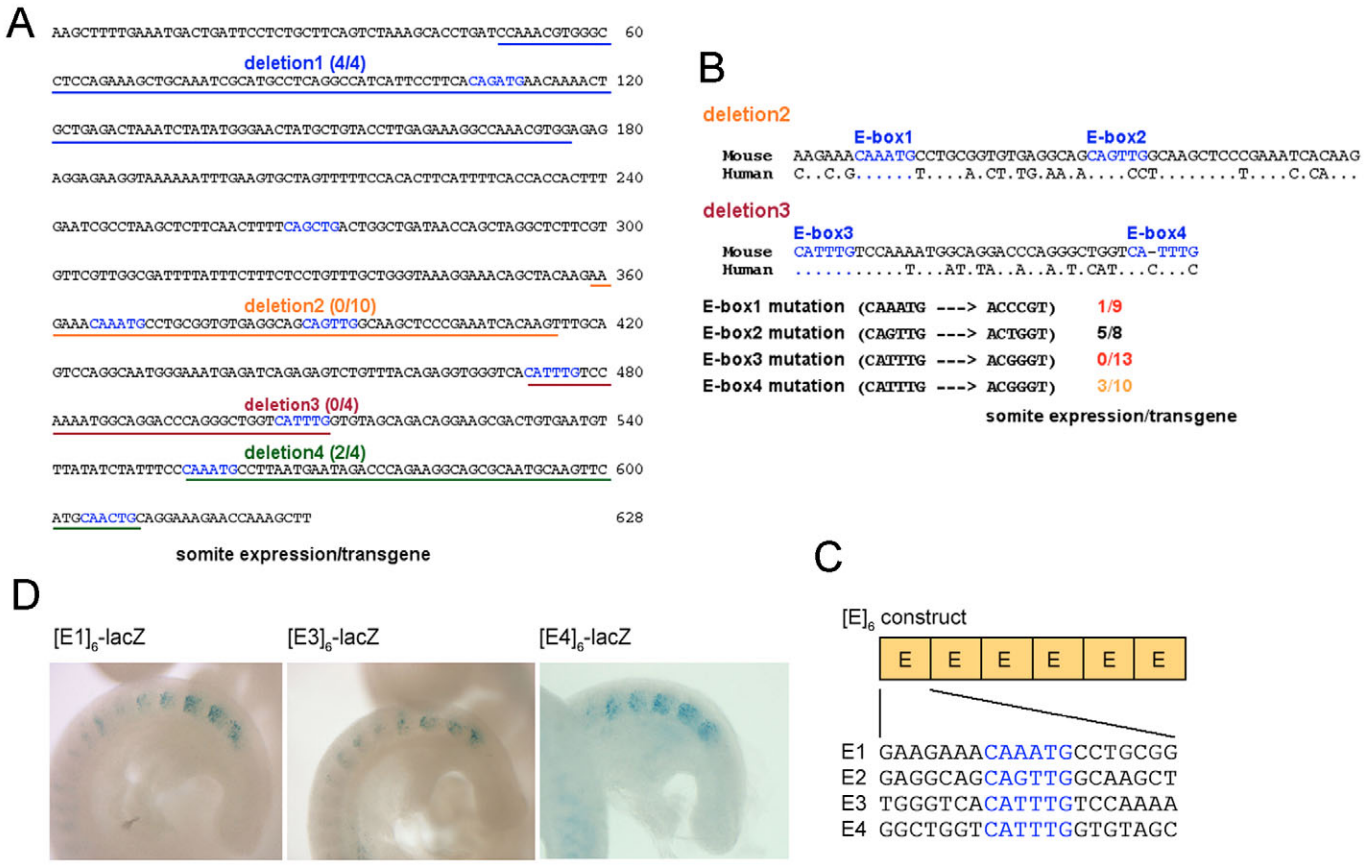

Fig. 2. Identification of the functional E-box motifs responsible for the somite-specific activation of *Epha4*. (**A**) Sequence of the 630 bp *Epha4* somite enhancer (core enhancer) region. The four indicated fragments (fragment 1-4) represent deleted sequences from the core enhancer region. The results of transgenic analyses using these deleted constructs are shown in parentheses. (**B**) Sequence alignment of somite enhancer regions (fragment 2 and fragment 3) of mouse *Epha4* with the corresponding regions of human *Epha4*. The mutations introduced in each E-box and the results of the subsequent transgenic analyses are shown. (**C**) Schematic representation of artificially constructed enhancers, containing six E-box motif repeats. (**D**) The artificial enhancers were cloned upstream of a *lacZ* reporter vector and the results of the subsequent transgenic analyses (representative images are shown) are indicated in parentheses. For the $[E2]_6$-lacZ artificial enhancer, no somite expression was observed among the 11 transgene-positive embryos. Blue letters indicate putative E-box motifs.

1B,C), and to confirm that this reflects endogenous *Epha4* expression, we compared the expression of *lacZ* RNA with the endogenous *Epha4* transcripts. Among several expression domains of endogenous *Epha4*, such as limb buds, branchial arches and rhombomeres, an identical expression pattern was revealed in both the anterior PSM and the rostral compartment of the S1 somites (Fig. 1D,E). Further transgenic analyses were conducted using DNA fragments that had been generated by several restriction enzymes. Although we detected one embryo which showed somite-specific expression using Sw-X region, we concentrated our analyses on the P-Sw region, which showed most consistent results. Further deletion identified a *Hin*dIII fragment of 630 bp that could sustain endogenous pattern of somite-specific *Epha4* expression (Fig. 1A). We established a permanent transgenic line using a *lacZ* reporter with the 630 bp enhancer. The somite-specific expression was observed during somitogenesis from 8.5 to 11.5 days post-coitum (dpc) as similar to the endogenous one (see Fig. S2 in the supplementary material). However, the transgene expression became weaker in the later stage embryo at 11.5 dpc and the somite-specific expression was not observed with both probes for endogenous *Epha4* and *lacZ* transgene after 13.5 dpc (data not shown). When the expression was examined in the *Mesp2*-null genetic background ($Mesp2^{L/L}$) (Takahashi et al., 2000), no β-gal activity was detected in the $Mesp2^{L/L}$ embryos (Fig. 1F,G), which confirms that the enhancer contains cis elements required for the *Epha4* activation downstream of the Mesp2.

Subsequent sequence analysis revealed that this 630 bp region contained eight E-boxes. As Mesp2 belongs to the bHLH family of transcription factors, which are known to bind E-box or N-box motifs, we initially performed deletions of some of the *Epha4* E-boxes. We generated four deletion constructs that lack a region (fragments 1-4) of the E-boxes (Fig. 2A) and examined the enhancer activities. Our results clearly showed that the enhancer activity was completely lost when using both fragment 2- and 3-deletion constructs, indicating that both regions are necessary for its expression (Fig. 2A). Both fragments 2 and 3 contain two E-boxes, designated E1- E4 (Fig. 2B). The core sequences of E1, E3 and E4 are identical (CAAATG or CATTTG) but only E1 and E3 are conserved in the human *EPHA4* gene. To determine whether these E-boxes are crucial, we next introduced mutations into their consensus sequences and transgenic analysis was conducted using the full 630 bp enhancer as a wild-type activity control. The E2 mutation did not affect enhancer activity but a considerable reduction in the activity was observed for the E1 and E3 mutations. In addition, mutation of E4 substantially disrupted enhancer activity, indicating that a mutation in a single E-box abrogates the enhancer

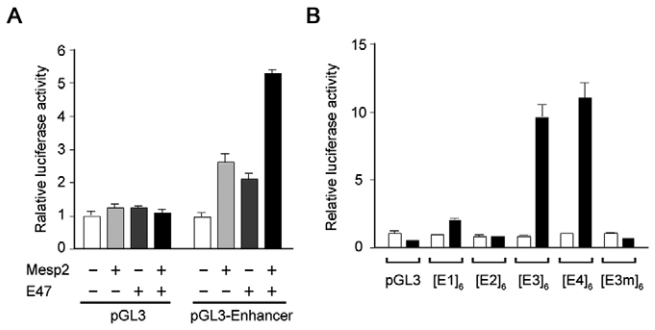

Fig. 3. Transactivation of the *Epha4* enhancer by the Mesp2/E47 heterodimer. *Epha4* somite enhancers (A, 630 bp core sequence; B, six tandem repeats of E-box sequences) were ligated to the pGL3 luciferase vector. Luciferase activity was measured at 36 hours after transfection into NIH3T3 cells. (**A**) The presence (+) or absence (–) of either Mesp2 or E47 are indicated in each column. (**B**) Luciferase activity was compared with (black bars) and without (white bars) Mesp2/E47. Mutations in E3 (5′-CATTTG-3′), that give rise to E3m (5′-ACGGGT-3′), results in the loss of reporter activity. The results shown are the mean values from three independent experiments. Standard deviations are indicated by error bars.

activity and that the presence of tandem repeats of the E-box consensus sites is important for the activity. To confirm this, we generated reporter constructs with artificial enhancers, composed of six tandem repeats of the E1, E2, E3 or E4 boxes and flanking sequences (Fig. 2C). Transient transgenic analysis revealed that each of these regions, with the exception of E2, showed weak but specific expression in the somitic region (Fig. 2D). This confirmed that these E-boxes are a minimum requirement for the specificity of *Epha4* expression in the somitic region and that the consensus sequence is CAAATG.

The *Epha4* enhancer is activated by Mesp2 in cultured cells

The identification of E-boxes as a vital component of the *Epha4* enhancer strongly indicates that this gene is directly regulated by Mesp2, as the bHLH-type transcription factor is known to bind E-boxes. In order to further elucidate whether Mesp2 can activate the *Epha4* enhancer, we established a luciferase assay system using NIH3T3 cultured cells. The reporter gene was constructed by ligating the 630 bp *Epha4* enhancer region to a luciferase gene. As B-type bHLH transcription factors are known to function as heterodimers with the so called A-type bHLH factors (Ledent and Vervoort, 2001), we analyzed reporter activity with or without E47, which is a typical A-type bHLH factor. As shown in Fig. 3A, Mesp2 and E47 alone exhibited weak transactivating activities when these expression vectors were separately transfected with the reporter construct, whereas strong activity was observed when Mesp2 and E47 were co-transfected. The transactivating activity of Mesp2 alone can be ascribed to its association with endogenous E47. Next, we constructed reporters with six repeats of each of the *Epha4* enhancer E-boxes (E1 to E4) used in our transgenic analyses (Fig. 2C). Interestingly, very strong activity was observed when the E3 and E4 constructs were used, and this was only observed upon co-transfection with E47 (Fig. 3B). This specificity was confirmed by the lack of activity resulting from a construct with a mutant-type E3 enhancer element. E2 showed no activity, which is consistent with the findings of our transgenic analysis. E1 did not have strong activity, although we obtained positive activity for this E-box via transgenic analysis.

The binding abilities of Mesp2 and E47 to the *Epha4* E-boxes were then analyzed using electrophoresis mobility shift assays (EMSA). Nuclear extracts were prepared from NIH3T3 cells, transfected with either FLAG-tagged Mesp2 or Myc-tagged E47, and these were used in the experiments either separately or in combination. The E3 motif was used in the EMSA experiments as it gave the most consistent results in both the transgenic and luciferase assays. As expected, a strong bandshift was observed when both Mesp2 and E47 were combined, although a faint band was detectable when E47 was incubated alone, indicating that it may form a homodimer that can bind the E3 E-box (Fig. 4A). The sequence specificity of the protein-DNA interactions was confirmed by competition assay using both intact and mutated sequences (Fig. 4B). The specificity of the heteroduplex complex was also confirmed by supershift experiments with anti-FLAG and anti-Myc antibodies (Fig. 4C). We also examined the binding specificity by applying other bHLH proteins. A family protein, Mesp1 also showed strong

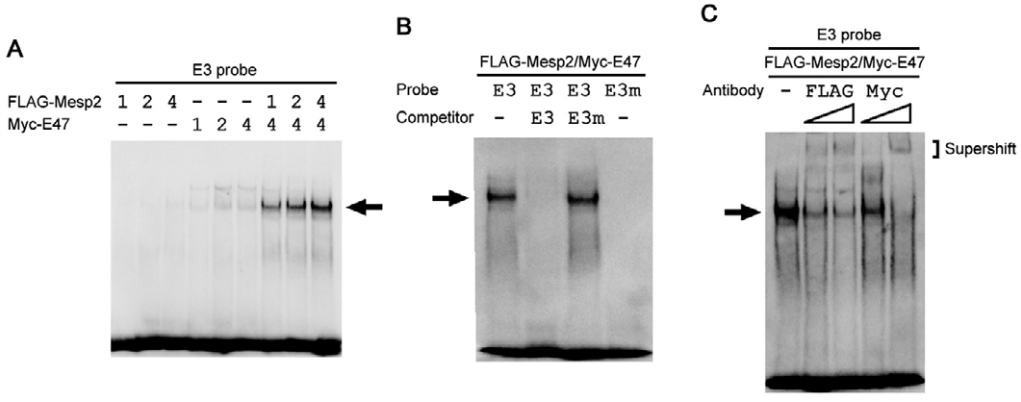

Fig. 4. The Mesp2/E47 heterodimer binds to the E3 site of the *Epha4* enhancer. (**A**) The results of EMSA using nuclear extracts from NIH3T3 cells transfected with FLAG-Mesp2 and/or Myc-E47 and incubated with E3 probe. The quantities of nuclear extracts used (μg) are indicated. (**B**) A competition assay indicating the specificity of the binding of the Mesp2/E47 (2 μg each) complex to the E3 probe. The addition of 100-fold excess of unlabeled E3 probe, but not the E3m mutant probe, abolished the binding. (**C**) Evidence for the heterodimer formation of FLAG-Mesp2/Myc-E47. The band containing E3 (arrow) was supershifted by the addition of either anti-FLAG or anti-Myc antibodies. The oligonucleotides used were as follows: E3, 5′-TGGGTCA**CATTTG**TCCAAAA-3′; E3m, 5′-TGGGTCAACGGGTTCCAAAA-3′ (E-box is shown in the bold; altered nucleotides in the mutant probe are underlined).

binding, but other bHLH proteins such as paraxis (Tcf15 – Mouse Genome Informatics), Myod1 and Twist (Twist1 – Mouse Genome Informatics) did not show significant binding to the E3 probe (see Fig. S3 in the supplementary material). These data strongly suggest that Mesp2 forms a heterodimer with E47, binds to the E-boxes within the *Epha4* enhancer and then activates *Epha4* transcription in the rostral region of somites.

The overexpression of *Mesp2* leads to the activation of *Epha4*

Epha4 has been implicated in border formation via the repulsive interaction with ephrin molecules, and this occurs during the formation of segmental boundaries in the hindbrain and in the somites (Barrios et al., 2003; Cooke et al., 2005; Durbin et al., 1998). However, loss-of-function experiments have failed to show any functional relevance for Epha4 in the mouse. In order to investigate whether Mesp2 functions as an activator of *Epha4*, and possibly to reveal the role of Epha4 during somitogenesis, we established a system that achieves the conditional expression of Mesp2 using Cre-loxP. A transgenic mouse line *CAG-floxed-CAT-Mesp2* was established in our laboratory, in which the *CAT* gene is inserted between two *loxP* sites and can therefore be excised by Cre recombinase. Hence, the *Mesp2* gene in this system will come under the control of the *CAG* promoter after this excision. To activate *Mesp2*, we generated and then used a *Mox1-Cre* mouse (see Fig. S1 in the supplementary material). *Mox1* expression initiates just prior to segment border formation, in a similar manner to endogenous *Mesp2*, but its expression persists after segmentation and is relatively higher in the caudal half of the somites (Mankoo et al., 2003; Saga et al., 1997) (Fig. 5A,B). The *Cre* expression was detected as early as 8.5 dpc and showed the similar pattern to the *Mox1* (Fig. 5C-E).

To confirm the presence of Cre recombinase activity, we crossed the *Mox1-Cre* mouse with the R26R reporter line and examined β-gal activity during the period 8.5-11.5 dpc (Fig. 5F-I; data not shown). The expression of the reporter was found to begin in the paraxial mesoderm and the most prominent levels were restricted to the somitic derivatives, at least up to 11.5 dpc. Some reporter expression in the rostral neural tube and in the intermediate mesoderm was also observed. We detected differences in the initial expression domain between the *Mox1* (Fig. 5B) or *Cre* transcripts (Fig. 5E), and β-gal reporter activity (Fig. 5G), which most likely reflects a time-lag for the activation of the reporter gene following the excision of the *CAT* gene by Cre recombinase. Histological sections revealed that the reporter activation was initiated in only a few somitic cells just after segmentation, but that the β-gal expression gradually expanded throughout the entire components of somite derivatives. Hence, this Cre line is a useful system to drive genes in the somitic cell lineage.

Mesp2 activation induces abnormal epithelialization

To activate *Mesp2* expression in the somitic lineage, we crossed the *CAG-CAT-Mesp2* and *Mox1-cre* lines. The double heterozygous mice died shortly after birth and their skeletal specimens revealed strong malformations (see below), indicating abnormal somitogenesis. Under a dissection microscope, the morphology of the somites was not found to have been disrupted, which was unexpected from the observations of the skeletal phenotype. Segmental boundaries were observed, although their width was not perfectly equal to the wild type and the surface appeared to be rough. At first, we analyzed *Mesp2* expression at 10.5 dpc (Fig. 6A,B). In

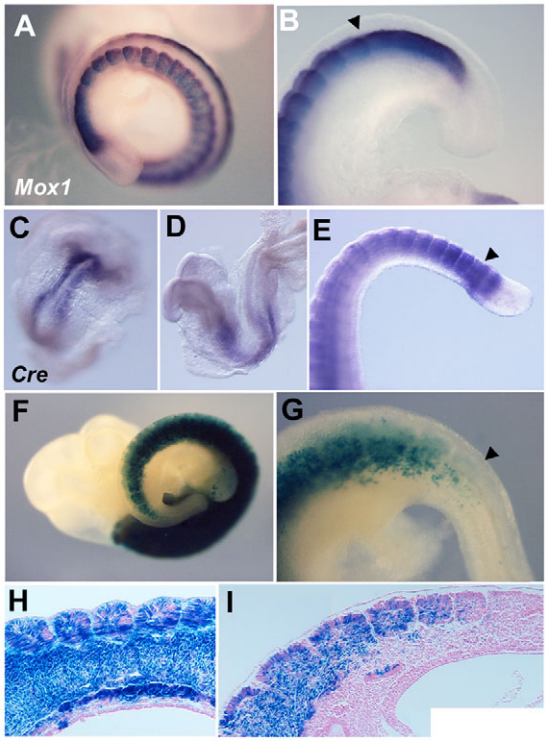

Fig. 5. *Mox1* and *Mox1-cre* expression and the lineage analysis. Whole-mount in situ hybridization analysis of *Mox1* expression in 9.5 dpc embryos (**A**,**B**) and *Mox1-cre* in 8.25 (**C**), 8.5 (**D**) and 11.5 (**E**) dpc embryos. (**F-I**) Whole-mount expression patterns and sagittal sections of β-gal-stained *R26R/Mox1-cre* double heterozygous embryos at 10.5 dpc. (H,I) The sections were counterstained with Eosin. Arrowheads indicate somite borders forming between S0 and S-1.

the wild-type and single heterozygous embryos, *Mesp2* is expressed in the anterior PSM as a single band, although the width and the strength of this expression differs from embryo to embryo as shown previously (Fig. 6A) (Takahashi et al., 2000). In double heterozygotes, however, the ectopic expression of *Mesp2* could be observed throughout the entire somitic region, in addition to its normal expression pattern in the anterior PSM (Fig. 6B). Moreover, the *Mesp2*-positive cells often formed clusters and were not localized in specific regions of somites (Fig. 6C,D).

A similar ectopic expression pattern was observed for *Epha4*, although the levels of ectopic expression were much lower than the endogenous gene expression (Fig. 6E-H). The spotty expression pattern in both *Mesp2* and *Epha4* indicates that the expression is suppressed or the transcripts are destabilized in many cells and only parts of cells maintain the expression. To further investigate the characteristics of the gene expression profiles and morphologies, serial sections were prepared and subjected to staining for Mesp2 protein, *Epha4* transcripts and actin filaments (Fig. 6I-N). The segmental borders were found to have generated but fluorescent phalloidin staining revealed cells showing abnormal epithelialized features and broken epithelial sheaths were also evident (Fig. 6N). In the cells nearby, both ectopic Mesp2 (Fig. 6K) and *Epha4* expression (Fig. 6L) could be observed. Although we could not conclude that Mesp2 directly induced *Epha4* using the serial sections, these observations indicate that the cells may have acquired repulsive properties that enable them to form abnormal cell borders within somites (Fig. 6O).

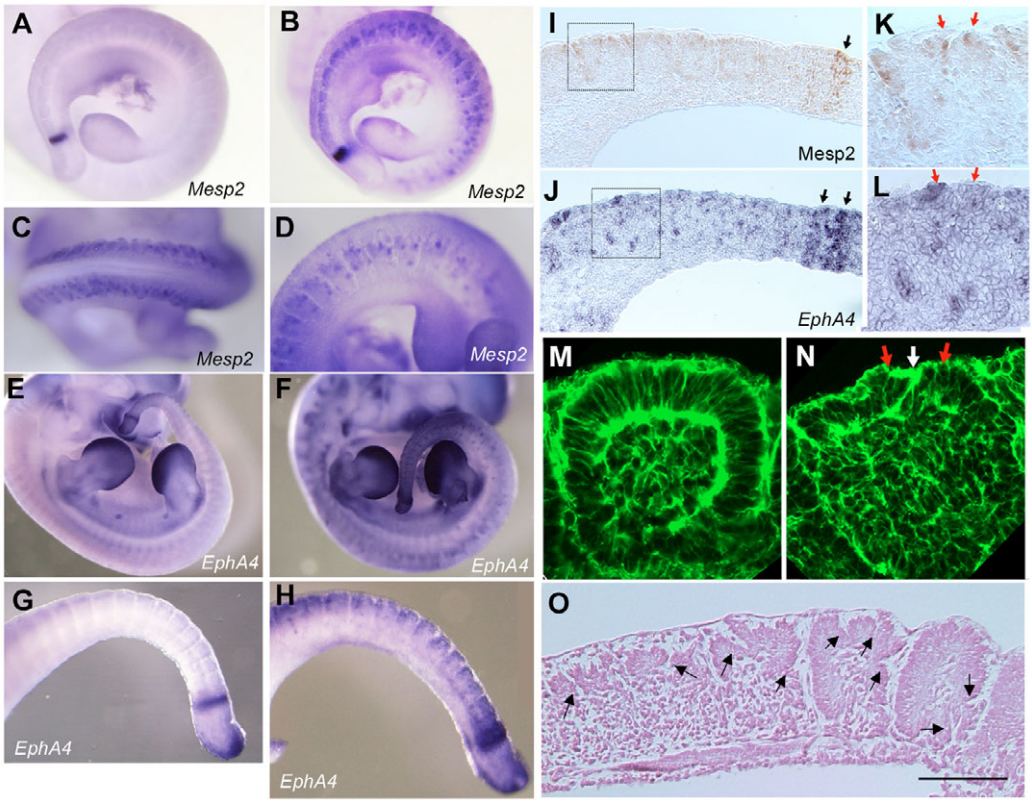

Fig. 6. Ectopic Mesp2 expression in somites leads to the activation of *Epha4*. Expression of *Mesp2* in 10.5 dpc wild-type (**A**) and *CAG-CAT-Mesp2*; *Mox1-Cre* double heterozygous embryos (**B-D**). In addition to the normal *Mesp2* expression in the anterior PSM, ectopic expression of *Mesp2* was observed throughout the entire somitic region (B-D). The expression pattern of *Epha4* at 11.5 dpc in wild-type (**E**,**G**) and double heterozygous embryos (**F**,**H**) is also shown. An expression pattern for *Epha4* that was similar to *Mesp2* was observed in the double heterozygote (F,H). Histological analyses of 10.5 dpc wild-type (**M**) and double heterozygous (**I-L**,**N**) embryos. Ectopic Mesp2 protein (I,K) and *Epha4* expression (J,L) were evident in serial sections of double heterozygotes. Magnified images of square parts of I and J are shown in K and L, respectively. Another consecutive section was stained with phalloidin (N) and a similar region of the wild-type embryo is shown in M. In double heterozygotes, abnormal epithelial cells were observed within the somite (N). A paraffin section stained with nuclear Fast Red revealed gaps in epithelialized somites in the double heterozygote at 10.5 dpc (**O**). Black arrows in I and J indicate the endogenous expression of Mesp2 (I) or *Epha4* (J). Red arrows in K,L,N indicate separated cell clusters. White arrow in N indicates an abnormal epithelialized feature. Black arrows in O indicate local gaps. Scale bar: 100 μm.

Mesp2 activation induces skeletal malformation

The *CAG-CAT-Mesp2/Mox1-Cre* double heterozygous fetus showed strong skeletal defects, which are restricted in the ribs and vertebra as expected from the somite-specific *Mox1* expression (Fig. 7A-H) (Mankoo et al., 2003). The vertebral bodies and the lamina of neural arches were present in these fetuses, although they displayed severe defects in both their morphology and patterning. By contrast, the pedicles of the neural arches were largely lost (Fig. 7C,G). In addition, the proximal region of the ribs did not form properly (Fig. 7D,H). This phenotype contrasts with Mesp2-null embryos and is somewhat similar to Psen1-null mutants (Takahashi et al., 2000), indicating that it is a rostralized phenotype. To gain insight into the morphogenetic failure underlying the skeletal defects observed in the double transgenic mice, cartilage formation was examined by whole-mount staining with Alcian Blue. Strikingly, rib as well as pedicle cartilages were severely affected even in the 13.5 dpc embryo (Fig. 8A,B).

Mesp2 is required for the establishment of the rostral properties within somites via the suppression of caudal genes. Therefore, we anticipated that the forced expression of Mesp2 may lead to the suppression of caudal properties, which would be the cause of the skeletal malformation. *Uncx4.1* is a molecular marker for caudal somites (Fig. 8C,E,G) and this gene is also known to be required for the pedicle formation of the neural arch (Leitges et al., 2000; Mansouri et al., 2000). In the *CAG-CAT-Mesp2/Mox1-Cre* double heterozygotes, the caudally restricted expression pattern was not disrupted but the levels of expression were much lower and the stripes were often interrupted (Fig. 8D,F). The histological section revealed the presence of signal-negative regions in the caudal compartments, which was often accompanied by the morphological abnormalities. We noticed local fusion between cells in the caudal compartment and the rostral ones in the posterior somite (Fig. 8H). Such a fusion was never observed in wild-type or single heterozygous embryos (Fig. 8G).

To explore more genes affected in Mesp2-activated embryos and to understand the cause of abnormalities, we examined expressions of several somitic markers at 11.5 dpc. The segmental expression of *Pax3* that is the marker of dermomyotome (Fig. 8I) (Denetclaw and Ordahl, 2000) was expanded in the double transgenic embryo (Fig. 8J), which may indicate expansion of the dermomyotomal progenitor. By contrast, *Sox9*-positive cell lineage appeared to be

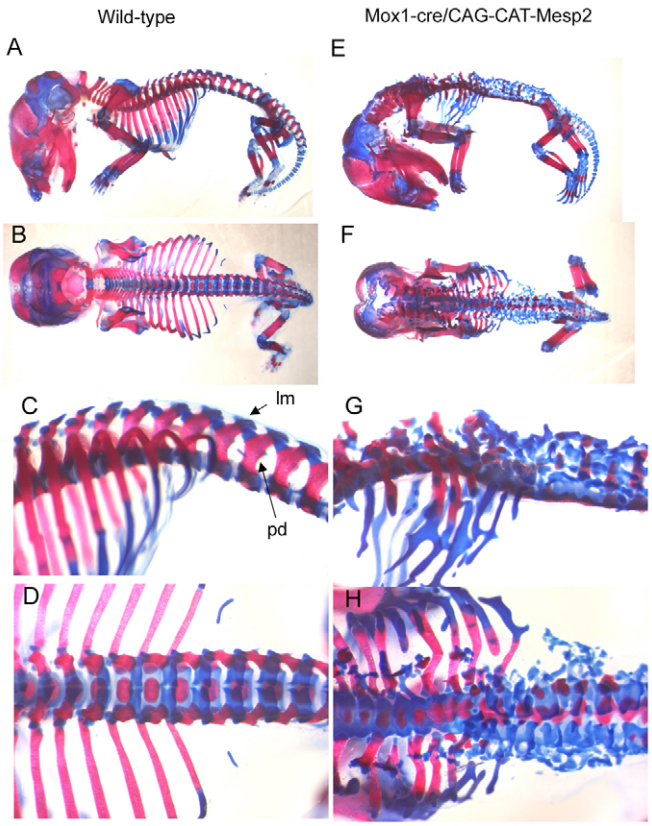

Fig. 7. Ectopic expression of Mesp2 leads to skeletal malformations. Skeletal preparations of 18.5 dpc wild-type (**A-D**) and *CAG-CAT-Mesp2*; *Mox1-Cre* fetuses (**E-H**) stained with Alcian Blue and Alizarin Red. Lateral (A,E) and dorsal (B,F) views of whole skeletons are shown. Higher magnifications of the lumber region from wild-type (C,D) and double heterozygous fetuses (G,H) are also shown. The lack of pedicles of the neural arches could be observed in the double heterozygotes (G,H). The rib structure was also severely affected in the double heterozygotes (G,H). pd, pedicle; lm, lamina.

relatively reduced in the transgenic embryo especially in the thoracic region (Fig. 8K,L), which may account for the underdevelopment of the rib cartilage. The expansion of the rostral compartment of somites was indicated by the expression of *Tbx18* (Fig. 8M-P), which is another target candidate of Mesp2 as its expression is lost in the *Mesp2*-null embryo (Bussen et al., 2004) (Y.T., unpublished).

These observations are consistent with the idea that ectopic Mesp2 expression is of sufficient strength to activate rostral genes such as *Epha4* and *Tbx18*, and to suppress expression of the caudal gene *Uncx4.1*.

DISCUSSION

In our current study, we have identified a cluster of E-boxes in the enhancer region of *Epha4*, incorporating the Mesp2 binding site, in which at least three crucial E-boxes (E1, E3 and E4 in Fig. 2C) are present. The loss of these motifs results in a substantial reduction in gene reporter activity, in both luciferase and transgenic reporter assays, indicating that there is an essential requirement of multiple E-boxes for *Epha4* activation by Mesp2. Interestingly, the co-expression of Mesp2 and E47 resulted in higher luciferase activity (tenfold) (Fig. 3B), whereas only weak activity (twofold) was obtained with Mesp2 alone (data not shown). Mesp2 alone could also not bind to E-box containing DNA sequences (Fig. 4A). Therefore, Mesp2 alone or Mesp2 homodimers appear to be inactive on *Epha4* somite enhancer.

The core E-box sequence appears to be CAAATG/CATTTG and synthetic enhancers generated by six repeats of the *Epha4* enhancer E1, E3 and E4 motifs, and flanking sequences, can recapitulate the segmental expression pattern of this gene in vivo. The differences that we observed in the measured luciferase activities for the multiple E-boxes may reflect the involvement of the sequences flanking the core enhancer region in promoting the binding of bHLH-type transcription factors, which has been observed in other cases (Powell et al., 2004). In addition, other factors may modulate the interactions between Mesp2/E47 and its target sequences. It has been reported that the phosphorylation of E47 is required for the formation of heterodimers with Myod1 and for the subsequent binding to the target sequence (Lluis et al., 2005). The methylation state of target sequences has also been implicated in the binding by another bHLH heterodimer, Max/Myc, in which methylation of the CpG dinucleotide within the E-box has been shown to prevent the access of the bHLH proteins (Perini et al., 2005). Further studies will be required to determine whether such modulations are involved in the binding of the Mesp2/E47 heterodimer to its target sites.

Epha4 is implicated in segmental border formation via its interaction with the Eph ligand ephrin, which is expressed in apposed cells in zebrafish (Barrios et al., 2003; Durbin et al., 1998). However, there is no direct evidence for this in the mouse, as the loss of Epha4 failed to reveal any role for this protein during somitogenesis, which may be due to functional redundancy among the several Eph and ephrin family proteins. In such a situation, a transgenic strategy of forced gene expression is an alternative and effective method. In the current study, we have tried the forced expression of *Mesp2* with expectation that *Epha4* should be induced under the control of Mesp2. The forced expression of Mesp2 not only activates *Epha4* expression but also induces the local segregation of somitic cells. Recently, we showed that Mesp2 establishes the segmental boundary by suppressing Notch signaling, which then generates a boundary between the Notch-active and Notch-negative domains (Morimoto et al., 2005). We have also shown that this boundary forms the next somite border. However, the precise molecular mechanisms involved in the generation of these morphological boundaries are not yet fully understood, although Cdc42 and Rac1 are known to play important roles in subsequent epithelial somite formation (Nakaya et al., 2004). Although the direct evidence was not presented, our current data indicate that Mesp2 activates *Epha4* in the anteriormost cells in the PSM and that this may activate reverse signaling though ephrin expression in opposing cells and generate a gap during normal somitogenesis. A similar mechanism has previously been proposed for the epithelialization of boundary cells in zebrafish (Barrios et al., 2003; Cooke et al., 2005). Nevertheless, we can not exclude the possibility that pathways other than Epha4 activation by Mesp2 are required for the induction of epithelialization.

Mesp2 is also known as a strong suppressor of the establishment of caudal properties, which is mediated by the suppression of both Notch signaling and *Dll1* and *Uncx4.1* expression (Nomura-Kitabayashi et al., 2002; Takahashi et al., 2000). We actually did observe suppression of *Uncx4.1* in our double heterozygotes, but the segmental pattern of *Uncx4.1* expression at 10.5 dpc was not found to be severely disrupted. Therefore, our finding of an extremely defective skeletal phenotype in the *CAG-CAT-Mesp2/Mox1-Cre*

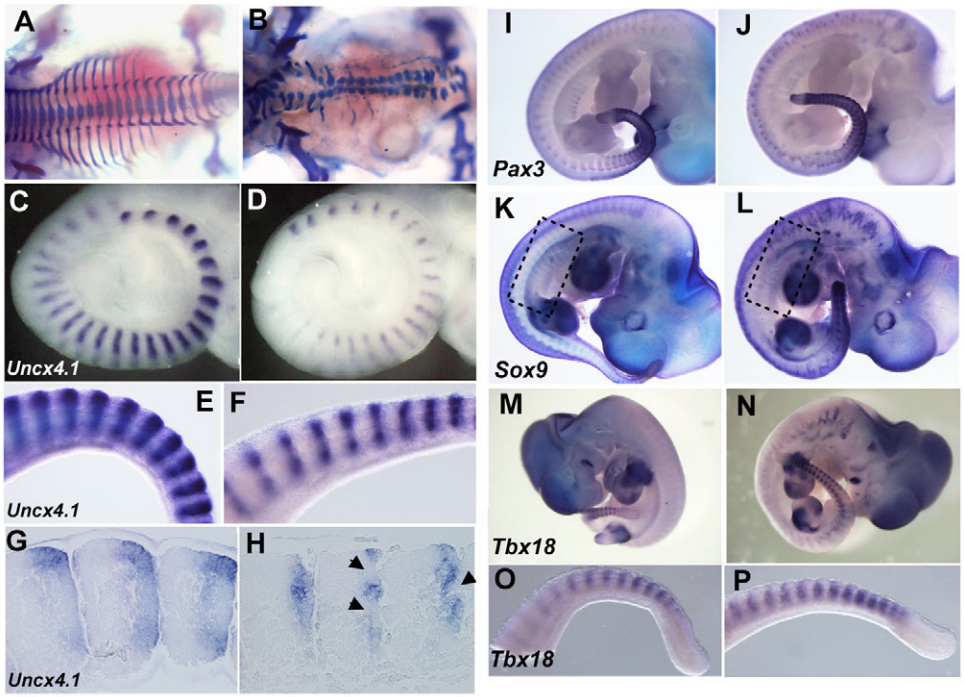

Fig. 8. Early defects in chondrogenesis and gene expressions affected in the *CAG-CAT-Mesp2;Mox1-Cre* double heterozygotes. Skeletal morphology was revealed by Alcian Blue staining in the wild-type (**A**) and double heterozygous (**B**) embryos at 13.5 dpc. (**C-H**) *Uncx4.1*. expression was reduced in double heterozygotes (D,F) at both 10.5 (C,D) and 11.5 dpc (E,F) compared with wild-type embryos (C,E). The section of 11.5 dpc embryonic tail revealed *Uncx4.1*-negative cells (arrowheads) in the caudal compartment of somites in the double heterozygote (H). Comparison of expression patterns of *Pax3* (**I**,**J**), *Sox9* (**K**,**L**) and *Tbx18* (**M-P**) between wild-type (I,K,M,O) and double heterozygous (J,L,N,P) embryos. Outlines in K and L show *Sox9* expression in the rib primordia.

mice was somewhat surprising. We postulate that prolonged Mesp2 expression, driven by the CAG promoter, must continuously attenuate *Uncx4.1* and the corresponding downstream gene expression in the later stages of development, which would lead to the almost complete suppression of chondrogenesis, as observed in the case of loss of Uncx4.1 (Leitges et al., 2000; Mansouri et al., 2000). One of target genes activated by Uncx4.1 and responsible for the phenotype would be *Sox9*, the product of which is known to be a key regulator of chondrogenesis (Akiyama et al., 2005). However, it remains to be investigated whether this suppression is directly mediated by Mesp2 or by other transcriptional suppressors that are activated by Mesp2.

We also show in our present study that the *Mox1-Cre* mouse is a useful tool for inducing either the disruption or activation of genes that are components of the somitic cell lineage. However, there is a delay in gene activation and the activation of *Mesp2* was 'spotty' and these may be the reason why we did not observe strong segmental defects. Gene reporter activity was also observed in other lineages, including parts of the neural tube and the intermediate mesoderm. Therefore, although a detailed lineage study will be required in future studies, the activity that we observed in our *CAG-CAT-Mesp2/Mox1-Cre* transgenics proved to be useful for the manipulation of gene expression, at least in the somitic cell lineages. Recently, a similar Cre line (*Meox1*^{cre}) was reported by another laboratory and the results of their study were consistent with our current data (Jukkola et al., 2005).

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/13/2517/DC1

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