A *Drosophila doublesex*-related gene, *terra*, is involved in somitogenesis in vertebrates

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**SUMMARY**

The *Drosophila doublesex (dsx)* gene encodes a transcription factor that mediates sex determination. We describe the characterization of a novel zebrafish zinc-finger gene, *terra*, which contains a DNA binding domain similar to that of the *Drosophila dsx* gene. However, unlike *dsx*, *terra* is transiently expressed in the presomitic mesoderm and newly formed somites. Expression of *terra* in presomitic mesoderm is restricted to cells that lack expression of *MyoD*. In vivo, *terra* expression is reduced by hedgehog but enhanced by BMP signals. Overexpression of *terra* induces rapid apoptosis both in vitro and in vivo, suggesting that a tight regulation of *terra* expression is required during embryogenesis. *Terra* has both human and mouse homologs and is specifically expressed in mouse somites. Taken together, our findings suggest that *terra* is a highly conserved protein that plays specific roles in early somitogenesis of vertebrates.

Key words: Zebrafish, *terra*, Somitogenesis, Transcription factor, Secreted signal

**INTRODUCTION**

During vertebrate embryogenesis, paraxial mesoderm differentiates into segmented somites that give rise to major axial structures such as vertebrae and skeletal muscles. Prior to the formation of somites in the paraxial mesoderm, somitomeres appear as spheroid clusters of mesenchymal cells in the presomitic mesoderm (Meier, 1979). The somitomeres then develop into easily distinguishable somites through an increase in cell number, cell packing density and epithelialization (Tam and Trainor, 1994).

The epithelialized cells in the newly formed somites undergo an epithelio-mesenchymal transition to become committed to different cell lineages. In amniotes, the medioventral portion of the somite is differentiated to form a large sclerotome that yields axial skeleton (Keynes and Stern, 1988). The remaining portion of the somite is converted into dermamyotome that contains precursors for muscle and dermis. The medial half of the dermamyotome gives rise to the deep muscles of the back (epaxial muscles), whereas its lateral half forms the muscles of the limbs and body wall (hypaxial muscles) (Ordahl and Le Douarin, 1992). In zebrafish, the myotome constitutes the major part of the somite whereas the sclerotome is only a small medioventral portion (Morin-Kensicki and Eisen, 1997). The zebrafish myotome has two populations of muscle precursors, slow and fast, that are discernible from their positions, morphologies and gene expression patterns (Devoto et al., 1996). The slow muscle precursors, also called adaxial cells, are located adjacent to the notochord in the presomitic mesoderm and newly formed somites. As somites mature, these cells migrate radially and eventually form a monolayer of superficial muscle cells that differentiate into slow muscle fibers in the adult fish. The fast muscle precursors are lateral to the adaxial cells prior to and during early somitogenesis. They later stay deep in the myotome and give rise to fast muscle fibers.

Although the morphogenetic events of vertebrate somitogenesis have been well depicted, molecular mechanisms controlling these events remain unclear. A number of genes, mainly identified as vertebrate homologues of *Drosophila* segmentation or neurogenic genes, are expressed in the presomitic mesoderm and developing somites (Christ et al., 1998; Tam and Trainor, 1994). These genes encode signaling molecules, transmembrane receptors or transcription factors. Recently, some of these genes have been implicated in somitogenesis in mice through a targeted gene-inactivation strategy. For example, disruption of *Delta-like gene 1* (Hrabe de Angelis et al., 1997), *Notch 1* (Conlon et al., 1996), *Presenilin 1* (Wong et al., 1997), *Mesp2* (Saga et al., 1997) or *paraxis* (Burgess et al., 1996) leads to the failure of epithelial somite formation and other abnormalities in somite-derived tissues in mutant mouse embryos. Extracellular matrix proteins surrounding somitomeres and somites, e.g. N-cadherin and fibronectin, also play important roles in mesenchymal-epithelial transition during somite segmentation (Duband et al., 1987; George et al., 1993; Radice et al., 1997).

Recent studies have also established that somitogenesis is regulated by signaling molecules derived from neighboring tissues. Signaling molecules secreted from the notochord, neural tube and surface ectoderm usually promote
somitogenesis, whereas signals from lateral mesoderm plates have negative effects. One of the best studied signaling proteins is ventral midline-derived Sonic hedgehog (Shh), a member of the hedgehog protein family. Mice deficient in Shh have sclerotome defects and reduced expression of the sclerotomal marker Pax-1 and the myotomal marker myf-5 (Chiang et al., 1996). In combination with Wnt family members, Shh can elevate the expression of myogenic genes in avian somitic tissues in vitro (Munsterberg et al., 1995). Overexpression of Shh in zebrafish expands the slow muscle domain at the expense of the fast muscle domain (Blagden et al., 1997; Du et al., 1997). Bone morphogenetic protein (BMP)-4, a member of the TGF-β family, is a negative regulator of somite differentiation. It has been shown that BMP-4 can inhibit the activation of the myogenic markers myoD and myf-5 in the dermamyotome of chicken somites (Reshef et al., 1998). Implantation of BMP-4-expressing cells between the neural tube and the paraxial mesoderm can convert the medial somite into a lateral somitic lineage (Pourquie et al., 1996). This BMP-4 activity can be antagonized by another secreted protein, Noggin, that acts downstream of the Shh signaling pathway (Capdevila and Johnson, 1998; Hirsinger et al., 1997). In zebrafish ectopic expression in the notochord of dorsalin-1, another TGF-β-related factor, can antagonize the induction of extra muscle pioneer cells, a set of the slow muscle precursors, by Shh or tiggy-winkle hedgehog (twih) (Du et al., 1997).

We report the identification and characterization of a novel zebrafish gene, terra, which encodes a putative zinc-finger protein, and is specifically expressed in the presomitic mesoderm and developing somites. In vivo, terra expression is inhibited by hedgehog family members and enhanced by BMP-2. Overexpression of terra causes rapid apoptosis both in vitro and in vivo. Since terra has both human and mouse homologs, and is specifically expressed in mouse somites, we propose that terra is a conserved somite-specific factor that mediates very early events of vertebrate somitogenesis and its activity is somehow directly or indirectly influenced by the relative activities of hedgehog and BMP signals.

**MATERIALS AND METHODS**

**Identification of terra**

We performed a systematic search for novel genes that have unique expression patterns during early zebrafish embryogenesis. An unamplified cDNA library was constructed in pcDNA3.0 (Invitrogen) using mRNA isolated from 12- to 16-hour embryos. Partial sequences obtained from individual clones in this library were used to search the GenBank database and those with novel sequences were selected for whole-mount RNA in situ hybridization. terra was identified as one of the cDNA clones with a tissue-specific expression pattern.

Mouse and human homologues of zebrafish terra were identified by searching the public EST databases using the amino acid sequence of terra. The mouse and human EST clones were purchased from Genome Systems, Inc.

** Constructs**

The original clone containing terra cDNA in pcDNA3.0 was named pCMV-terra. The construct pCMV-terra-gfp was generated by ligating the 5' untranslated region and coding sequence of terra in frame to gfp using the vector pEGFP-N2 (Clontech). To facilitate preparation of capped mRNA, the terra-gfp fusion cassette was inserted into the multiple cloning region of pcDNA3.0 to generate the construct pCMV/T7-terra-gfp. The pXT7-terra was made by cloning the full-length cDNA into pXT7 that has S' and 3' untranslated regions derived from the Xenopus major beta-globin gene. Construct pKS-GFP was generated by inserting a GFP cDNA with a SV40 polyadenylation signal into pBluescript KS(−). This construct was used to produce control gfp mRNA.

**In vitro translation**

The pCMV-terra was linearized with XbaI and used as a template to synthesize capped mRNA using the T7 Cap Scribe (Boehringer-Mannheim). The mRNA was translated in the presence of [35S]methionine using the Reticulocyte Translation Kit (Boehringer-Mannheim). The translation product was separated on a 10% SDS-polyacrylamide gel and exposed to X-ray film.

**Northern and Southern hybridizations**

Total RNA was isolated from embryos using Trizol (GIBCO/BRL) and 20 μg RNA per lane were run on a 1.2% formaldehyde gel that was then blotted onto a nylon membrane. Full-length cDNA of terra or zebrafish e-f-Iα, a housekeeping gene, was radiolabeled and hybridized using standard procedures (Sambrook et al., 1989), except that hybridization and washes were carried out at 56°C. Genomic DNA was isolated from an adult fish as described by M. Westerfield (1995) and digested with BamHI, EcoRI, HindIII, PstI or XbaI, enzymes that have no recognition sites in the cDNA sequence of terra. 15 μg of each digest was separated on a 1% agarose gel and Southern hybridization was performed at 55°C using a 32p-labeled 605 bp EcoRV/XhoI fragment (extending from positions 645 to 1250) of the terra cDNA.

**RNA injection**

Templates for transcription were prepared by linearizing plasmids with the appropriate restriction enzymes: pCMV/t7-terra-gfp and pXT7-terra with XbaI; zebrafish Shh construct zshh T7TS and twih construct twih T7TS with BamHI; zebrafish BMP-2 construct zBMP-2 with XhoI; pKS-GFP with NotI. Capped mRNA was synthesized in vitro using the mMESSAGE mMACHINE Kit (Ambion). After purification as recommended by the manufacturer, RNA was dissolved in nuclease-free 0.1 M KCl at a concentration of approximately 200 μg/ml. Unless otherwise stated, the RNA was diluted to a final concentration of 100 μg/ml for injection and 1 μl was used to inject approximately 400 embryos. RNA was injected into the yolk of one-cell stage wild-type embryos. For each construct, RNA injection was performed 2-4 times and the data were pooled.

**Whole-mount RNA in situ hybridization**

Digoxigenin-UTP labeled sense and antisense RNA probes were generated for zebrafish terra, mouse terra and zebrafish myoD genes by in vitro transcription. For double staining, the antisense myoD RNA probe was labeled with fluorescein-UTP, RNA in situ hybridizations were performed essentially as described by Westerfield (1995). For double staining for zebrafish terra and myoD mRNA, embryos were simultaneously hybridized to fluorescein-labeled terra and digoxigenin-labeled terra antisense RNA probes. The hybridized embryos were incubated with AP-conjugated anti-fluorescein antibody (Boehringer-Mannheim) and the first color reaction was performed using a Fast Red substrate (Boehringer-Mannheim). After purification as recommended by the manufacturer, RNA was dissolved in nuclease-free 0.1 M KCl at a concentration of approximately 200 μg/ml. Unless otherwise stated, the RNA was diluted to a final concentration of 100 μg/ml for injection and 1 μl was used to inject approximately 400 embryos. RNA was injected into the yolk of one-cell stage wild-type embryos. For each construct, RNA injection was performed 2-4 times and the data were pooled.
Cell culture and transfection

Mouse 3T3 cells and human HeLa cells were grown in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. Zebrafish PAC2 cells were maintained at 32°C in L-15 medium (Gibco/BRL) supplemented with 10% fetal bovine serum and 10% embryo extract (Westerfield, 1995). Transfection of plasmid DNA into 3T3 and HeLa cells was performed using the Calcium Phosphate Transfection System (Gibco/BRL) according to the manufacturer’s recommendations. For transfecting zebrafish cells, the following modifications were introduced. After incubation of cells at 32°C in medium containing DNA/CaPO4 precipitate for 4 hours, the precipitate was removed, and the cells were washed once with medium. For a 6-cm tissue culture plate, 1 ml of 15% glycerol in 1× Hepses-buffered saline was added to the cells, which were then incubated for 3 minutes at 37°C. The cells were washed once with medium and cultured in fresh L-15 medium. 24 hours after transfection, the cells were split 1:5 into 10-cm diameter plates containing appropriate medium and 0.6 mg/ml G418 (Geneticin, Gibco). This selection continued for 2-3 weeks. Co-transfection of pCMV-terra and pEGFP-N2 was done at a 10:1 ratio. Transfected cells were visualized for GFP using a fluorescence microscope under regular FITC filter conditions.

Detection of apoptosis in whole-mount embryos

Fixation, rehydration, proteinase digestion, and post-fixation of the injected embryos were done as for whole-mount RNA in situ hybridization (Westerfield, 1995). After post-fixation, the embryos were washed in PBS for 30 minutes, followed by rinsing in 0.1% sodium citrate for 1 hour. The TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) reaction mixture, which was prepared using the In Situ Cell Death Detection (AP) Kit (Boehringer-Mannheim) according to the manufacturer’s recommendations, was added to the embryos and incubated at 37°C for 1 hour. The embryos were washed three times with PBST and once with PBS. The color substrate Fast Red solution (Boehringer-Mannheim) was added to the embryos and incubated at room temperature for 10-15 minutes. The embryos were washed 3 times with PBST and stored in 4% paraformaldehyde.

RESULTS

Terra encodes a putative DNA binding protein

We identified terra through a systematic search for genes with tissue-specific expression patterns during early zebrafish development (see Materials and methods). The zebrafish terra cDNA clone, selected because of its somite-specific expression pattern (see below), has an insert of 2,086 bp with a major open reading frame (ORF) encoding a putative polypeptide of 506 amino acids (Fig. 1A). In vitro translation experiments demonstrated that a major translational product matched the predicted 56 kDa molecular mass of the terra protein (Fig. 1B). Western blot analysis using polyclonal antibodies raised against a synthetic peptide based on the predicted protein sequence also detected a protein band of 56 kDa (data not shown). These results suggest that we have identified the authentic protein sequence that is translated in vivo during zebrafish embryogenesis. Low stringency Southern blotting of zebrafish genomic DNA digested with various restriction enzymes showed that terra is a single copy gene (Fig. 1C).

The predicted polypeptide has an acidic domain at the N terminus, a feature common to the region responsible for protein-protein interactions in many transcription factors. This domain consists of 43 amino acid residues, of which 37.2% are aspartate and glutamate residues. Following the acidic domain is a basic domain containing 58 amino acid residues between positions 57 and 114, as is similarly seen in the DNA-binding domains of many transcription factors. This basic domain shows 63.2% identity to the DNA binding domain of Drosophila melanogaster doublesex (DSX) protein (Baker and Wolfer, 1988) and 77.6% identity to a similar domain of a Caenorhabditis elegans sequenced clone (Fig. 1D), as revealed by a BLAST search using the amino acid sequence of terra. In Drosophila, dsx is specifically expressed in germ cells and is required for sex determination through alternative splicing (Burris and Baker, 1989). However, because no sequence homology is found outside the DNA binding domain and, as described below, terra is expressed in a tissue different from germ cells, we classify terra as a novel protein rather than a vertebrate homologue of Drosophila DSX.

In the putative DNA binding domain of terra, all the cysteine and histidine residues that are critical for forming zinc finger-like DNA binding structures are well conserved (Fig. 1D). As a putative DNA binding protein, terra should be localized mainly in the nucleus. To visualize the localization of terra, we generated an expression construct pCMV/T7-terra-gfp by fusing terra to the green fluorescent protein (GFP) reporter gene. Capped mRNA encoding the terra-GFP fusion protein was produced in vitro and injected into 1-cell stage embryos. As shown in Fig. 1E, green fluorescence was only observed in the nuclei of cells, indicating that terra is indeed a nuclear protein.

Terra expression is restricted to presomitic mesoderm and newly formed somites

We performed northern blot analysis for terra transcripts using total RNA isolated from zebrafish embryos at different stages of development. As shown in Fig. 1F, terra transcripts first become detectable at the 1-somite stage and are maintained at high levels through the 15-somite stage of development. The size of the transcript is similar to the insert size of our cDNA clone, suggesting that we have isolated a full length clone of terra.

To reveal further the temporal and spatial expression pattern of terra during embryogenesis, we performed whole-mount RNA in situ hybridization. Terra was first detected in tail bud-stage embryos as two pairs of rectangular patches along the rostral-caudal axis (Fig. 2A,B), in the lateral presomitic mesoderm on either side of the prospective notochord and neural tube. During the period in which the first 20 somites are formed, terra transcripts were detected in a series of bands of cells in somites on both sides of the notochord (Fig. 2C-F). The width of these bands and the staining intensity decreases progressively from the caudal to rostral direction. By 24 hours, the level of terra expression has dropped considerably throughout the trunk and the strongest region of expression is restricted to the 2-3 posteriormost somites near the tail (Fig. 2G,H). By approximately 35 hours, no expression can be detected in somites or other tissues. We estimate that expression of terra in each somite persists for approximately 5 hours. Transverse sections show that terra mRNA is evenly distributed in the lateral domains of the first 2-3 newly formed somites, and then the amount of transcripts starts to decrease mediolaterally and caudorostrally. In older somites, expression is restricted to the lateralmost cells and occurs at a very low level (Fig. 2IJ).
To position *terra* in the somitogenic regulatory pathway, we compared its expression pattern with *myoD*, a well-known regulatory gene expressed in developing somites (Weinberg et al., 1996). We found that *terra* expression lags about 30 minutes behind that of *myoD*, which starts at approximately the 80% epiboly stage. During the period when the first five somites are formed, *myoD* expression is restricted to adaxial cells and the posterior part of the lateral domain. The spatial relationship between *terra* and *myoD* expression was also examined by simultaneously staining the same embryos with two different color substrates (Fig. 2K). This double-staining experiment demonstrates that *terra* (in purple) is expressed in lateral presomitic cells that do not express *myoD* (in red). In zebrafish, these lateral presomitic cells are thought to be precursors of fast muscles whereas *myoD*-positive adaxial cells are precursors of slow muscles (Devoto et al., 1996).

**Hedgehog proteins inhibit *terra* expression**

During early embryogenesis, vertebrate hedgehog genes are primarily expressed in ventral midline tissues such as the notochord, neural tube and floor plate. Hedgehog proteins secreted from the midline often play important roles in patterning adjacent tissues, including the brain, spinal cord and somites (Chiang et al., 1996; Echelard et al., 1993; Ekker et al., 1995; Lassar and Munsterberg, 1996). In zebrafish, Shh, twhh and echidna hedgehog can induce slow muscle and affect somite formation (Blagden et al., 1997; Concordet et al., 1996; Currie and Ingham, 1996; Du et al., 1997; Hammerschmidt et al., 1996; Weinberg et al., 1996).

To determine if hedgehog signaling molecules influence *terra* expression, ectopic expression of zebrafish *Shh* and *twhh* was performed by injecting in vitro synthesized mRNA into 1-cell embryos. As previously reported (Blagden et al., 1997; Du et al., 1997; Hammerschmidt et al., 1996), we observed that injection of *Shh* or *twhh* mRNA can induce expansion of *myoD* expression throughout the segmentation plate (91.3% for *Shh* and 97.8% for *twhh*) (Fig. 3A-C). When the injected embryos were examined for *terra* mRNA, 96.3% of *Shh* mRNA-injected embryos (*n*=108) and 97.7% of *twhh* mRNA-injected embryos (*n*=174) showed a significant reduction or complete elimination of *terra* expression at the 6-somite stage (Fig. 3D-F). At the 16-somite stage, the affected embryos still exhibit a reduction in the level of *terra* expression (Fig. 3G-I). This effect is specific to hedgehog mRNAs because no significant change of *terra* expression was observed in the embryos injected with *gfp* mRNA (*n*=123). Inhibition of *terra* expression by the ectopic expression of *Shh* and *twhh* suggests that *terra* acts downstream of a signaling factor that is antagonized by either of the hedgehog genes to control the formation and/or differentiation of somites. Alternatively, expansion of
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hedgehog-induced MyoD-positive somite domains is achieved at the expense of terra expressing cells. Many zebrafish mutants with defects in middle line structures have been identified (Brand et al., 1996; Odenthal et al., 1996; Stemple et al., 1996). One of them, bozozok (boz), has a deficiency in axial mesoderm development. Embryos with a strong boz phenotype do not have a visible notochord, and therefore lack notochord-derived Shh. It has been shown that these embryos have defects in somites and lack differentiated slow muscles (Blagden et al., 1997; Stemple et al., 1996). To investigate whether the expression of terra is affected by the boz mutation, embryos from a boz<sup>m168</sup> heterozygous cross were hybridized with a terra antisense RNA probe. Compared with wild-type sibling embryos (Fig. 3J), the mutant embryos have two expanded staining stripes that are fused in the midline (Fig. 3K,L). The increase in staining signal is more notable in the anterior part of the mutant embryos. This indicates that elimination of notochord-derived signaling molecules, including Shh, can expand or assist to maintain terra expression in the medial domain of older somites.

**BMP-2 upregulates terra expression**

BMPs are signaling molecules that play an important role in inducing ventral mesoderm (Graff, 1997; Hogan, 1996). Recent studies have demonstrated that BMP-4 derived from the lateral plate mesoderm is involved in specifying the lateral somitic fate during early somitogenesis. The activity of BMP-4 is antagonized by dorsal midline signals such as Noggin or Shh (Hirsinger et al., 1997; McMahon et al., 1998; Pourquie et al., 1998; Reshef et al., 1998). Since the expression of terra is downregulated by the hedgehogs, we tested whether BMPs, as antagonists of hedgehogs, may enhance terra expression. We injected single-cell embryos with zebrafish BMP-2 mRNA and analyzed the expression of terra in the injected embryos. We found that 44.1% of the embryos injected with BMP-2 mRNA (<i>n</i>=68) had expanded expression of terra in the medial domain of rostral somites (Fig. 4B). However, this increase was not observed in embryos injected with gfp control mRNA (Fig. 4A).

We next examined terra expression in swirl, a zebrafish mutant that has mutations in the BMP-2 gene (Kishimoto et al., 1997; Nguyen et al., 1998). In swirl mutant embryos, dorsal structures such as notochord and somites are expanded whereas most of the ventral tissues are missing (Kishimoto et al., 1997; Mullins et al., 1996). Expression of myogenic marker genes, including myoD, is also significantly expanded in swirl embryos. As revealed by RNA whole-mount in situ hybridization, swirl mutant embryos do not have any detectable terra RNA transcripts under staining conditions where the wild-type siblings have already shown clearly visible signals (Fig. 4C,D). However, if the staining time is extended, faint terra signals are detected in the mutant embryos (data not shown). This suggests that BMP signals are important for enhancing expression of terra or maintaining the cells that express terra.

**Overexpression of terra induces apoptosis in vitro and in vivo**

To determine whether terra, like myoD, could induce myogenic gene expression in non-muscle cells, we transfected the construct pCMV-terra-gfp, which encodes a terra-GFP fusion protein, into mouse NIH3T3 cells, human HeLa cells and zebrafish PAC2 fibroblast cells. Surprisingly, more than 80% of the GFP-positive cells exhibited apoptotic characteristics such as detachment from their neighbors and rounded and shrunken appearance by 24 hours after transfection (Fig. 5B,D,F,M). By 48-60 hours, no viable...
GFP-positive cells were observed. In contrast, only about 20% of the GFP-positive cells appeared to be dying following transfection with a GFP control construct, pEGFP-N2 (Fig. 5A,C,E,M). After selection of transfected cells with G418 for 2-3 weeks, only a few surviving clones were obtained from pCMV-terra-gfp transfections and none of them were GFP-positive, whereas over 85% of surviving clones in the control experiment expressed GFP. This supports the results observed in transient transfection experiments. We performed TUNEL assays on the transfected cells and detected a significantly higher number of apoptotic cells compared with the control cells (data not shown). To assure that apoptosis was not caused by a function derived from the fusion of terra and GFP protein, we co-transfected these cells with constructs pCMV-terra and pEGFP-N2. For all three cell lines, we observed death rates similar to that described as above (Fig. 5G,H).

When zebrafish embryos were injected with terra or terra-gfp mRNA at a concentration of 100 µg/ml (approximately 200 pg per embryo), more than 80% of the embryos died by 24 hours, apparently due to extensive cell death in a variety of tissues. When the concentration of injected terra mRNA was reduced to 25 µg/ml (approximately 50 pg per embryo), approximately 50% of the injected embryos developed normally. A TUNEL assay was performed on these embryos to detect the internucleosomal cleavage of chromosomal DNA, which is a typical characteristic of apoptosis. As shown in Fig. 5, the embryos injected with terra mRNA (Fig. 5I,K) have more apoptotic cells compared with the control embryos injected with gfp mRNA (Fig. 5J,L). This indicates that overexpression of terra also induces apoptosis in living embryos.

**Terra is conserved in human and mouse**

To determine if terra is conserved between species, we searched the public human and mouse EST databases and identified five mouse and two human EST clones that show amino acid sequence homology with zebrafish terra. We sequenced a portion of the mouse clone containing the largest insert (1,689 bp). Its 5' region encodes a putative peptide of 237 amino acids before encountering a stop codon. Alignment analysis shows that this putative peptide shares approximately 50% amino acid identity (84% similarity) to the C-terminal domain of zebrafish terra (Fig. 6A). However, this mouse terra sequence must be incomplete because the translation start codon and DNA binding domain have not been found.

Whole-mount in situ hybridization of mouse embryos was performed by using the mouse terra antisense RNA probe. Mouse terra is strongly expressed during the segmentation period, and the expression is restricted to early somites (Fig. 6B). Transverse sections of the stained embryos show that the expression occurs in the dermamyotome of the differentiating somites (Fig. 6C). This expression pattern suggests that terra is also likely to play a role in somitogenesis during mouse embryogenesis.

**DISCUSSION**

**Terra may represent a novel class of zinc-finger nuclear proteins**

We have isolated a novel zebrafish gene, terra, encoding a nuclear-localized somite-specific protein. A portion of the amino acid sequence of terra shows high homology with the DNA binding domain of the Drosophila DSX protein transcription factor (Baker and Wolfner, 1988; Erdman and Burtis, 1993). A third protein that has this type of DNA binding domain is identified as a sequenced clone isolated from Caenorhabditis elegans. For these three proteins, no sequence homology can be found in the regions outside the DNA binding domain. In addition, terra is expressed in different tissues than that of DSX, which has its own homologs in C. elegans and humans (Raymond et al., 1998). We therefore believe that these are three different genes that share a common type of DNA binding domain. Although the DNA binding domain of DSX is capable of binding zinc, its primary structure does not belong to any known classes of DNA binding zinc-finger proteins, suggesting that DSX represents a new class of zinc-finger proteins.
proteins (Erdman and Burtis, 1993). Considering the divergence in expression patterns and possible functions between the zebrafish terra and Drosophila DSX, we speculate that more members of this class may be identified and that their functions may differ considerably. Since the mouse homologue of terra is also specifically expressed in somites during early embryogenesis, the function of terra may be well conserved between species. We noted that the mouse terra EST clones were isolated from a cDNA library constructed using mRNA from mammary glands and the human terra EST clones from a fetal heart cDNA library. Thus, it is likely that terra is also involved in organogenesis at later developmental stages.

**Terra is reciprocally regulated by hedgehog and BMP signals**

Ectopic expression of hedgehog genes in zebrafish embryos eliminates or significantly reduces the expression of terra. In boz mutant embryos that lack a notochord and therefore lack the notochord-derived hedgehog proteins, the expression of terra is enhanced, most notably in the medial domains of anterior somites. These results suggest that terra expression is somehow negatively regulated by hedgehog proteins. Previous studies have shown that hedgehog proteins are involved in the formation of slow muscle and muscle pioneer cells in zebrafish (Blagden et al., 1997; Currie and Ingham, 1996; Du et al., 1997; Hammerschmidt et al., 1996). It is possible that hedgehog proteins mediate these aspects of somitogenesis in part by negatively regulating terra expression.

BMPs, members of the TGF-β superfamily, have been implicated in the specification of lateral somite fate during avian somitogenesis (Pourquie et al., 1996; Reshef et al., 1998). Effects of BMPs on somite patterning are antagonized by signaling molecules in the hedgehog signaling pathway (Capdevila and Johnson, 1998; Hirsinger et al., 1997). Although BMPs have been shown to ventralize zebrafish embryos (Kishimoto et al., 1997; Nikaido et al., 1997), their...
expression observed in our studies. Evidence of a direct role for hedgehog or BMP in regulating terra expression should employ the direct assay of reporter gene constructs driven by terra regulatory sequences in cells transfected with or without hedgehog or BMP. We have isolated terra genomic sequences and intend to conduct such experiments in the near future.

**Terra may play a role in somitic apoptosis**

Apoptotic cell death has been well-documented in the primitive streak (Sanders et al., 1997) and in the myotome and sclerotome domains of differentiating somites in chick embryos (Chung et al., 1989; Jeffs and Osmond, 1992; Sanders, 1997; Wride et al., 1994). Additionally, BMP signals have been implicated in apoptosis during development (Buckland et al., 1998; Ferrari et al., 1998; Song et al., 1998). Recently, BMP-induced apoptosis in paraxial and lateral plate mesoderm have also been reported (Schmidt et al., 1998). However, none of these studies have identified any somite-specific apoptotic genes. We demonstrate that overexpression of terra induces rapid apoptosis in zebrafish, mouse and human cultured cells and in living zebrafish embryos. We also showed that BMP signals can expand terra expression in embryos. It is tempting to speculate that terra might play a role in somite-specific apoptosis, which might be regulated by BMPs. It should be noted that no extensive cell death has been detected, by using the TUNEL assay, in the normal developing somites of zebrafish (Abdelilah et al., 1996 and our unpublished data). It is possible that the sensitivity of this technique is too low to detect apoptosis in the zebrafish somites. Alternatively, the apoptosis induced by a constant high level of terra expression may not represent a normal physiological condition and the transient expression of terra in somites may only serve to control cell cycle or proliferation. The role of terra in these processes requires further investigation.

**Terra mediates the earliest events of somitogenesis**

Zebrafish terra transcripts are most abundant in the presomitic mesoderm and the first 2-3 newly formed somites and rapidly disappear after the segmented somites are formed. The expression pattern of terra suggests that this putative transcription factor plays a role in zebrafish somitogenesis. Indeed, some genes that have a similar expression pattern in developing somites such as paraxis (Burgess et al., 1995), Notch1 (Rausch et al., 1992) and delta-like gene 1 (Bettenhausen et al., 1995) have been shown to play critical roles in the formation of somites by gene knock-out experiments (Burgess et al., 1996; Conlon et al., 1995; Hrabe de Angelis et al., 1997). We have preliminary data indicating that inhibition of terra activity in zebrafish embryos by a transient dominant interference approach resulted in the absence of epithelial somites on one or both sides of the neural tube, the formation of irregularly shaped somites or a truncated tail (data not shown). This result indicates the importance of terra expression during early zebrafish somitogenesis. Mouse terra expression also occurs early and is restricted to the dermamyotome in developing somites, suggesting a role in the initial compartmentalization of somites. We are currently generating transgenic zebrafish that will express stable dominant interference alleles of terra. We are also using a gene targeting approach to disrupt terra in mice. Together, these effects on somites have not been reported. However, a protein related to the BMPs, Dorsalin, has been shown to antagonize the inductive effect of hedgehog proteins on slow muscle (Du et al., 1997). In this study, we have demonstrated that BMP-2 is required for the expression of terra, indicating that BMPs play a role in zebrafish somitogenesis. Together, our findings suggest that terra may mediate zebrafish somitogenesis in response, directly or indirectly, to the relative levels of hedgehog and BMP proteins.

It should be noted that the regulation of terra by hedgehog and BMP may only reflect an indirect effect of these signals. Global overexpression of hedgehog and BMP in zebrafish embryos have reciprocal effect on axial and paraxial mesodermal fates. Loss or gain of structural domains expressing terra can also account for the changes of terra expression. Identical amino acid residues (in red) are connected with a vertical line. Dashes indicate gaps introduced for optimal alignment.
approaches should provide further insight into how tera functions to mediate the early events of somitogenesis.

Expression screen of tissue-specific genes using zebrafish

To date, more than 1.3 million mouse, rat and human ESTs have been obtained. A major challenge that lies ahead is the determination of the function of these EST sequences. Identification of the expression pattern of novel genes can often shed light on their function. However, large-scale expression studies using higher vertebrates are both unwieldy and cost-prohibitive for such a vast collection of genes. Zebrafish can provide an alternative approach to this problem. Zebrafish embryos for expression studies can be produced very inexpensively. Hundreds of whole-mount RNA in situ hybridizations to staged embryos using zebrafish EST sequences can be performed simultaneously to reveal their expression patterns. As exemplified by tera, this approach is especially promising since the novel genes identified using zebrafish ESTs already have human and mouse homologues in the EST databases.

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References


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