Functional importance of evolutionally conserved Tbx6 binding sites in the presomitic mesoderm-specific enhancer of Mesp2

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The T-box transcription factor Tbx6 controls the expression of Mesp2, which encodes a basic helix-loop-helix transcription factor that has crucial roles in somitogenesis. In cultured cells, Tbx6 binding to the Mesp2 enhancer region is essential for the activation of Mesp2 by Notch signaling. However, it is not known whether this binding is required in vivo. Here we report that an Mesp2 enhancer knockout mouse bearing mutations in two crucial Tbx6 binding sites does not express Mesp2 in the presomitic mesoderm. This absence leads to impaired skeletal segmentation identical to that reported for Mesp2-null mice, indicating that these Tbx6 binding sites are indispensable for Mesp2 expression. T-box binding to the consensus sequences in the Mesp2 upstream region was confirmed by chromatin immunoprecipitation assays. Further enhancer analyses indicated that the number and spatial organization of the T-box binding sites are critical for initiating Mesp2 transcription via Notch signaling. We also generated a knock-in mouse in which the endogenous Mesp2 enhancer was replaced by the core enhancer of medaka mespb, an ortholog of mouse Mesp2. The homozygous enhancer knock-in mouse was viable and showed normal skeletal segmentation, indicating that the medaka mespb enhancer functionally replaced the mouse Mesp2 enhancer. These results demonstrate that there is significant evolutionary conservation of Mesp regulatory mechanisms between fish and mice.

KEY WORDS: T-box transcription factor, Enhancer, Targeted disruption, Somitogenesis

INTRODUCTION

Somitogenesis is an important morphogenetic process that generates metameric structures in vertebrates, including vertebra, muscles and motoneurons. The segmental boundary of each somite forms at the anterior end of the presomitic mesoderm (PSM) or unsegmented paraxial mesoderm, which are supplied from the primitive streak or tailbud at a later stage of development (Saga and Takeda, 2001). This process proceeds through the interaction of a number of signaling cascades, including Notch, Wnt and Fgf (Delfini et al., 2005; Dunty et al., 2008; Galceran et al., 2004; Hofmann et al., 2004; Moreno and Kintner, 2004; Takahashi et al., 2000). Thus, somitogenesis could be a very useful model system in which to study the interactions among the various signaling cascades that facilitate periodic pattern formation.

The basic helix-loop-helix transcription factor Mesp2 plays a crucial role in both somite segment border formation and in the establishment of the rostrocaudal patterning of each somite (Saga et al., 1997). Mesp2 shows dynamic and periodic expression in the anterior PSM. This expression pattern defines the positioning of the newly forming somite by suppressing Notch signaling, in part through the activation of lunatic fringe (Lfrng) (Morimoto et al., 2005). Genetic analyses have revealed that Mesp2 expression is itself controlled by Notch signaling, indicating the existence of complicated feedback circuitry (Takahashi et al., 2003; Takahashi et al., 2000). We have previously identified the minimal PSM-specific Mesp2 enhancer (denoted P2PSME) that is sufficient to reproduce the normal Mesp2 expression pattern in transgenic animals (Haraguchi et al., 2001). We have also demonstrated that the T-box transcriptional regulator Tbx6 directly binds to P2PSME and is essential for P2PSME activity (Yasuhiko et al., 2006). We also showed that Notch signaling strongly enhanced Mesp2 activation via Tbx6 and we identified the sequences that are important for this enhancement using an in vitro reporter assay (Yasuhiko et al., 2006). However, the question of whether P2PSME is indispensable for Mesp2 expression during somitogenesis remained to be addressed. Because of differences in the expression patterns of Mesp2 and Tbx6 – Tbx6 is expressed throughout the PSM and tailbud (Chapman et al., 1996; White and Chapman, 2005) whereas Mesp2 expression is observed only in the anterior PSM (Saga et al., 1997) – another open question was whether Tbx6 actually binds to P2PSME.

The evolutionary aspect of this system is also noteworthy. We previously identified the mespb PSM-specific enhancer in the teleost fish medaka, and reported that the mutation of two T-box binding sites therein diminished its PSM-specific enhancer activity in transgenic embryos (Terasaki et al., 2006). However, definitive evidence as to whether the T-box-factor-dependent regulation is a conserved mechanism among vertebrates remains elusive.

In this study, we established Mesp2 enhancer knockout mice and confirmed that Tbx6 binding sequences are essential for Mesp2 expression. The in vivo association of Tbx6 with P2PSME was confirmed in chromatin immunoprecipitation assays, and reporter assays further showed that the number and spatial organization of Tbx6 binding sites are important for P2PSME activity. Furthermore, using a knock-in mouse that harbors the medaka mespb enhancer in place of the mouse Mesp2 enhancer, we show that the T-box-factor-dependent regulation of the Mesp gene is evolutionally conserved between fish and mice.

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Materials and Methods

Site-directed mutagenesis

Site-directed mutagenesis of each Tbx6 binding site was performed using previously reported PCR-based procedures (Yasuhioko et al., 2006) with the following primers (mutated nucleotides in lower case): mB1, 5’-CTCTGAGGGGTCGAAACTgaAATGTGCAGAATGTCGCAATGAGTCGACCAGTG-3’; mB2, 5’-CTCTTGAGgaGaTGAATCCACACCTCTGCATCGAATG-GGCCGCTTTT-3’; mD, 5’-AACCTGGCAGGGAACACCTCggCATTTAGTCCAGATATAAAGCTT-3’; mG, 5’-CGGGCTCTGTGGGCTGTTTTTGTTTG-AattCTCTGTGCGAGCTCGGCA-3’. The mutated Tbx6 binding sites are indicated for each construct, such that P2EmB1D represents a P2PSME-GCCCGCTTT-3’ containing both mB1 and mD.

Gene targeting

For targeted disruption of P2PSME, a 356-bp DNA fragment containing mutated Site B and Site D was generated by PCR using primers mB1 and mD. As a negative control, the wild-type P2PSME fragment was also generated by PCR. To construct the targeting vectors, a floxed PGK-neoR selection marker cassette was inserted between a 6-kb long arm and the 356-bp DNA fragment with or without mutations (Fig. 1A). The region corresponding to Mesp2 exon 1, intron 1 and a part of exon 2 served as the short homology arm. The targeting vector was introduced into mouse ES cells (strain TT2) by electroporation. Resulting G418-resistant ES clones were characterized by PCR using primers: Fesneo, 5’-GCCTCTCTCTGATTGTCGCGGATCTGGGCTGTTTTG-GAATCCACACCTCTGCAATGAGTCGACCAGTG-3’ and RP213, 5’-CAGGACAGGCACTCGAGTCGAGCCGTCTAGA-3’. Southern blots were performed to confirm homologous recombination. Positive ES clones were then aggregated with 8-cell stage ICR mouse embryos in order to produce chimeric mice. The ES selection marker PGK-neoR was removed by crossing the chimeric mice with CAG-Cre mice, which express Cre recombinase ubiquitously. The resulting mouse strains, with insertions of either mutated P2PSME or wild-type P2PSME, were designated P2EmB1D or P2EmCont, respectively. Although the knockout mice were established using an ES cell line (TT2) obtained from a C57BL/6 J background unless otherwise stated.

Skeletal preparation

Embryonic day 17.5 (E17.5) mouse embryos were obtained by crossing the mutants of interest. Embryos were then fixed with 90% ethanol. For genotyping, PCR was performed using a piece of embryonic liver digested with proteinase K (Roche). Alcian Blue and Alizarin Red staining were performed as described (Saga et al., 1992) and were subsequently examined for the presence of the transgene by PCR (Sasaki and Hogan, 1996).

Generation of anti-Tbx6 antibody

His-tagged fragments of Tbx6 protein (N-terminal antigen, amino acids 2-78; internal antigen, amino acids 311-408) (White and Chapman, 2005) were produced using the pET system (Novagen) and Escherichia coli Rosetta-gamiB (Novagen) as a host strain. The Tbx6 fragments were extracted from bacterial culture using the MagneHis system (Promega), purified by thrombin digestion to remove the His-tag, followed by affinity column purification (Novagen) and dialysis using a semipermeable membrane cassette (Pierce). Rabbits (two animals for each antigen) were immunized with the purified Tbx6 fragments and processed for antibody purification following the standard procedures of Hokudo Bio (Abuta, Hokkaido, Japan).

Protein and mRNA expression analyses

Whole-mount RNA in situ hybridization was performed as described (Saga et al., 1997). Whole-mount immunohistochemistry and simultaneous staining of Mesp2 mRNA and Tbx6 protein were as previously described (Morimoto et al., 2005; Oginuma et al., 2008).

Chromatin immunoprecipitation (ChIP) assay

Embryonic tail buds were dissected along the anteroposterior axis into three parts using a tungsten needle. Sonite part (s) corresponds to SIV to SII, anterior PSM (ap) is from S1 to S-1, and posterior PSM (pp) corresponds to the region posterior to S-2. A total of 120 embryos were dissected, the samples treated with trypsin and dispersed cells counted (around 1×10^6 cells for each sample). Cells were fixed in 1% formaldehyde in PBS for 10 minutes at 37°C. The preparation of cell lysates and ChIP assay were performed using the Chromatin Immunoprecipitation Assay Kit (Upstate biology) according to the manufacturer’s protocol. PCR primers used for ChIP assays were: LP286, 5’-AGACATCCAGGATCCTCGAGGTC-3’; LP287, 5’-CGGGATGACATCCAGGATCCTCGAGGTC-3’ and RP287, 5’-GGCTGTTGTGACCTGCTGGGAACT-3’. LP286 and RP287 were used for detection of mutated P2PSME, whereas LP287 and RP287 were used for detection of wild-type P2PSME. As a positive control, the Dll1 mesoderm (msd) enhancer was amplified using the following primers: LP259, 5’-CCCAACACAGATGTTCTGGCCGACTAACT-3’ and RP255, 5’-GCTTTGTGGTGTGAGACCTCAGACTGCTGA-3’. A sequence 22 kb from P2PSME was amplified by PCR as a negative control, using the following primers: LP285, 5’-GGCTGTTGTGAGACCTCAGACTGCTGA-3’; and RP286, 5’-CGCTCTCACGCTTGCTCTAGTGA-3’.

Electromobility shift assay (EMSA)

The full-length Tbx6 ORF was obtained from the pACT-Tbx6 construct, which was previously isolated from a yeast one-hybrid screen (Yasuhioko et al., 2006). After ligation to a 3xFLAG tag (Sigma), the tagged Tbx6 insert was cloned into pC52+ (Rupp et al., 1994). In vitro transcription/translation was then performed using the TNT In Vitro Translation Kit (Promega) according to the manufacturer’s protocol. Oligonucleotide probes were labeled with DIG-11-ddUTP using recombinant TdT (Roche Diagnostics). Five microliters of crude in vitro translated product was subjected to EMSA. As a negative control, reticulocyt e lysate without Tbx6 template was used. The oligonucleotide probes are as follows (mutated nucleotides are indicated in lower case): SiteF, 5’-GCAAATACGGGTATAGGACACACCTGATACGGTCACCC-3’; SiteG, 5’-CTGGGCTCTGTGGGTTTTG-GTCTTCTGAACTCAGGCGCAATGAGTCGACCAGTG-3’; SiteGmut, 5’-CTGGGCTCTGTGGGTTTTG-GTCTTCTGAACTCAGGCGCAATGAGTCGACCAGTG-3’. Southern blots were performed to confirm homologous recombination. Positive ES clones were then aggregated with 8-cell stage ICR mouse embryos in order to produce chimeric mice. The ES selection marker PGK-neoR was removed by crossing the chimeric mice with CAG-Cre mice, which express Cre recombinase ubiquitously. The resulting mouse strains, with insertions of either mutated P2PSME or wild-type P2PSME, were designated P2EmB1D or P2EmCont, respectively. Although the knockout mice were established using an ES cell line (TT2) obtained from a C57BL/6 J background unless otherwise stated.

Transgenic assay

DNA fragments with and without mutations in conserved upstream sites were generated from an Mesp2 genomic fragment using a standard PCR-based protocol. Each transgene comprised the lacZ reporter and a 6-kb genomic fragment upstream of the Mesp2 first ATG, including P2PSME with and without mutated Tbx6 binding sites. The transgenes were injected into the male pronucleus of a fertilized egg as described (Hogan et al., 1994). Embryos recovered at E9.5-10.5 were analyzed for lacZ expression by X-Gal staining (Saga et al., 1992) and were subsequently examined for the presence of the transgene by PCR (Sasaki and Hogan, 1996).

Luciferase assay

The KpnI-Ncol fragments (356 bp) corresponding to P2PSME, with and without mutations in the Tbx6 binding sites, were subcloned into the pGL3- Basic (Promega) vector to generate luciferase reporter constructs. The expression vectors for the proteins to be assessed were constructed in the same way as those used in the EMSA assays described above. The luciferase assay using COS-7 cells was conducted as described previously (Yasuhioko et al., 2006). Each assay was performed in triplicate and repeated at least twice.

Results

Mutations in the Tbx6 binding site of the Mesp2 enhancer result in the complete loss of Mesp2 expression in the presomitic mesoderm

We have shown previously that nucleotide substitutions in two Tbx6 binding motifs in the Mesp2 PSM enhancer (P2PSME) eliminate Tbx6 binding activity in vitro (Yasuhioko et al., 2006). To establish the function of these Tbx6 binding sites in vivo, we...
introduced nucleotide substitutions into the mouse genome using a gene-targeting technique. These mutations disrupted two Tbx6 binding sites, denoted Site B and Site D, that were shown to be sufficient to activate Mesp2 expression in vitro (Yasuhiko et al., 2006) (Fig. 1A). After the establishment of a neoP2EmB1D mouse line, the neoR cassette was removed (Δneo) by a cross with the deleter mouse line CAG-Cre. Interbreeding of the Δneo mutants gave rise to homozygotes (P2EmB1D/P2EmB1D) that retained a loxP site after neoR removal (Fig. 1B). This residual loxP site appears to have no effect on Mesp2 expression or somitogenesis because another knock-in mouse, P2EmCont, in which wild-type P2PSME is knocked-in using the same strategy, had viable homozygous offspring without any morphological defects (data not shown).

The homozygous P2EmB1D/P2EmB1D embryos showed distinct skeletal defects (Fig. 1C) and perinatal lethality, features identical to the previously reported Δneo-type Mesp2-null mouse (Fig. 1C, P2MCM/P2MCM). As expected from the phenotype, Mesp2 expression in P2EmB1D/P2EmB1D embryos was eliminated (Fig. 1D). Segmental borders were generated during an early stage of

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**Fig. 1. Disruption of Tbx6 binding sites eliminates Mesp2 expression.**

(A) Targeting strategy to generate the Mesp2 enhancer knockout mouse (P2EmB1D). A DNA fragment containing mutated Tbx6 binding sites (black ovals with X) was substituted for the wild-type sequence (white ovals) by homologous recombination. The PGK-neoR selection marker was replaced by the Cre-loxP system to obtain a Δneo allele. (B) PCR detection of homozygotes in the P2EmB1D intercross. (C) Impaired skeletal segmentation in the Mesp2 enhancer knockout mouse. The P2EmB1D/P2EmB1D mouse exhibits severe skeletal malformation at E17.5 (centre) identical to that of the Mesp2-null mouse (P2MCM/P2MCM, right). Note the shortened spine with incompletely segmented vertebrae (upper panels) and fused ribs (bracket in lower panels). (D) Expression of Mesp2 and the somite-specific genes Mesp1, Eph4 and Tbx18 in P2EmB1D/+ (left column) and P2EmB1D/P2EmB1D (right column) embryos. Mesp2 mRNA expression is eliminated in the P2EmB1D/P2EmB1D homozygotes. Wild-type (+/+) and heterozygote (P2EmB1D/+) embryos showed varying Mesp2 expression patterns owing to its cyclic expression. Mesp1 is upregulated and Eph4 is not affected, whereas Tbx18 is completely abolished in P2EmB1D/P2EmB1D.
somitogenesis (Fig. 1D, right-hand panels). However, the borders were unlikely to be maintained because the vertebral bodies were fused along the anteroposterior axis at later developmental stages (Fig. 1C).

To further characterize the phenotypes in these embryos, we first examined the expression of Mesp1, which is known to be upregulated and to partially rescue somitogenesis in the absence of Mesp2 (Morimoto et al., 2006; Takahashi et al., 2007). Mesp1 expression was upregulated in the P2EmB1D/P2EmB1D embryo, but its expression domain was broader than normal (Fig. 1D). EphA4, which is required for proper border formation, was expressed normally. However, Tbx18, which is implicated in the maintenance of segmental border and somite patterning (Bussen et al., 2004), was not expressed (Fig. 1D). These gene expression patterns were similar to those reported for the Δneo-type Mesp2-null mouse (Morimoto et al., 2006; Takahashi et al., 2007). These results confirmed that the Tbx6 binding sites are bona fide enhancer elements required for Mesp2 expression.

**Tbx6 binds the Mesp2 PSM enhancer in vivo**

The Tbx6 protein is normally broadly distributed in the PSM and tailbud (White and Chapman, 2005), whereas Mesp2 is expressed only in the anterior PSM (Saga et al., 1997). This discrepancy between the Tbx6 and Mesp2 expression patterns prompted us to investigate whether Tbx6 actually binds to the Mesp2 enhancer in vivo. We raised an anti-Tbx6 antibody using two different antigens: an N-terminal portion of Tbx6 and an internal portion. The internal antigen yielded an antibody with good specificity and sensitivity. Embryo whole-mount immunohistochemistry confirmed the previously reported distinct Tbx6 staining pattern in the PSM and tailbud (Fig. 2A) (White and Chapman, 2005). Western blot analyses further revealed that this antibody identifies a single band of approximately 58 kDa in cell lysates prepared from the posterior region (PSM and tailbud), but not from the anterior region (formed somites), of E11.5 tails (Fig. 2B). We also performed double staining of Mesp2 mRNA and Tbx6 protein and confirmed colocalization only in the anterior-most region of the PSM (Fig. 2C). For ChIP assays, we dissected E11.5 embryo tails into three regions: the tailbud and posterior PSM (pp), the anterior PSM and newly formed somites (ap), and formed somites (s). Protein-DNA complexes were prepared from each pool and used in ChIP assays, which revealed that Tbx6 binds to the Mesp2 PSM enhancer in the ap and pp regions, but not in the s region, which is consistent with the expression pattern of Tbx6 (Fig. 2E). These results indicate that Tbx6 binds to the P2PSME uniformly in its expression domain, suggesting that Tbx6 alone cannot activate Mesp2 in the posterior PSM where it binds. Dll1 is known to be a downstream target of Tbx6 and putative binding sites have been identified in its mesoderm

**Fig. 2. Tbx6 binds to P2PSME in the PSM and tailbud.**

(A, B) Characterization of the anti-Tbx6 antibody produced in this study. (A) Whole-mount immunohistochemistry demonstrating the localization of Tbx6 protein in the mouse PSM and tailbud. (B) Western blot analysis showing that the anti-Tbx6 antibody detected a protein of expected molecular weight (58 kDa) in the PSM and tailbud (p) but not in formed somites (s). The asterisk indicates non-specific binding. (C) Double staining of Mesp2 mRNA (purple) and Tbx6 protein (green) demonstrating the coexistence of both signals in the anterior-most part of the Tbx6-positive region (white in merged image). (D) Design of in vivo technique for detecting Tbx6 binding to the P2PSME by ChIP. Arrows represent primers for the ChIP assay for the Mesp2 and Dll1 genes. Dll1 is known to be downstream of Tbx6 and was therefore used as a positive control. Gray and black boxes represent the P2PSME and Dll1 mesoderm (msd) enhancers, respectively. White ovals indicate Tbx6 binding sites. P2Em and P2Ewt, mutated and wild-type P2PSME regions, respectively; NC, unrelated sequence as negative control. (E) Tbx6 associates with P2PSME in the anterior and posterior PSM. (F) The association of Tbx6 with mutated P2PSME as detected by ChIP assay. Mutated and wild-type P2PSME regions were differentially detected by PCR with different sets of primers in the tails of E10.5 embryos obtained from the crossing of P2EmB1D/+ and ICR mice.
(msd) enhancer (White and Chapman, 2005). ChIP assays using the putative Dll1 msd enhancer (Fig. 2E, column Dll1) revealed that Tbx6 also binds to the Dll1 enhancer in both the ap and pp regions, which is consistent with the expression pattern of Dll1. In all cases, the negative control (PCR amplification of an unrelated sequence in the mouse genome) gave no signal in ChIP assays with the anti-Tbx6 antibody (Fig. 2E,F, column NC). Thus, these results confirm our previous finding that Tbx6 binding is required for Mesp2 expression, but is not sufficient for full transcriptional activation.

Mutated P2PSME contains Tbx6 binding sites that are inactive in vivo

We next applied the ChIP assay system to confirm that the phenotype of our enhancer-specific knockout mouse was due to the lack of Tbx6 binding in the Mesp2 enhancer region. We performed ChIP assays using the tails of P2EmB1D heterozygous embryos and specific primer sets in order to distinguish the mutated DNA fragment from its wild-type counterpart, expecting that Tbx6 would not bind to the mutated enhancer. Surprisingly, mutated P2PSME, which has no PSM-specific transcriptional activity (Fig. 1), gave rise to a band that co-precipitated with the anti-Tbx6 antibody. This indicated that Tbx6 still binds to the mutated PSM enhancer in vivo (Fig. 2F). To identify the Tbx6 binding site within the mutated P2PSME, we re-examined this region for a consensus Tbx6 binding sequence (White and Chapman, 2005) and found two additional candidate sites, denoted Site F and Site G, in and upstream of P2PSME (Fig. 3A). EMSA demonstrated that Site G was strongly associated with Tbx6 in vitro (Fig. 3B).

The number and spatial organization of the T-box binding sites are important for initiating Mesp2 transcription via Notch signaling

We reported previously that the simultaneous mutation of two Tbx6 binding sites, Site B and Site D, eliminates PSM-specific activation of a reporter gene by P2PSME in transgenic embryos (Yasuhioko et al., 2006). To confirm this finding and also investigate the possible involvement of the new Tbx6 binding site, Site G, in enhancer activity, we generated a series of reporter constructs with P2PSME harboring serial mutations in the Tbx6 binding sites. We tested two types of reporter assay: a luciferase assay using cultured cells, and transgenic analyses. In the luciferase assay, the loss of any single Tbx6 binding site among Sites B, D and G, caused a 10-fold decrease in luciferase reporter activity, we generated a series of reporter constructs with P2PSME harboring serial mutations in the Tbx6 binding sites. We tested two types of reporter assay: a lacZ dependent reporter activation (Fig. 3C, right). Conversely, Tbx6 binding site among Sites B, D and G, caused a 10-fold decrease in luciferase reporter activity, when Notch signaling was applied (Fig. 3D). Taken together, these data indicate that the four Tbx6 binding sites have equal importance in regulating P2PSME activity, and at least two neighboring sites are required for the Notch signaling-dependent induction of Mesp2 expression.

The medaka mespB PSM enhancer regulates Mesp2 expression and normal somite formation in the mouse embryo

mespB, the zebrafish homolog of Mesp2, shows a similar expression pattern to mouse Mesp2 during embryogenesis and we speculated that it might exert a similar function in the mouse (Nomura-Kitabayashi et al., 2002). We have previously identified the PSM-specific enhancer of medaka mespB, which contains T-box binding sites. Two of these sites, T1 and T2, are important for PSM-specific mespB expression (Terasaki et al., 2006) (Fig. 4A). These data suggest that the T-box-protein-dependent expression mechanism is evolutionarily conserved between mammals and teleosts (zebrafish, medaka). We demonstrated that zebrafish Tbx24, a T-box protein that is homologous to mouse Tbx6 and is responsible for the fss mutant phenotype, binds to the medaka mespB PSM enhancer (Fig. 4B). A sequence comparison revealed three putative T-box binding sites in the medaka mespB PSM (Fig. 4A). Two of these had the ability to bind two Tbx24 molecules each, whereas in the mouse P2PSME, only Site B can bind two Tbx6 molecules (Fig. 4B).

To more directly demonstrate the evolutionary conservation of this regulatory mechanism, we generated a knock-in mouse with a medaka mespB upstream sequence inserted in place of the endogenous Mesp2 PSME. For this purpose, we substituted the 356-bp sequence upstream of the Mesp2 first ATG with 2.8 kb of sequence upstream of the mespB first ATG, generating a medakaP2 mouse (Fig. 4C). Heterozygous mice (medakaP2/medakaP2) were viable and appeared normal (data not shown). Homozygous mice (medakaP2/medakaP2) were also viable and showed no physical malformations (Fig. 4D). In skeletal preparations, we observed that medakaP2 homozygous fetuses were indistinguishable from heterozygous or wild-type littermates (Fig. 4E), indicating that the PSMEs of medaka mespB and mouse Mesp2 are functionally equivalent, despite some differences in their structural features.

DISCUSSION

The activation of Mesp2 expression requires at least two Tbx6 binding sites in P2PSME

In our current study, we have shown that Tbx6 binding sites are fundamentally important for P2PSME function and that P2PSME is necessary and sufficient for Mesp2 expression during somite formation in mouse embryogenesis. However, ChIP assays revealed that Tbx6 binds to P2PSME not only in Mesp2-expressing cells, but also in non-expressing cells such as those in the tailbud and posterior
This indicates that Tbx6 binding alone is not sufficient to activate Mesp2 expression. Previously, we showed in vitro that Mesp2 was activated weakly, if at all, by Tbx6 alone, but rigorously by a coexisting Notch signal (Yasuhiko et al., 2006). Taken together, these data suggest that it is highly likely that the restricted expression pattern of Mesp2 in the anterior PSM is regulated by a combination of Tbx6 and Notch signaling in vivo.

Similarly, Tbx6 activatesDll1 together with Wnt signaling (Hofmann et al., 2004) and activates Ripply in cooperation with Mesp2 (Hitachi et al., 2008). Although the molecular mechanisms...
by which Tbx6 regulates its target genes together with various partners remain elusive, it is possible that the number and spatial organization of Tbx6 binding sites facilitate the response of P2PSME to Notch signaling. In total, there are four Tbx6 binding sequences in this region: two palindrome-like sequences in Site B and one each in Sites D and G. Importantly, the P2PSME reporters with only one intact Tbx6 binding site were inactive in both the luciferase assay and the transgenic analyses (Fig. 3D), suggesting that a single P2PSME-bound Tbx6 molecule might not act as a mediator of Notch signaling in the regulatory mechanism controlling Mesp2 expression. This is consistent with the observation that Site G fails to activate Mesp2 expression by itself in the P2EmB1D mouse.

The loss of two or more of the four Tbx6 binding sites greatly diminishes P2PSME activity in both luciferase and transgenic assays (Fig. 3D). Interestingly, the reporters with two intact Tbx6 binding sites showed varied levels of activity depending upon the position of the intact sites. Two intact Tbx6 binding sites in Site B resulted in the highest reporter activity (Fig. 3D). These data indicate that Site B may be of predominant importance in the function of P2PSME,

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**Fig. 4.** The medaka *mespb* PSM enhancer is functionally equivalent to its counterpart in the mouse. (A) A comparison of the medaka *mespb* and mouse *Mesp2* PSM regions. Black and gray boxes represent presumptive T-box binding sites. The numbers above the boxes represent the nucleotide positions from the first ATG. The nucleotide sequences of the putative T-box binding sequences are shown beneath. Consensus Tbx6 binding sequences and their directions are indicated by arrows. The dashed arrow in Site B of the Mesp2 PSMPE depicts an incomplete Tbx6 binding sequence that only binds to Tbx6 if an adjoining complete Tbx6 binding sequence is present. The T-box proteins that might bind to these sequences are indicated. (B) EMSA analysis of the T-box binding sites in the medaka *mespb* PSMPE. T-box binding site T1 associates with a single Tbx24 molecule and T2 and T3 with two Tbx24 molecules, which is consistent with their nucleotide sequences as shown in A. (C) The targeting strategy used to generate the medaka *mespb* PSM knock-in mouse (*medakaP2*). A 2.8-kb fragment of *mespb* genomic DNA that is required for PSM-specific *mespb* expression was substituted for *Mesp2* PSMPE by homologous recombination. The neoR selection marker was removed by recombination using the Cre-loxP system. (D) *medakaP2* homozygotes are viable and have normal external features. (E) Homozygotes are indistinguishable from heterozygotes and wild-type littermates in skeletal preparations.
binding site contributes equally to activity by the same amount (Fig. 3C), suggesting that each Tbx6 might shed light on these novel regulatory mechanisms that operate together, these results indicate that the multiple Tbx6 binding sites in mouse embryos (Yasuhiko et al., 2006). Further analyses of κ independent Notch signaling machinery [disruption of potential possible that two neighboring Tbx6 molecules on the palindrome-like site has thus far been found only in the PSME domain (Hofmann et al., 2004) and Msgn1 (Wittler et al., 2007), the palindromic-like site has therefore been found only in the mouse PSME of Mesp2 and its medaka ortholog mespb (Fig. 4A). It is therefore possible that two neighboring Tbx6 molecules on the palindromic-like site are specifically recognized by as yet unidentified factor(s) (Fig. 5, ‘X’) that together with Tbx6 constitute an RBPJ-κ (Rbpj) independent Notch signaling machinery [disruption of potential RBPJ-κ binding sites does not affect P2PSME activity in transgenic embryos (Yasuhiko et al., 2006)]. Further analyses of Mesp2 PSME might shed light on these novel regulatory mechanisms that operate during development.

Mutations that removed any one of the Tbx6 binding sites in P2PSME, regardless of which, diminished luciferase reporter activity by the same amount (Fig. 3C), suggesting that each Tbx6 binding site contributes equally to Mesp2 expression in vitro. In vivo, by contrast, the mutation of a single Tbx6 binding site did not seem to affect PSM-specific gene expression (Fig. 3C). Taken together, these results indicate that the multiple Tbx6 binding sites confer a functional robustness to P2PSME that ensures proper Mesp2 expression during embryogenesis.

An evolutionally conserved mechanism regulating Mesp expression through multiple T-box binding sites

We previously found that the deletion of two T-box binding sites in the mespb PSME greatly reduced its PSM-specific enhancer activity in transgenic medaka embryos (Terasaki et al., 2006), similar to our findings in transgenic mouse embryos. The medaka mespb PSME harbors three T-box binding sites (T1-T3), which is similar to the complement of the mouse Mesp2 PSME (Fig. 4A). However, the total length of the PSME is very different between mouse Mesp2 and medaka mespb (356 bp versus 2.8 kb, respectively) (Terasaki et al., 2006). The number of T-box proteins that bind to the medaka and mouse PSMEs is also different (Fig. 4A,B), and the distance between each element is greater in the mespb PSME than in its mouse counterpart.

We have demonstrated, however, that the medaka mespb PSME is functionally equivalent to the mouse Mesp2 PSME. In our transgenic assay, a mutation in the double T-box binding site (Site B in mouse and Site T2 in medaka) had the most profound effect upon PSME activity. Consistent with these results, deletion of medaka Site T1 (harboring a single T-box binding sequence) did not affect reporter gene expression. However, deletion of one of the sites within the double T-box binding sequence (T2) caused a 50% decrease in reporter expression (Terasaki et al., 2006), again demonstrating the importance of the binding to the double T-box site for PSM enhancer function.

In the teleost fish, zebrafish, the T-box transcription factor Tbx24 was identified as responsible for the fused somite (fss) mutant phenotype. Tbx24 has a T-box domain that is homologous to that of mouse Tbx6 (Nikaido et al., 2002). The segmentation of somites and expression of mespb are eliminated in the fss mutant (Sawada et al., 2000), implying that mespb is a downstream target of Tbx24, similar to the relationship between Mesp2 and Tbx6 in mice. However, fss mutant fish are viable and fertile (van Eeden et al., 1996), whereas Tbx6-null mouse embryos fail to form a mesoderm and die early in development (Chapman and Papaioannou, 1998). This difference might be due to the presence in zebrafish of a Tbx6 counterpart gene, spadetail, which supports paraxial mesoderm formation. Despite this difference, our data clearly demonstrate that the mechanism regulating the PSM-specific expression of Mesp2 and mespb is evolutionarily well conserved between fish and mice.

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