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The genes for the helix-loop-helix proteins Id6a, Id6b, Id1 and Id2 are specifically expressed in the ventral and dorsal domains of the fish developing somites

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Summary

Muscle differentiation is inhibited by members of the Id family that block the transcriptional effect of myogenic bHLH regulators by forming inactive heterodimers with them. Also, Id proteins promote cell proliferation by interacting with key regulators of the cell cycle. In order to determine the role of Id-encoding genes during fish development and especially in early myogenesis, we examined the expression patterns of Id1, Id2 and two non-allelic Id6 (Id6a and Id6b)-encoding genes in developing trout embryos. These four *Id* paralogs were found to exhibit discrete expression in the developing nervous system and in the eye rudiment. During the segmentation process, *Id6a*, *Id6b* and *Id1* were expressed in the tail bud, the paraxial mesoderm and the ventral and dorsal domains of neoformed somites. As the somite matured in a rostrocaudal progression, the labelling for *Id1* transcripts rapidly faded whereas labelling for *Id6* transcripts was found to persist until at least the completion of segmentation. By contrast, *Id2* transcripts were visualised transiently only in dorsal domains of neoformed somites and strongly accumulated in the pronephros. The preferential localisation of *Id6a*, *Id6b*, *Id1* and *Id2* transcripts within ventral and/or dorsal extremes of the developing somites, suggests that these areas, which were the last ones to express muscle-specific genes, contain dividing cells involved in somite expansion.

Key words: *Id*, somite, helix-loop-helix protein, myogenesis, muscle differentiation, gene expression, trout.

Introduction

Myogenesis is induced by the activity of basic-helix-loop-helix (bHLH) transcription factors of the MyoD family. These factors (MyoD, Myf5, myogenin and MRF4) exert their transcriptional effect by binding as homo- or heterodimers to E-box a DNA motif present in numerous promoters of skeletal muscle-specific genes (Edmondson and Olson, 1993). The positive effect of the bHLH factor on transcription is inhibited by Id proteins. These belong to a class of helix-loop-helix proteins lacking a basic amino acid domain necessary for binding DNA. Id proteins are thought to function in a dominant negative manner by sequestering active bHLH transcriptional regulators (Norton et al., 1998). The role of Id proteins in inhibiting cell differentiation and stimulating cell growth is furthermore demonstrated by their integration within cell-cycle-regulatory pathways orchestrated by cyclin-dependent kinases and the retinoblastoma protein (Norton et al., 1998).

In higher vertebrates, four different Id-encoding genes have been described: *Id1* (Benezra et al., 1990), *Id2* (Sun et al., 1991), *Id3* (Christy et al., 1991) and *Id4* (Riechmann et al., 1994). The proteins encoded by these genes have a high degree of conservation in the HLH domain but diverge almost totally outside this region. Orthologs of these genes have been isolated in frogs and fish, indicating that the ancestral *Id*-like gene duplicated and diverged early in vertebrate evolution (Rescan, 2001).

Expression of muscle differentiation genes begins in trout embryos from the 20-somite stage onwards (Rescan et al., 2001; Thiébaud et al., 2001). At these stages, the somite size also increases, especially in height (Bobe et al., 2000), suggesting that undifferentiated somitic cells that continue to proliferate persist alongside differentiating myocytes. One of the molecular mechanisms that maintains these somitic cells in a proliferative state could be the expression of *Id* genes. To test this hypothesis, we sought to examine the transcription of *Id* genes in developing somites and to compare *Id* gene expression pattern with that of muscle-specific genes.

In this study, we report the characterisation of two distinct rainbow trout *(Oncorhynchus mykiss* Walbaum) cDNAs that are both orthologous to *Id6* identified in zebrafish (*Danio rerio*; Sawai and Campos-Ortega, 1997). *In situ* hybridisation on whole trout embryos shows that these two *Id6* orthologs, as well as *Id1* and *Id2*, which have been previously characterized (Rescan, 1997), are selectively expressed in ventral and/or

dorsal domains of the developing somite. These *Id*-positive areas may correspond to proliferative somitic cells involved in somite growth.

Materials and methods

Whole-mount in situ *hybridisation*

TId1 and TId2 have been previously characterized (Rescan, 1997). TId6a and TId6b have been identified from a large-scale trout 3′ and 5′ sequencing project (AGENAE Research Programs; Institut National de la Recherche Agronomique). Digoxigenin-labelled antisense RNA probes were synthesised from a PCR-amplified template using T3 RNA polymerase. The embryos were dechorionated with fine forceps and fixed overnight at 4°C in paraformaldehyde in phosphate-buffered saline (PBS). Specimens were dehydrated and stored in methanol at –20°C. Following rehydration in graded methanol/PBS baths, embryos were processed according to established procedures (Joly et al., 1993) with minor modifications. Depending on the embryonic stage, different times, temperatures and concentrations were chosen for proteinase K treatment.

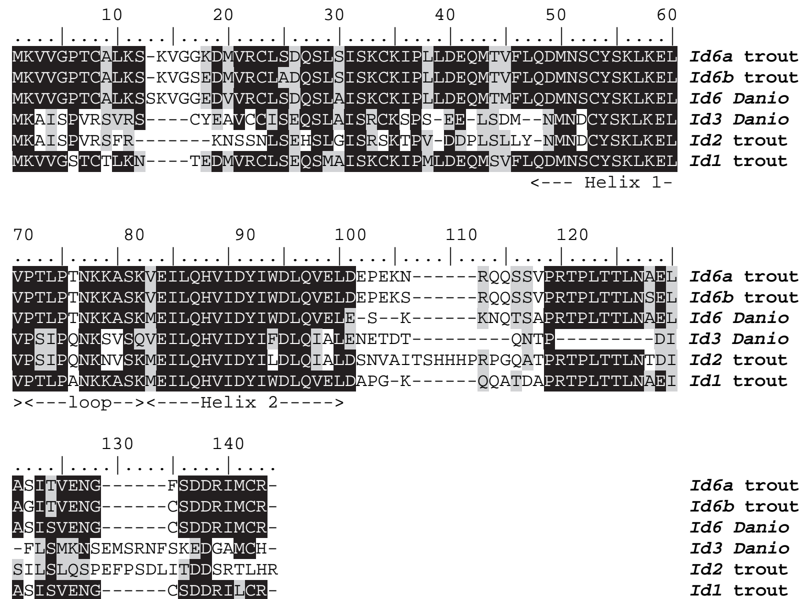

Fig. 1. Comparison of amino acid sequence of trout *Id6a*, *Id6b*, *Id1* (Y08368), *Id2* (Y08369) and zebrafish *Id6* (AF007414) and *Id3* (AY065841). Dark shading indicates identity; light shading indicates similarity. The helix-loop-helix region is indicated.

Histological methods

For histological examinations, embryos were dehydrated and mounted in paraffin, and 10 µm sections were cut. Sections were counterstained with nuclear fast red, mounted in Eukitt (WWR International SAS, Westchester, USA) and observed using a Zeiss 47.50.57 stereo microscope.

Results

Identification of two trout Id6 *orthologs*

In a large-scale trout cDNA sequencing project (AGENAE Research Programs), we identified two cDNAs encoding novel Id proteins [accession numbers BX084393 (TId6a) and BX085138 (TId6b)]. These two cDNAs were quite distinct from those encoding Id1 and Id2 trout orthologs (Rescan, 1997). The proteins (TId6a and TId6b) encoded by these cDNAs are 95% identical and appear to be more closely related to zebrafish Id6 (87 and 85% identity, respectively) than to other Id proteins identified previously (Fig. 1). Given that TId6a cDNA exhibits multiple gaps in the 3′ untranslated region that are not observed in TId6b cDNA (not shown), it is very likely that the two trout Id6-encoding genes are not alleles but originate from two loci that were duplicated during the tetraploidisation of the salmonid genome.

Expression of the TI6a *and* TId6b *genes*

We examined the expression pattern of *TId6a* and *TId6b* on whole trout embryos using digoxigenin-labelled riboprobes. Our observations are based on the Ballard (1973) development table. The expression patterns were similar using the full-length or the 3′ UTR riboprobes (not shown). *TId6a* and *TId6b* transcripts were found to exhibit a similar expression pattern in all developmental stages we examined. *TId6a* and *TId6*b transcripts were first detected at stage 11 when approximately 15 somites had been formed. Around this stage, the labelling was observed in the most rostral part of the paraxial mesoderm, in neoformed somites as well as in the tail bud and the dorsal domain of the neural keel (Fig. 2A). During the rostrocaudal wave of somite formation, *TId6a* and *TId6b* transcripts accumulated selectively in the ventral and dorsal regions of the somite (Figs 2B, 3A). The persistence of *TId6a* and *TId6b* transcripts in ventral and dorsal domains of the myotome was observed at least until stage 20, when the segmentation is complete to the tip of the tail (Fig. 2C). Labelling for *TId6a* and *Tid6b* genes was also evident in the neural tube (Fig. 3A), the cerebellum, the optic tectum and the telencephalon (Fig. 4A). The lens and the retina were both labelled for these two transcripts (Fig. 4A).

Expression of the TId1 *gene*

As for *TId6a* and *Tid6b*, *TId1* was also transcribed in the tail bud (Fig. 5A) and the dorsal part of the neural keel. As somitogenesis proceeded, *TId1* mRNA was detected in the rostral paraxial mesoderm as well as in ventral and dorsal parts of the neoformed somites (Figs 3B, 5A). In contrast to *TId6*, *TId1* expression at the periphery of the somites was rapidly downregulated (Fig. 5A). Staining for *TId1* was also observed in the dorsal domain of the neural tube (Fig. 3B), the cerebellum, the optic tectum, the telencephalon, the lens and the retina (Fig. 4B).

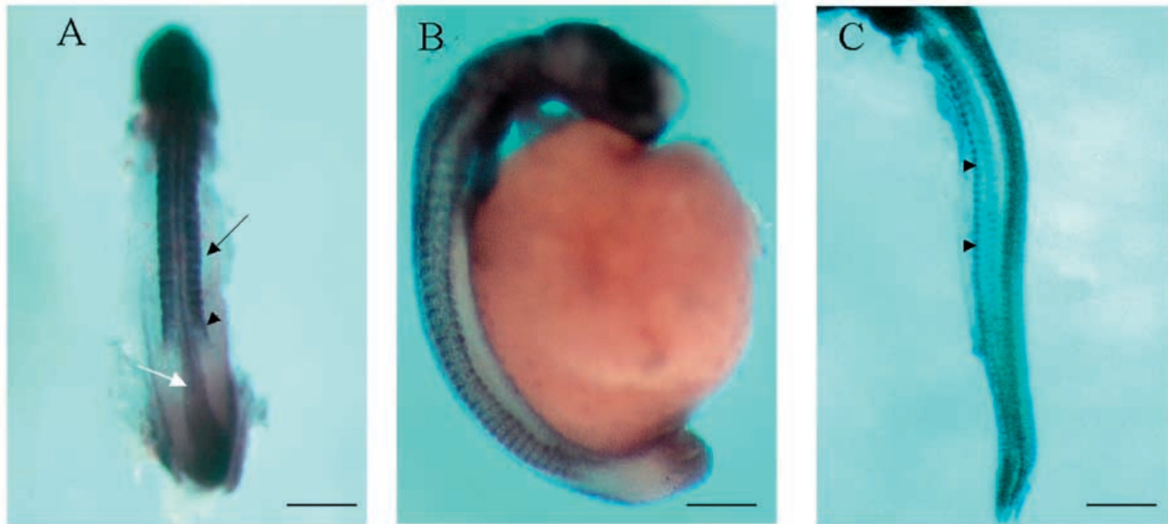

Fig. 2. Expression of *TId6* in trout embryos. The expression was identical using *Id6a* or *Id6b* riboprobe. (A) Stage 12 embryo (approximately 20 somites), dorsal view. The label is observed in the tail bud, the neural keel (white arrow), the rostral presomitic mesoderm (arrowhead) and the somites (arrow). (B) Stage 15 embryo (35 somites), lateral view. The label progresses caudally as somites form and is higher in the ventral and dorsal domains of the somites. (C) Stage 20 embryo (the segmentation is complete), lateral view. Axial structures as well as ventral (arrowheads) and dorsal domains of the somites are labelled. Scale bars: A, 230 μm; B, 400 μm; C, 600 μm.

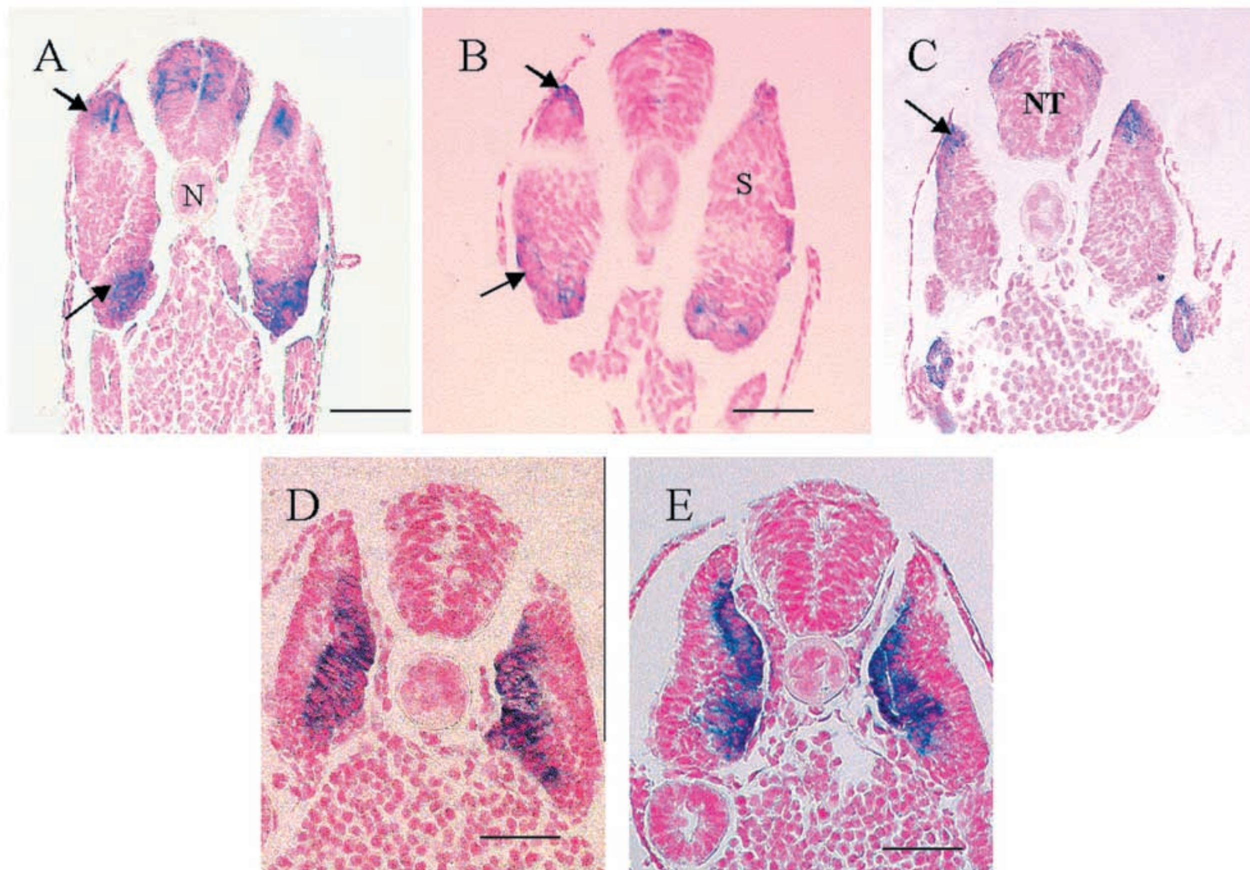

Fig. 3. Expression of *TId6* (A), *TId1* (B), *TId2* (C), troponin C (D) and desmin (E) in stage 15 trout embryos. (A–E) Transverse sections. (A) *TId6* transcripts are concentrated in the ventral and dorsal extremes of the somite (arrows) as well as in the neural tube. (B) *TId1* transcripts are observed in the dorsal part of the neural tube and in the ventral and dorsal somitic cells (arrows). (C) *TId2* transcripts accumulate in the dorsal part of the neural tube, in the pronephros and in dorsal extremes of the somite (arrow). (D,E) Troponin C (D) and desmin (E) transcripts are visualized in the deep part of the somite. S, somite; N, notochord; NT, neural tube; P, pronephros. Scale bars: A–C, 30 μm; D,E, 20 μm.

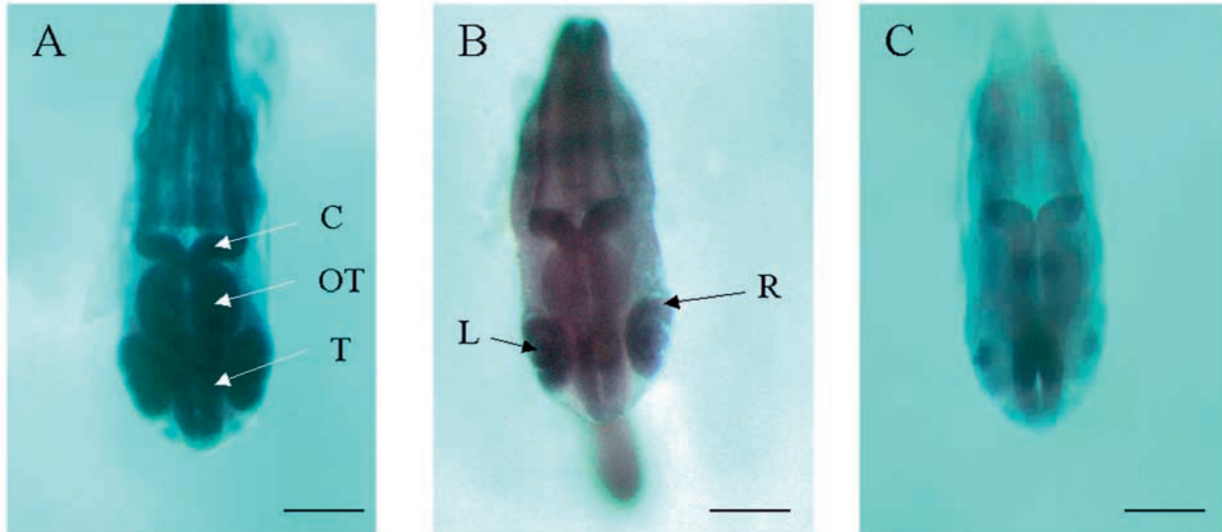

Fig. 4. Expression of *TId6* (A), *TId1* (B) and *TId2* (C) in the head region of a stage 16 embryo. (A–C) Dorsal views. (A,B) Transcripts for *TId6* and *TId1* accumulate in the cerebellum, the optic tectum, the telencephalon, the lens and the retina. (C) Transcripts for *TId2* accumulate mainly in the telencephalon and the lens. C, cerebellum; OT, optic tectum; T, telencephalon; L, lens; R, retina. Scale bars: A–C, 250 μm.

Expression of the TId2 *gene*

In contrast to *TId6a*, *TId6b* and *TId1*, the *TId2* transcript was not visualized in the tail bud nor in the paraxial mesoderm but accumulated in somites that had already been formed (Fig. 5B). Observation of both whole-mount embryos and transverse sections indicated that the expression of *TId2* within somites was present in a narrow domain situated in the apical zones of the somites/myotome while no labelling was evident in the ventral domain of the somites (Figs 3C, 5B). The labelling for *TId2* appeared transient in somites and was no longer observed after the end of the segmentation. A strong and lasting staining was detected in the pronephros that flanked the trunk (Figs 3C, 5B). Otherwise, in the developing brain, *TId2* transcripts were found to accumulate preferentially in telencephalon. Only the lens was labelled in the eye rudiment (Fig. 4C).

Somitic subdomains expressing Ids *correspond to regions that do not exhibit terminal differentiation*

To more fully understand the function of *Id* genes in developing somites, we analysed the early somitic expression of muscular markers including troponin C (Fig. 3D), myosins, tropomyosins, α actin, desmin (Fig. 3E), and muscular isoforms of aldolase A, enolase and creatine kinase. We observed that all these muscle-specific genes were initially expressed in a medial somitic domain that is complementary to the expression domain of the *Id* genes. This indicated that *Id* genes are mostly transcribed in areas (i.e. ventrally and/or dorsally) where the myoblasts are not yet fully differentiated.

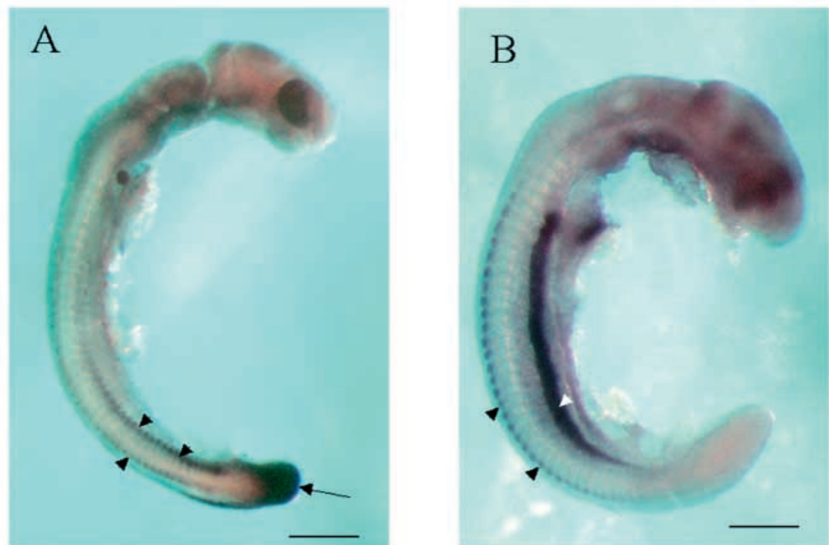

Fig. 5. Expression of *TId1* (A) and *TId2* (B) in trout embryo. (A) Stage 16 embryo (45 somites), lateral view. Transcripts for *TId1* are localized in the tail bud (arrow), paraxial mesoderm and transiently in the ventral and dorsal extremes of the neoformed somites (arrowheads). (B) Stage 16 embryo (40 somites), lateral view. Transcripts for *TId2* concentrate in the dorsal domain of the somites (black arrowheads). A strong signal is also apparent in the pronephros (white arrowheads). Scale bars: A, 300 μm; B, 250 μm.

Discussion

Id1 *and* Id6 *genes coexist in the salmonid genome*

The present study reports the identification in the trout of two novel *Id* cDNAs. Comparison of the polypeptide sequences among members of the Id family indicates that these two cDNAs encode orthologs of Id6 identified in zebrafish (Sawai and Campos-Ortega, 1997). The presence of two Id6 orthologs in trout probably results from the tetraploidisation of the salmonid genome. In contrast to other duplicated genes identified in the trout genome, such as MyoD (Delalande and Rescan, 1999), we did not find any evidence of a differential expression of the two *Id6* genes in developing trout embryos. This indicates that their *cis*-regulatory sequences did not strongly diverge during evolution. The functional significance, if any, of this genetic redundancy is unknown. Although quite distinct, *TId1* and *TId6* genes are both more closely related to mammalian *Id1* than to other mammalian *Ids*. This suggests that *TId1* and the gene from which the two *Id6* trout orthologs arose were probably derived by duplication of an *Id1*-like ancestral gene. It remains uncertain whether the duplication resulting in *Id1* and *Id6* paralogs is specific to salmonids or occurred earlier in the fish lineage. The current large-scale sequencing of cDNAs and genomic DNA in numerous fish species will elucidate the evolutionary relationship of Id family members.

Distinct and overlapping expression of the Id *genes in non-muscle tissues*

Our *in situ* studies are consistent with an important role for the dominant negative helix-loop-helix Id proteins in the development of non-muscle tissues in fish. A strong accumulation of *Id1*, *Id2*, *Id6a* and *Id6b* transcripts is observed in several discrete domains of the brain and the spinal cord as well as in the eye rudiment. These observations, which are reminiscent of numerous data on mammals, birds and amphibians, emphasize the functional involvement of Id proteins in regulating nervous system and eye development (Jen et al., 1997; Zhang et al., 1995; Wilson and Mohun, 1995; Liu and Harland, 2003; Kee and Bronner-Fraser, 2001). Candidate regulators of neural differentiation that can interact with Id are orthologs of achaete-scute. An examination of the expressed sequence tags identified in the AGENAE programs reveals the existence of such an ortholog in the trout (accession no. BX874588), supporting the notion that an antagonism between HLH and bHLH proteins is probably required for proper neurogenesis in the trout.

Among the four *Id* transcripts examined in the present study, only *TId2* was found to be expressed in the pronephros. The role of Id2 in regulating kidney morphogenesis and homeostasis remains unclear. The experimental inactivation of the *Id2*-gene locus does not lead to an apparent alteration of kidney development (Yokota et al., 1999). Nevertheless, our observation emphasizing a high level of *Id2* transcription in the pronephros is in agreement with the strong accumulation of *Id2* transcript reported in the developing kidney of *Xenopus* (Wilson and Mohun, 1995) and humans (Biggs et al., 1992) as well as in the adult kidney of trout (Rescan, 1997). Interestingly, an involvement of Id2 in regulating gene transcription in the kidney is suggested by *in vitro* data showing that Id2 interacts directly with Pax-2 and Pax-8 proteins, both of which are expressed in the kidney (Roberts et al., 2001).

Selective expression of Id *genes in subdomains of the developing somite*

All four mammalian members of the Id family interact with E-proteins and with bHLH proteins of the MyoD family, disrupting their transcriptional activity. Thus, it has been proposed that members of the Id family play a regulatory role during myogenesis in mammals (Kadesch, 1993). In the present study, we show that the transcription of fish *Id* genes occurs in forming somites at stages where myogenic regulator factors (MRFs) are expressed (Delalande and Rescan, 1999) and starts well before the activation of muscle structural genes (Rescan et al., 2001). This raises the possibility that *Id* paralogs impose temporal and spatial limits on bHLH myogenic regulator activity in fish embryos, leading to a delay in muscle differentiation. Supporting this view, Sawai and Campos-Ortega (1997) have shown that zebrafish Id6 protein antagonizes bHLH heterodimer function *in vitro*. On the other hand, *Id* paralogs may also promote cell proliferation in the somite subdomains, where they are expressed by interacting with key regulators of the cell cycle (Ruzinova and Benezra, 2003).

Somitic transcription seems to be an ancient and conserved feature of *Id* genes; indeed, the presence of *Id* transcripts in somites has been observed in different phyla including not only lower and higher vertebrates but also primitive chordates such as *Amphioxus* (Meulemans et al., 2003). In addition, it is interesting to note that *Id6a*, *Id6b*, *Id1* and *Id2* expression in dorsal and/or ventral domains of the somite is reminiscent of that of *Id2*, *Id3* and *Id4* in developing somites of *Xenopus* embryos (Zhang et al., 1995; Wilson and Mohun, 1995; Liu and Harland, 2003). This emphasizes the conservative aspects of the transcriptional network that regulates muscle growth pattern in lower vertebrates. Although *Id1*, *Id2*, *Id6a* and *Id6b* genes are all restrictedly transcribed in most dorsal and ventral parts of the myotomes, there are some differences in the temporal expression of these genes, the two *Id6* genes being the only ones that display a transcription after the completion of segmentation. This suggests that the consequences of dominant negative regulation of transcription factor activity within somitic cells may be different for the different *Id* paralogs.

In situ hybridisation of transcripts encoding myosin heavy chains (MyHC; Rescan et al., 2001) or other muscle-specific proteins (present study) shows that the activation of genes involved in muscular differentiation always starts in the medial domain of the somite before spreading from the inside to the outside. Such an expression pattern is complementary to that of *Id1*, *Id2*, *Id6a* and *Id6b*. Thus, it is likely that the regionalized *Id* expression in the lateral domains of the somite accounts at least in part for the delay in muscular differentiation observed at this level. In keeping with the

regulation of *Id* expression in the developing myotome, it is worth mentioning that *Id* expression is upregulated i*n vitro* by bone morphogenetic proteins (BMPs; Hollnagel et al., 1999), so it would be of interest to examine whether the expression of BMPs overlaps with that of *Ids* in developing trout somites. In this regard, it is interesting to note that a BMP-like signal restricted to dorsal and ventral regions of the fish somite would be consistent with the somite patterning model involving opposing actions of lateral BMPs and axial hedgehogs (Du et al., 1997).

Bobe et al. (2000) have observed, using scanning electron microscopy, that somite size increases, especially in height, as soon as they form. In the light of the work presented here, it is tempting to speculate that the germinative domains involved in somite growth are situated in the *Id*-positive ventral and dorsal regions that are the last to differentiate. Such a growth pattern involving ventral and dorsal subdomains of the somite raises the possibility that a growth process similar to the stratified hyperplasia observed in late embryos and in larvae (Rowlerson and Veggetti, 2001) may occur as soon as the somite forms.

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