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A *Sox5* gene is expressed in the myogenic lineage during trout embryonic development

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ABSTRACT Sox proteins form a family of transcription factors characterized by the presence of a DNA-binding domain called the high-mobility-group domain (HMG). The presence of a large number of potential Sox5 binding sites in the trout promoter of Pax7, a gene which has emerged as an important regulator of neural and somite development, prompted us to clone trout *Sox5* and to examine its expression pattern in the developing trout embryo. Using whole mount *in situ* hybridisation, we show here that the *Sox5* transcript is first expressed before segmentation in the whole presomitic mesoderm. In newly formed somites, *Sox5* labelling was observed in myogenic progenitor cells of the posterior and anterior walls. As the somite matured rostrocaudally, *Sox5* expression disappeared from the differentiating embryonic myotome, deep in the somite, to become restricted to the undifferentiated myogenic precursors forming the dermomyotome-like epithelium at the surface of the embryonic myotome. *Sox5* was also expressed in the developing nervous system and in pectoral fin buds. On the whole, this work suggests a hitherto unappreciated role for Sox5 in regulating myogenic cells destined to muscle formation and growth.

KEY WORDS: *Sox5, somite, myotome, external cell layer, dermomyotome, teleost*

Intoduction

Sox proteins are transcription factors which contain a DNA-binding domain called the high-mobility-group domain (HMG). They bind to the minor groove of DNA and recognize the consensus motif (A/T)(A/T)CAA(A/T) (Mertin *et al.* 1999). Several Sox proteins have been shown to play major role in vertebrate development including early embryogenesis, gastrulation and neural induction, and to contribute to differentiation in many lineages (Guth and Wegner, 2008). Sox proteins are categorized into several subgroups on the basis of sequence similarity within the HMG box and other domains (Schepers *et al.* 2002).

Myogenic differentiation is controlled by a complex transcriptional regulatory network involving the four myogenic regulatory factors (MRFs) MyoD, myf5, myogenin and MRF4, which direct the transcription of muscle structural genes (Buckingham, 1992). MRFs act downstream of, or in parallel with, the paired domain and homeobox-containing transcription factors Pax3 and Pax7 which are precociously expressed in the dermomyotome, a somite derivative from which arise the myogenic precursors necessary for embryonic myotome development and muscle growth (Buckingham and Relaix, 2007). An external cell layer of undifferentiated Pax-7 positive myogenic cells surrounds the primary myotome in the fish embryo. This external cell layer, that exhibits many features of the amniote dermomyotome (Devoto *et al.* 2006), derives from the anterior somitic domain by a cell arrangement and provides myogenic cell precursors necessary for mediolateral expansion of the embryonic myotome (Hollway *et al.* 2007; Stellabotte *et al.* 2007; Steinbacher *et al.* 2008).

As with MRF and Pax7 genes, several Sox genes have been shown to regulate muscle differentiation. Thus Sox8 and Sox15 are expressed in muscle satellite cells and are down- regulated during myogenic differentiation (Schmidt *et al.* 2003; Beranger *et al.* 2000). Mice lacking Sox15 are viable but appear to have impaired skeletal muscle regeneration (Lee *et al.* 2004). On the other hand, Sox6 has been shown to play a critical role in the fiber type differentiation of skeletal muscle both in both the mouse and fish. Indeed, all fetal muscle fibers in Sox6 null mouse embryos

Abbreviations used in this paper: HMG, high mobility group (domain); MRF, myogenic regulatory factor.

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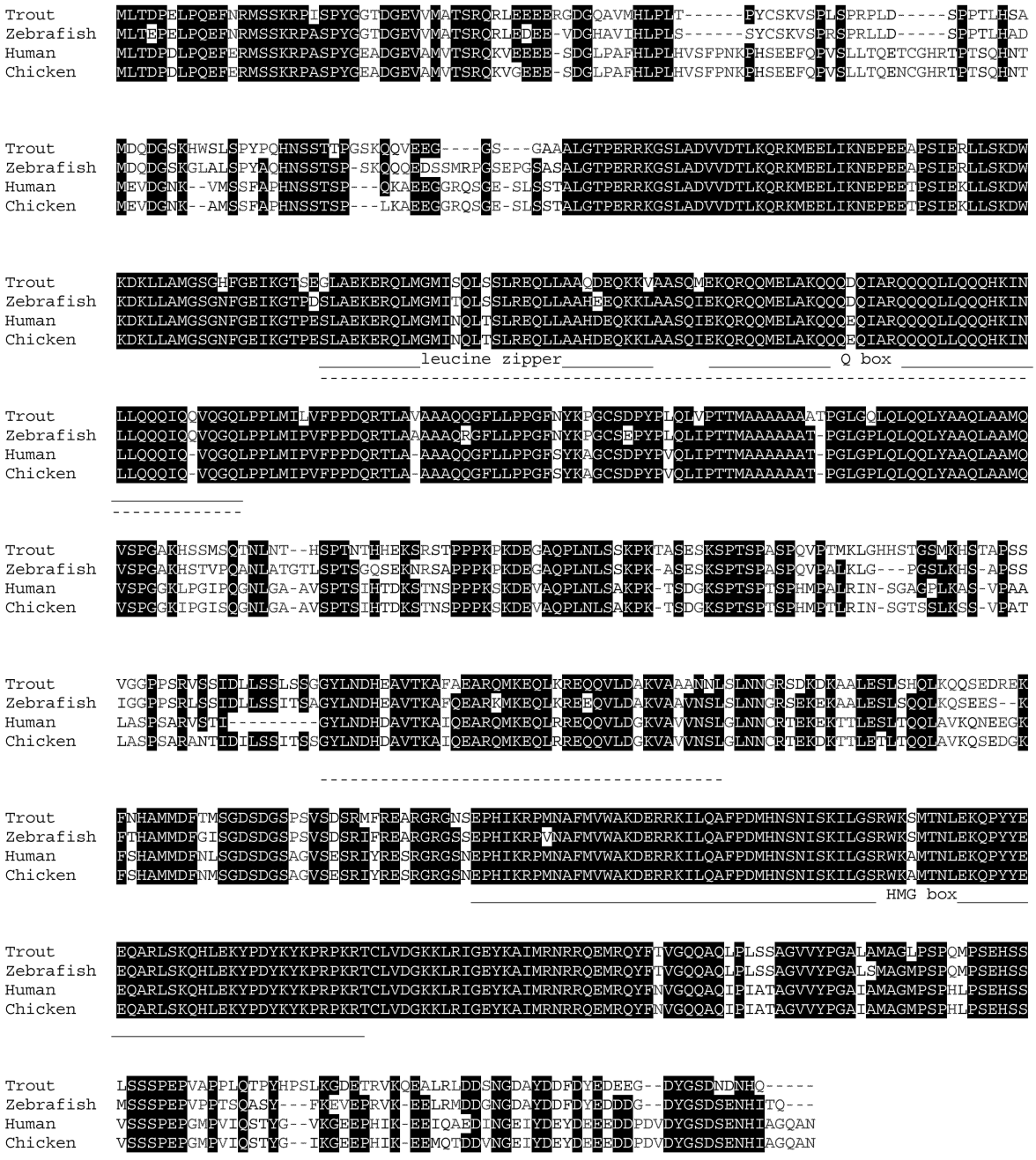

Fig. 1. Sequence comparison of Sox5 aminoacids between different species. *The sequence of the trout Sox5 gene product (FJ713023) is compared to that of the zebrafish (AY730586), human (NP_008871) and chicken (CAF25288). The shading indicates identity. Conserved regions are shown as lines (Leucine zipper, Q box and HMG box) or dotted lines (two coiled coil regions).*

maintain slow fiber characteristics (Hagiwara *et al*. 2007), while forcing the expression of Sox6 in zebrafish embryo adaxial cells inhibits the expression of Prox1, a gene involved slow fiber maturation (Hofsten *et al*. 2008).

Sox5 belongs to the SoxD subgroup together with Sox6 and Sox13 and is primarily expressed in cartilage, its expression being associated with the chondrocyte phenotype (Lefebvre *et al.* 1998). Sox5 transcript has also been found in many adult tissues, including the brain, kidney, lung and skeletal muscle (Ikeda *et al.* 2002). The identification of several potential Sox5-binding sites in the promoter of Pax7, which is a major developmental gene, led us to analyze the expression of Sox5 in trout embryos, particularly in relation with somite development.

Results and Discussion

The trout Pax7 proximal promoter contains several Sox5 binding sites

In an attempt to identify genes precociously expressed in myogenic progenitor cells of the trout embryo we searched for

potential transcription factor binding motifs present in the trout promoter of Pax7, a gene that marks myogenic cells (Buckingham and Relaix, 2007). For this purpose, the trout Pax7 gene was isolated by screening a rainbow trout bacterial artificial chromosome (BAC) library and then sequenced upstream to the initiation codon (GenBank accession no. FJ713022). Computational characterisation of the promoter led to the identification of five TRANSFAC motifs for Sox5 (ATTGTT) (Denny *et al.* 1992) in position -2665, -2639,-2617, -2018 and -1338 relative to the translation initiation codon (Supp. Fig. S1). We thus considered that Sox5 was a candidate gene to be transcribed in embryonic myogenic precursor cells, so its expression in developing embryo was therefore examined.

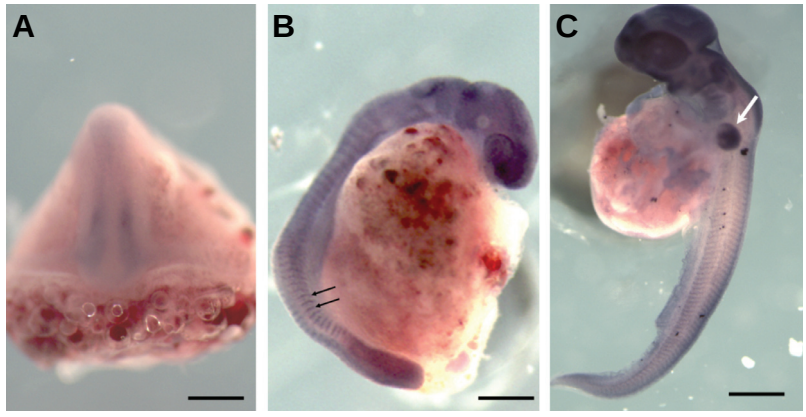

Fig. 3. Expression of *Sox5* in the trout embryo. (A) *Stage 10A embryo, dorsal view:* Sox5 *labelling is observed in the paraxial mesoderm on either side of the elongating embryonic shield.* **(B)** *Stage 15 embryo (approximately 35 somites), lateral view:* Sox5 *transcript accumulates in the paraxial mesoderm, somites (arrows) and developing brain.* **(C)** *Stage 20 embryo (segmentation is complete to the tip of the tail), lateral view:* Sox5 *transcript is evidenced in somite, branchial arches and pectoral fins (white arrow). Scale bar, 120 μm in (A), 300 μm in (B) and 400 μm in (D).*

Molecular cloning of trout Sox5 cDNA

All the PCR products generated in course of this study were overlapping and resulted in a 2289 nt cDNA (GeneBank accession no. FJ713023) with an open reading frame encoding a 762 amino acid protein (Fig. 1). The BlastP best match of this protein was zebrafish Sox5. The identity of putative trout Sox5 protein was further confirmed using the Figenix platform which unambiguously clustered it with Sox5 proteins within the SoxD subgroup (fig. 2). Alignment of trout Sox5 with other vertebrate Sox5 proteins revealed several common characteristics such as an HMG box, two coiled coil region, a leucine zipper and a Q box (Fig. 1).

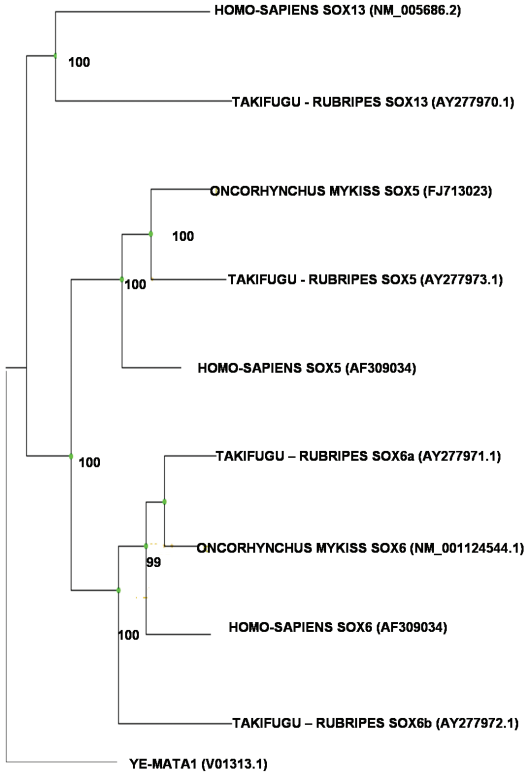

Fig. 2. Phylogeneic tree showing the relationship of the trout Sox5 protein to other Sox proteins belonging to the SoxD subgroup. *This tree was constructed using the phylogenomic analysis pipeline available in Figenix automated genomic annotation platform (Gouret* et al.*, 2005); Bootstrap values are given at each branch. Mata-1 was used as an outgroup.*

Expression pattern of Sox5 in developing trout embryo

Whole mount *in situ* hybridisation using an antisense Sox5 digoxigenin-labeled probe showed that Sox5 transcript was first present in two presomitic bands prior to the onset of somitogenesis (stage 10A of Ballard, 1973) (Fig. 3A). At the beginning of the somitogenesis, 6-10 somites formed simultaneously in the rostral paraxial mesoderm (stage 10B). At this stage Sox5 transcript was observed within the elongating presomitic mesoderm and in somites. As somitogenesis proceeded along an anteroposterior axis (stages 10B to 19), labelling extended progressively to more caudal somites (Fig. 3 B,C). Transverse sections showed that Sox5 transcript was present throughout the rostral presomitic mesoderm including adaxial and lateral cells (Fig. 5A). In the newly formed somites, Sox5 labelling appeared to be intense in a central stripe (Fig. 3B). Frontal sections indicated that this aspect resulted from the marked accumulation of Sox5 transcript in the cytoplasm of myogenic cell precursors of the posterior and anterior walls and its weak accumulation in the large nuclei mostly located at the apical pole of these cells (Fig 4A,B). Shortly after their incorporation into somite, the adaxial cells which had already started to express myogenin (Fig. 4C) ceased to express Sox5 (Fig. 4B). It is now established that adaxial cells, which are the first to differentiate, migrate radially through the somite to form a superficial layer of embryonic slow muscle fibres covering the fast embryonic muscle fibres that originate from the posterior somitic compartment (Devoto *et al.* 1996; Hollway *et al.* 2007; Stellabotte *et al.* 2007). As somite maturation proceeded, Sox5 expression gradually disappeared from the differentiating embryonic myotome, deep in the somite (Fig. 5B), to become restricted to the surrounding external cell layer (Fig. 5 C,D). This external epithelium, produced by the displacement of somitic anterior cells towards the outermost part of the somite (Hollway *et al.* 2007; Stellabotte *et al.* 2007), exhibits many features of the amniote

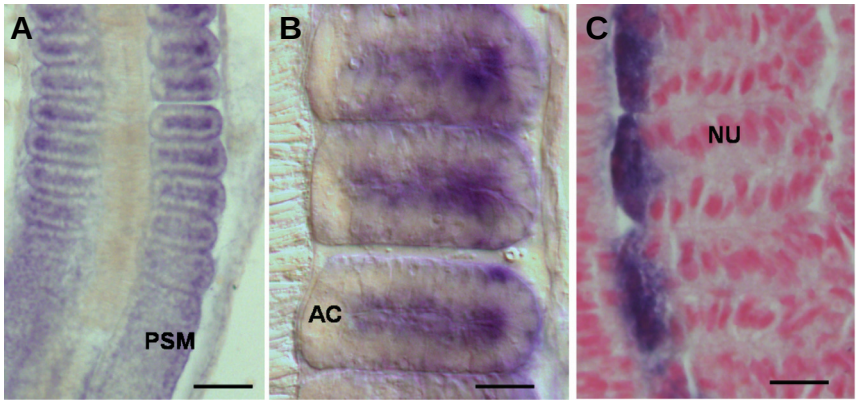

Fig. 4. Expression of *Sox5* and *myogenin* in newly-formed trout somites. **(A-C)** *Stage 15 embryo, frontal sections.* **(A)** Sox5 *labelling is intense in a stripe in the middle of the neoformed somites.* **(B)** *Higher magnification of* (A), Sox *5 transcript strongly accumulates in the cytoplasm of the somitic cells in the posterior and anterior walls, but is hardly detected in nuclei.* **(C)** Myogenin *is expressed in adaxial cells, nuclei are large and nearly fill the apical domain of the somitic cells. Scale bar, 50 μm in (A) and 15 μm in (B,C). psm, presomitic mesoderm, nu, nucleus; ac, adaxial cell.*

dermomyotome, including the expression of equivalent gene orthologs (Devoto *et al.* 2006; Dumont *et al.* 2008) and the ability to provide myogenic cells for myotome expansion (Hollway *et al.* 2007; Stellabotte *et al.* 2007; Steinbacher *et al.* 2008). That Sox5 expression faded in differentiating cells of the primary myotome while being maintained in somitic external cells indicated that Sox5 expression is related to an undifferentiated state of the embryonic myogenic cells. In line this observation, it is interesting to note that Sox8 and Sox15 are also expressed in mouse myogenic cells prior to their differentiation into myotubes, and have been found to inhibit myogenesis (Beranger *et al.* 2000; Schmidt *et al.* 2003). At the end of somitogenesis, Sox5 labelling appeared in pectoral fin buds which at this stage are evident as MRF-negative oval structures budding from the ventral portion of rostral somites (Macqueen and Johnston, 2008). An expression of Sox5 appeared in brain structures and in few cell subpopulations of the neural tube around the 20 somite stage (Fig. 5A). At the‘end of somitogenesis, Sox5 transcript was evidenced in the cerebellum, the optic tectum, the telencephalon, the lens, the retina, and branchial arches (Fig. 3C). In mouse embryo Sox5 has been shown to be expressed in notochord cells and in sclerotome cells surrounding the notochord and neural tube (Smits and Lefebvre, 2003). The Sox5 gene identified in this study was not expressed in notochord and sclerotome cells of the trout embryo (Fig. 5 B,C,D). Given the ancient whole-genome duplication that occurred in the teleost fish lineage, after split of lobe and ray finned lineages (Jaillon *et al.* 2004), we can not exclude that an additional Sox 5 gene copy with an expression in the forming vertebral column exists in the trout genome. Alternatively, the acquisition of a chondrogenic function by Sox5 may be specific to mammal evolution.

In conclusion, and on the basis of the presence of multiple potential Sox5 binding sites on the promoter of the Pax7 developmental gene, we examined the expression pattern of Sox5 in developing trout embryos. The expression data showed that Sox5 was transcribed in somite myogenic precursors giving rise to the embryonic myotome and in those forming the dermomyotome-like epithelium at the surface of the embryonic myotome. This work thus suggests a hitherto unappreciated role for Sox5 in regulating myogenic progenitor cells destined to muscle formation and growth.

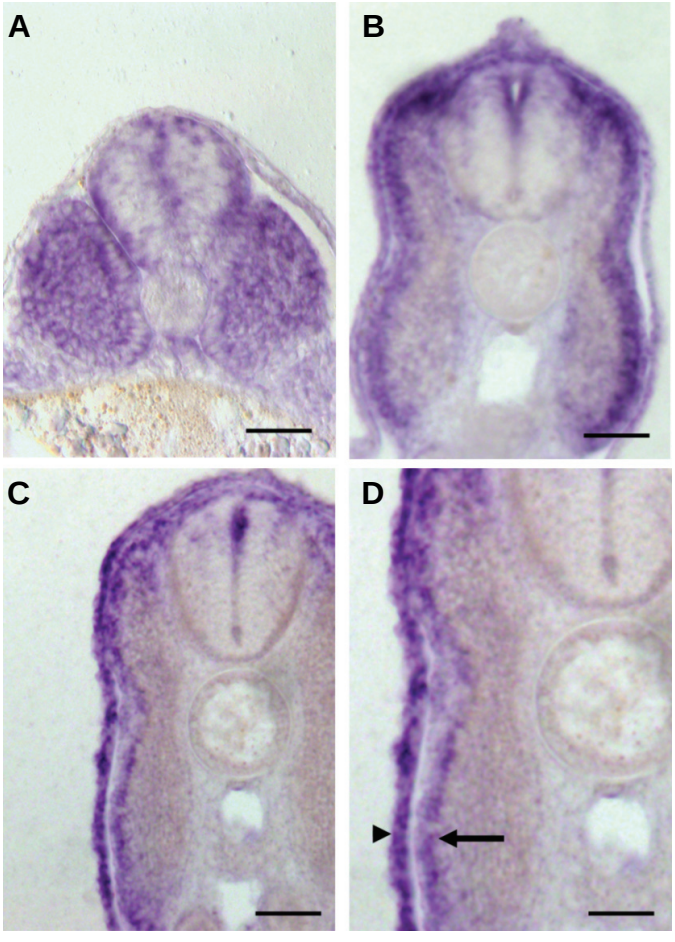

Fig. 5. *Sox5* somitic expression gradually becomes restricted to external cells. **(A)** *Stage 25 somite embryo, transverse section through the anterior presomitic mesoderm.* **(B)** *Stage 55-somite embryo, transverse section through posterior trunk.* **(C)** *Eyed-stage trout embryo, transverse section through posterior trunk.* **(D)** *Higher magnification of* (C)*;* Sox5 *transcript is restrictedly present in the external cell layer situated at the outermost domain of the somite (arrow) just beneath the epidermis (arrowhead). Scale bars: 50 μm in (A,B,C) and 30 μm in (D).*

Materials and Methods

Fish maintenance

All experiments were carried out on the rainbow trout *Oncorhynchus mykiss* (Walbaum). Eggs were collected at the experimental facilities of the INRA Drennec fish farm (Finistère, France). After artificial insemination, the eggs were incubated at 10°C in recirculating dechlorinated water. Chemical water parameters were regularly monitored. Oxygen levels were always above 98% saturation.

BAC library screening and sequencing of the 5'-flanking regulatory region of Pax7

To isolate the trout Pax7 promoter, a 5.3X genome coverage rainbow trout bacterial artificial chromosome (BAC) library (Palti *et al.* 2004) was screened by PCR using primers designed from the salmon Pax7 cDNA sequence (Gottenspare *et al.* 2006). DNA from positive BACs was isolated using the Nucleobond BAC Maxi kit (BD Bioscience). The primer walking method was then used to obtain a genomic sequence directly from the selected BACs. The samples were sequenced using an automatic sequencing system (ABI Prism 310, PE Biosystems). Sequence analysis of the 5'regulatory region was performed with the on-line program TRES (transcription regulatory element search: http://bioportal.bic.nus.edu.sg/tres/) using transfac weight matrices (Wingender *et al.* 2000) with a stringent matrix cut-off score >99.

Isolation of trout Sox5 cDNA

Two rainbow trout ESTs (CX252793 and CX 252794) were selected after a tblastn search using the zebrafish (CAK04950) Sox5 sequence. The sequencing of the corresponding clone and blastx analysis led to the identification of a cDNA fragment (position 1135-2082 in the full length sequence (GeneBank accession no. FJ713023)) encoding a partial protein sequence highly related to Sox5. The missing 5'part of the cDNA was amplified from a rainbow trout embryonic cDNA libraries (Uni-ZAP Custom cDNA Library; Stratagene, La Jolla, CA) using the reverse primer ATGACAGCA- GGTCTATGGAGCTGAC (position: 1336-1312) and the forward primer AGTTCCCACAGACCTATTCCAGC that flanks in 5' the presumptive open reading frame of salmon Sox5 cDNA (EST: GE773617). The missing 3' part of the cDNA was amplified using the forward primer GCCCAGCTGCCCTTGTCCTCGGCG (position 2005-2028) and the reverse primer 5'-TTCTGATTGGCTCTGCTTCCAGA that flanks in 3' the sequence of the presumptive open reading frame of salmon Sox5 cDNA (DY693671). Additional PCR fragments were then produced (positions—398-1234 and 1312-2028) to confirm the overlapping of the 5'and 3' ends with the initial truncated trout Sox5 cDNA. Purified PCR products were cloned onto the pGEM-T vector (Promega) and sequenced using an automatic sequencing system (ABI Prism 310, PE Biosystems). Multiple amino acid sequence alignments were constructed using Clustal W software. The phylogenetic analysis was performed using the phylogenomic analysis pipeline available in the FIGENIX platform (http://www.up.univ-mrs.fr/evol/figenix/) (Gouret *et al.* 2005). For this purpose Sox proteins of the group D, to which Sox5 belongs (Koopman *et al.*, 2004), were entered.

Whole-mount in situ hybridisation

Embryos were dechorionated with fine forceps and fixed overnight at 4°C in paraformaldehyde in phosphate buffered saline (PBS). The specimens were then dehydrated and stored in methanol at -20°C. After rehydration in graded methanol-PBS baths, the embryos were processed according to established automated procedures. Hybridizations were performed with Sox5 and myogenin digoxigenin-11UTP* labelled antisense riboprobes. Myogenin riboprobe was complementary of the 3'untranslated region and the 3' two thirds of the coding sequence of the trout myogenin transcript (Rescan *et al.* 1995). Sox5 antisense RNA probes was synthesised from a PCR-amplified template that included the HMG domain (position 1136- 2082 in the full length sequence). This Sox5 riboprobe did not cross-hybridize with its closest paralog Sox6 as shown by the distinct expression pattern of Sox6 (data not shown).

Histological methods

For histological examinations, embryos were embedded in 30% ovalbumin, 0.5% gelatine and 1% gluteraldehyde in PBS. Blocks were sectioned at 30 μm on a Leica vibratome. The resulting sections were mounted in Mowiol

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