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her7 and *hey1*, but not *lunatic fringe* show dynamic expression during somitogenesis in medaka (*Oryzias latipes*)

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Abstract

Epithelialized somites form repeatedly from the unsegmented presomitic mesoderm (PSM) in the tailbud of vertebrate embryos. Mutant analysis has shown that the Delta-Notch pathway is essential for the temporal and spatial control of somite formation. Several components of this pathway show cyclic transcription, which is driven by a molecular oscillator. This oscillator is thought to act similarly in different vertebrates. In this study, we used the Japanese Medaka (*Oryzias latipes*) to examine the expression of three factors of the Delta-Notch cascade that are known to show cyclic expression in the PSM of higher vertebrates. We report that in contrast to the situation in mice, *lunatic fringe* (*lfng*) in medaka is expressed in a non-dynamic fashion in the rostral halves of the formed somites and the anteriormost PSM. On the other hand, *her7*, a member of the *hairy/Enhancer-of-split related* (*Her*) gene family, shows cyclic expression in the medaka PSM. Although this is similar in zebrafish, there are important differences in the distribution of transcripts in the PSM indicating different modes of regulation in both fish species. Finally, we show that *hey1*, another Delta-Notch regulated bHLH gene, is dynamically expressed in the PSM of medaka, similar to *hey1* in zebrafish and the *hey2* orthologs in mice and chicken. Interestingly, medaka *hey1* is also expressed in the dorsal aorta and the heart, two tissues where *hey2*, but not *hey1*, is expressed in zebrafish. This shows that several components of the Delta-Notch pathway are differently regulated during somitogenesis in different species.

Keywords: Medaka; Embryogenesis; Notch; Delta; Fringe; Hey; Segmentation; Somite; bHLH factors; Hairy/enhancer-of-split

1. Results and discussion

A molecular oscillator is postulated to regulate the periodic transcription of a subset of genes in the presomitic mesoderm (PSM) of vertebrates. The expression of these 'cyclic genes' is synchronized between neighboring cells which subsequently results in waves of gene expression that sweep the PSM in a caudal to rostral direction. This is thought to provide the molecular basis of the segmentation clock that controls the repeated formation of epithelialized somites at the anterior edge of the PSM. Expression analyses in mouse, chicken and zebrafish have identified components of the Delta-Notch and the Wnt pathway that show rhythmic expression in the PSM (reviewed in Pourquie, 2003). Several of these components are indispensable for maintaining oscillating gene expression and consequently somite formation, as has been shown by mutant analysis (Conlon et al., 1995; Hrabe de Angelis et al., 1997; Leimeister et al., 2000a,b). Zebrafish mutants that are deficient for *notch1* (*deadly seven*; *des*) and one of its ligands *deltaD* (*after eight*; *aei*) fail to form somite boundaries after the first seven to nine pairs and also lack dynamic gene expression in the PSM (Holley et al., 2000, 2002). Insights into the molecular mechanisms came from gene knock-down and mutant analyses of *her1* and *her7*, two members of the *hairy/enhancer-of-split* gene family that encode basic helix-loop-helix transcription factors (Henry et al., 2002; Oates and Ho, 2002; Holley et al., 2002). These suggested that both factors act as putative transcriptional repressors at the center of autoregulatory feedback loops that also involve Notch ligands. Transcriptional and translational delays of Her1 and Her7 are thought to contribute to the periodic appearance of Delta/Notch activity in the PSM (Lewis, 2003).

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Although it is speculated that similar molecular mechanisms underlie segmentation in different vertebrates, and even spiders (Stollewerk et al., 2003), there are some important differences in the expression of postulated key regulators of somitogenesis. This is most obvious for Lunatic fringe (Lfng), a glycosyltransferase that modulates Notch activity. *lfng* shows oscillating expression in the PSM of chick (McGrew et al., 1998) and mouse (Forsberg et al., 1998), whereas robust and non-oscillating expression is found in the anterior-most PSM of zebrafish (Prince et al., 2001; Leve et al., 2001). Recent studies in chick and mouse suggested that *lfng* plays a central role in establishing the negative feedback loop needed for periodic gene expression in the PSM (Dale et al., 2003; Serth et al., 2003). In zebrafish, *lfng* is involved in a variety of Notch-dependent processes like e.g. hypochord formation (Appel et al., 2003), but cannot have a central function in the segmentation clock due to its static expression in the PSM. In order to test whether this is a general feature for teleosts, we analyzed *lfng* expression in the related fish medaka (*Oryzias latipes*) that recently emerged as model for developmental studies complementary to zebrafish (Wittbrodt et al., 2002).

Using RNA whole mount in situ hybridization, *lfng* expression was detected throughout the central nervous system at the onset of somitogenesis (stage 19; 27 h postfertilization, hpf; 2-3 somite stage; Fig. 1A,B). Some areas showed up-regulated transcript levels like the boundary between fore- and midbrain and the presumptive rhombomeres in the hindbrain. Importantly, no expression was observed in the PSM except a narrow stripe at its anterior-most end (arrowhead in Fig. 1B). Weak expression

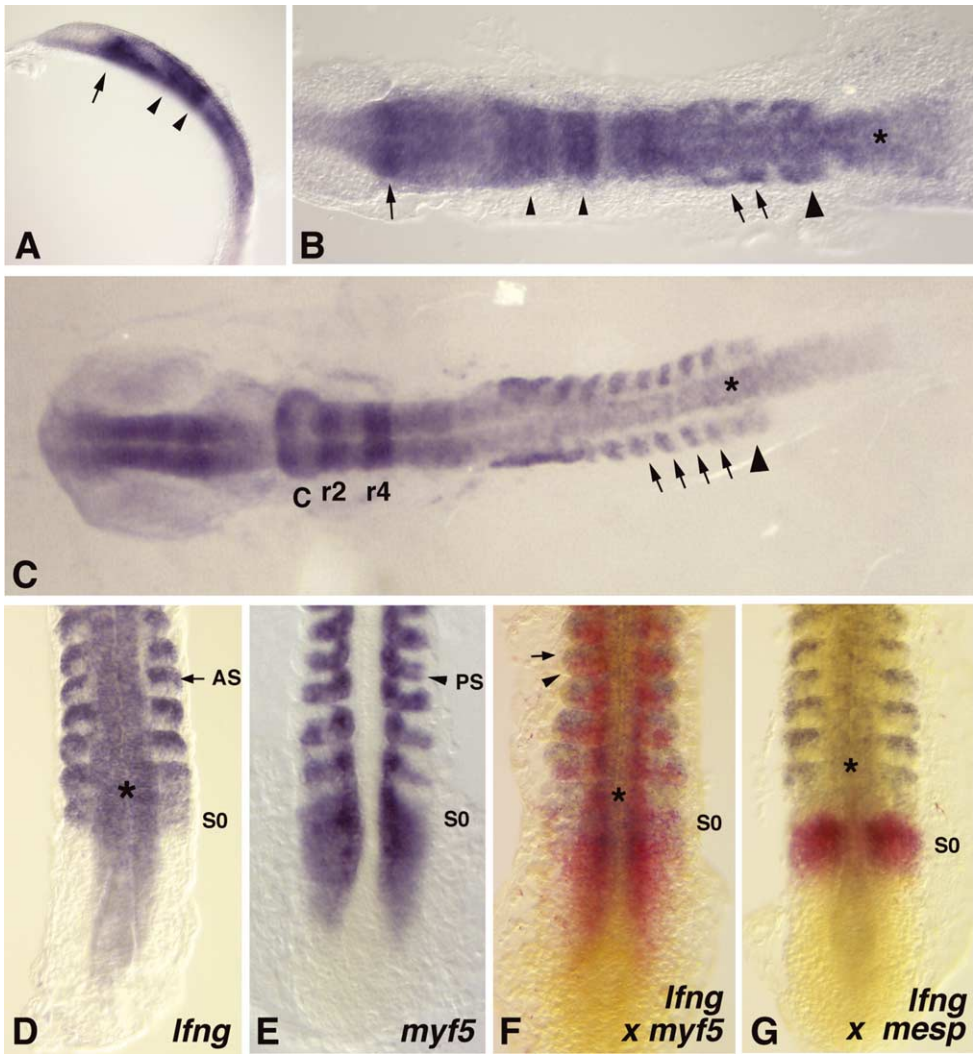

Fig. 1. Expression of *lunatic fringe* (*lfng*) during medaka embryogenesis. Lateral (A) and dorsal (B) views of an embryo at the 2-3 somite stage. Elevated levels of *lfng* expression at the fore-midbrain boundary (arrows) and in the hindbrain (arrowheads) are indicated. In B, *lfng* expression in the somites (right arrows) and the anterior presomitic mesoderm (PSM; large arrowhead) is highlighted. (C), Dorsal view of an embryo at stage 22 (10-somite stage) showing expression in the presumptive cerebellum (c), rhombomeres 2 and 4 (r2, r4), somites (arrows) and anterior PSM (arrowhead). (D–G), Dorsal views of trunk regions at the 10-somite stage showing *lfng* (D, F, G; blue), *myf5* (E, blue; F, red) and *mesp* (G, red) expression. Asterisks indicate *lfng* expression in the neural tube. Anterior is left in A–C and top in D–G.

was also seen at the position of the first forming somites (arrows in Fig. 1B). Later, at stage 22 (38 hpf, 9-10 somites), *lfng* expression persisted in the nervous system with elevated levels in the telencephalon, cerebellum and rhombomeres r2 and r4 (Fig. 1C). At this and subsequent stages, no expression was detected in the intermediate or posterior PSM (Fig. 1C,D). Instead, non-dynamic *lfng* expression was observed at the anterior edge in the PSM at the location where the next somite will form (somitomere S0). Also, strong expression was found in the rostral halves of the epithelialized somites. Two-color in situ hybridization was performed to analyze the antero-posterior (AP) distribution of *lfng* transcripts in formed somites. As reference, *myf5* was used that is expressed in the posterior halve of each somite and in the PSM of zebrafish (Coutelle et al., 2001) and medaka (Fig. 1E). Simultaneous detection of *myf5* and *lfng* revealed that *lfng* is expressed in the anterior halves of already epithelialized somites, directly adjacent to, but not overlapping with the *myf5* domains (Fig. 1F). Comparison with *mesp*, which is expressed in the rostral domain of the next forming somite S0, confirmed that *lfng* is expressed in the anterior-most PSM (Fig. 1G). Therefore, medaka *lfng* is expressed similar to its ortholog in zebrafish (Leve et al., 2001; Prince et al., 2001), but clearly different from those in chick and mouse (Cole et al., 2002; Dale et al., 2003). Most importantly, while *lfng* shows oscillating expression in the PSM of chick and mouse, this is not the case in zebrafish and medaka. This suggests that in fish there is no contribution of oscillating *lfng* to the establishment of feedback loops that set up the molecular oscillator.

As so far there is no *her1* gene identified in the medaka, we next analyzed the expression of medaka *her7* to test whether this gene shows cyclic expression in the PSM comparable to the situation in zebrafish. In zebrafish, *her7* expression is initiated at the caudal end of the PSM and progresses rostrally. In the anterior PSM, this wave is terminated and *her7* expression is stabilized at a position where the next somites will form (Oates and Ho, 2002). At the onset of somitogenesis, a similar pattern of *her7* expression was observed in medaka when compared to zebrafish. During gastrulation at stage 17 (25 hpf), when the first morphologically distinct somites appear, *her7* transcripts are detected around the margin and in the prospective PSM region (Fig. 2A). Therefore, like in zebrafish, *her7* expression is found in the progenitor cells of the paraxial mesoderm before they have started to converge in the tailbud. Already, at this stage, expression in the PSM is highly dynamic and regionally restricted (Fig. 2B–G). A wave-like expression is found that starts to progress towards the anterior edge of the PSM. As the first cycle of somite formation is completed, expression in the anterior domain ceases and a new transcriptional wave is initiated (Fig. 2G). At later somitogenesis stages, medaka *her7* expression is confined to the PSM and cannot be detected in the remaining embryos (Fig. 2H). Expression in the PSM remains dynamic (Fig. 2I–M), but in contrast to zebrafish (Oates and Ho, 2002; Gajewski et al., 2003) is not completely down-regulated in the posterior PSM as its expression extends rostrally. Consequently, medaka *her7* expression does not show a segmental pattern in the posterior PSM, as it does in zebrafish (Fig. 2N; also compare to Oates and Ho, 2002; Gajewski et al., 2003). Instead, medaka *her7* transcripts remain rather uniformly distributed throughout the posterior PSM during all phases of somite formation. There never is more than one discrete *her7* domain dispatched from the posterior domain in medaka, while there are up to two stripes in the zebrafish PSM, depending on the phase of cycling (Fig. 2N). Two-color in situ hybridization was performed to determine the anterior-most extent of the *her7* transcription wave in medaka. For this, *mesp* was used as reference marker. *mesp* genes are expressed in the anterior PSM in zebrafish (Sawada et al., 2000) and medaka in one or two stripes depending on the phase of somite formation (Fig. 2O–T). The anterior-most expression of medaka *her7* was localized adjacent to *mesp* indicating that *her7* is expressed in the posterior domain of the next forming somite. This suggests that the stabilization of anterior *her7* expression occurs at the same anterior PSM level as in zebrafish. Therefore, the additional *her7* stripe observed in the intermediate PSM in zebrafish (Oates and Ho, 2002; Gajewski et al., 2003) is missing in medaka. The formation of dynamic stripes and interstripes is an important feature of the Delta/Notch oscillator model in zebrafish. Therefore, the expression of medaka *her7* indicates fundamental differences in the mechanisms driving the propagation of expression waves in the PSM of these fish species. It remains to be tested whether the models proposed for oscillating gene expression in the PSM of zebrafish (see Holley and Takeda, 2002; Lewis, 2003) are also compatible with the observed pattern of *her7* expression in medaka.

Based on these differences, we next wanted to analyze *hey1* as member of a gene family with highly divergent expression and function in different vertebrate species (Fischer et al., 2002; Winkler et al., 2003). *Hey* genes encode a subfamily of Hairy/Enhancer-of-split bHLH transcription factors that are involved in somitogenesis, blood vessel and heart development. They represent mediators of Notch signaling and show broad expression in mice (Leimeister et al., 1999, 2000a) and restricted expression in zebrafish (Winkler et al., 2003). In mouse, all three known *hey* genes, *hey1*, *hey2* and *heyL*, are expressed in the PSM, while *hey1* is the only *hey* gene in zebrafish expressed in somites and PSM. Interestingly, *hey2* shows oscillating expression in the PSM of mice and chick (Leimeister et al., 2000b) and the expression of zebrafish *hey1* is dynamic in the anterior PSM (Winkler et al., 2003). In medaka, *hey1* shows a generally broader expression than in zebrafish. At the onset of somitogenesis (stage 19, 27 hpf, 2-somite stage), expression is found in the posterior halves

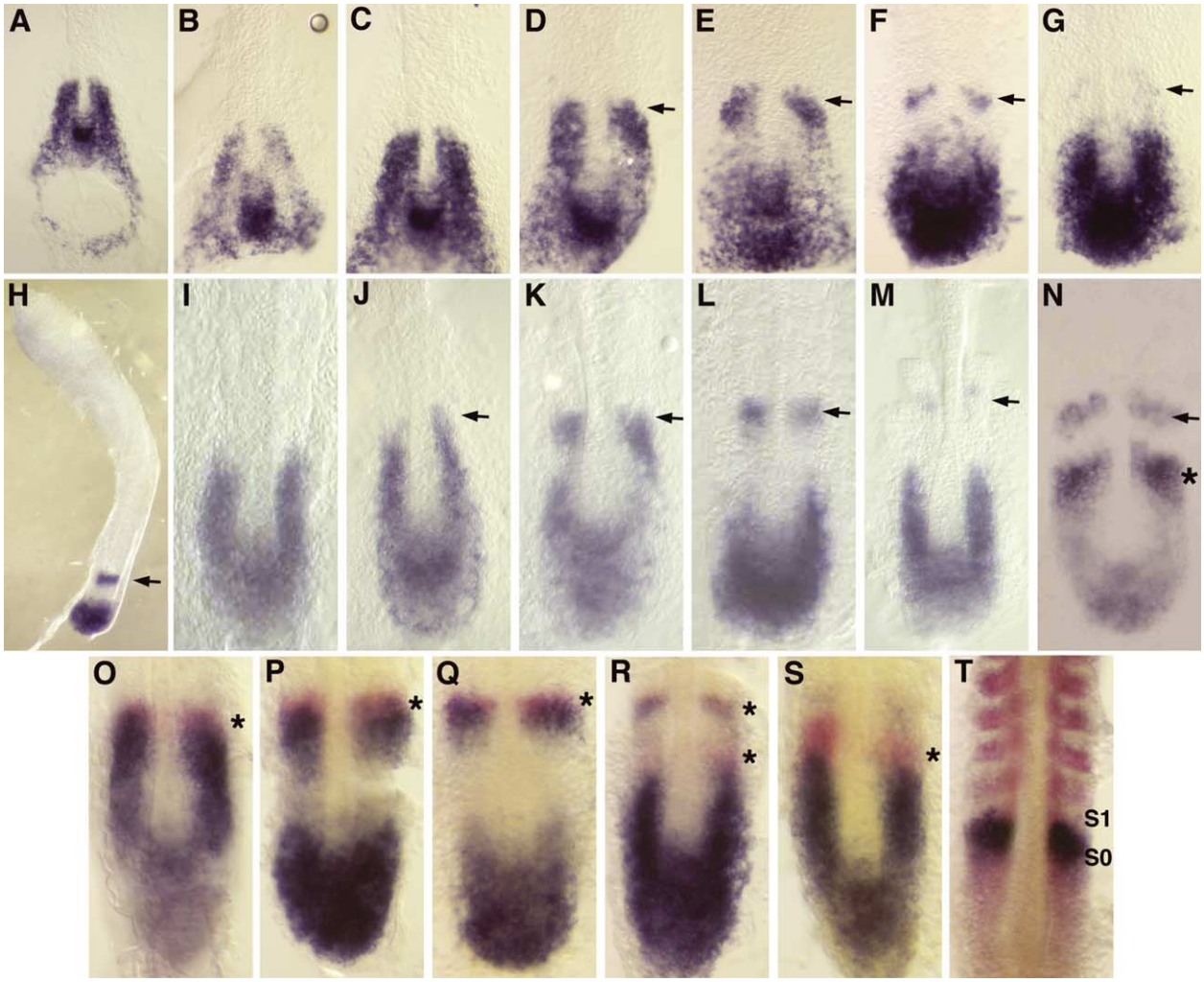

Fig. 2. Expression of *her7* during medaka embryogenesis. (A–G), Onset of expression and dynamic phases of *her7* expression during the 1-somite stage. (H), Lateral view of a medaka embryo at the 9-somite stage showing *her7* expression exclusively in the PSM. (I–M), Dorsal views of medaka tailbud regions showing different phases of *her7* expression at the 9 to 10-somite stage. The anterior-most edge of expression is marked by an arrow. (N), Expression of *her7* in a zebrafish embryo at a stage and phase corresponding to L. (O–S), Different phases of dynamic *her7* expression (in blue) and *mesp* expression (in red; marked by asterisks) in embryos at the 9 to 10-somite stage. Note appearance of two *mesp* stripes at the phase of breakdown of the anterior *her7* domain (R, compare to S). T. Medaka *mesp* (in blue) is expressed in the anterior domain of somitomere S0, as shown by double in situ analysis with *myf5* (in red) in a 10-somite stage embryo.

of the first forming somites (Fig. 3A–C,H). In addition, restricted expression is found in the ventral hindbrain and in the area of the presumptive heart field (arrows in Fig. 3B,C). At the 12-somite stage (stage 23, 41 hpf), *hey1* expression persists in all formed somites and the heart (Fig. 3D). Expression in the head becomes complex and shows elevated levels in the mesencephalon and segmental expression in the hindbrain. At this stage, medaka *hey1* expression is now also found in the dorsal aorta (Fig. 3D,E). After somite formation is completed at stage 32 (4.2 dpf), *hey1* expression in the somites ceases and only remains in the most ventral somite portions (Fig. 3F). At this stage, strong *hey1* expression also persists in the dorsal aorta and is now also found in the mesenchymal cells of the developing pectoral fin (Fig. 3G). To determine dynamic *hey1* expression in the PSM, two-color in situ hybridization was performed with *mesp* (Fig. 3I–L; in red) as a reference to *hey1* (in blue). Interestingly, distinct differences were observed when *hey1* expression was examined in several embryos of the same stage, but different somite phases. In some embryos, expression was found throughout the anterior PSM (Fig. 3I), while others showed a progressive loss of expression in this area (Fig. 3J–L). This suggests a dynamic mode of *hey1* expression in the anterior PSM very similar to that observed in zebrafish (Winkler et al., 2003). On the other hand, *hey1* expression in the developing heart and dorsal aorta marks an important difference between medaka and zebrafish.

In zebrafish, *hey2* but not *hey1* is expressed in dorsal aorta and heart and a mutation in *hey2* leads to the *gridlock* phenotype with defective aortic development (Zhong et al., 2000). At present, no medaka *hey2* sequence has been identified in the available sequence databases. Likewise, PCR with different degenerate primer combinations was

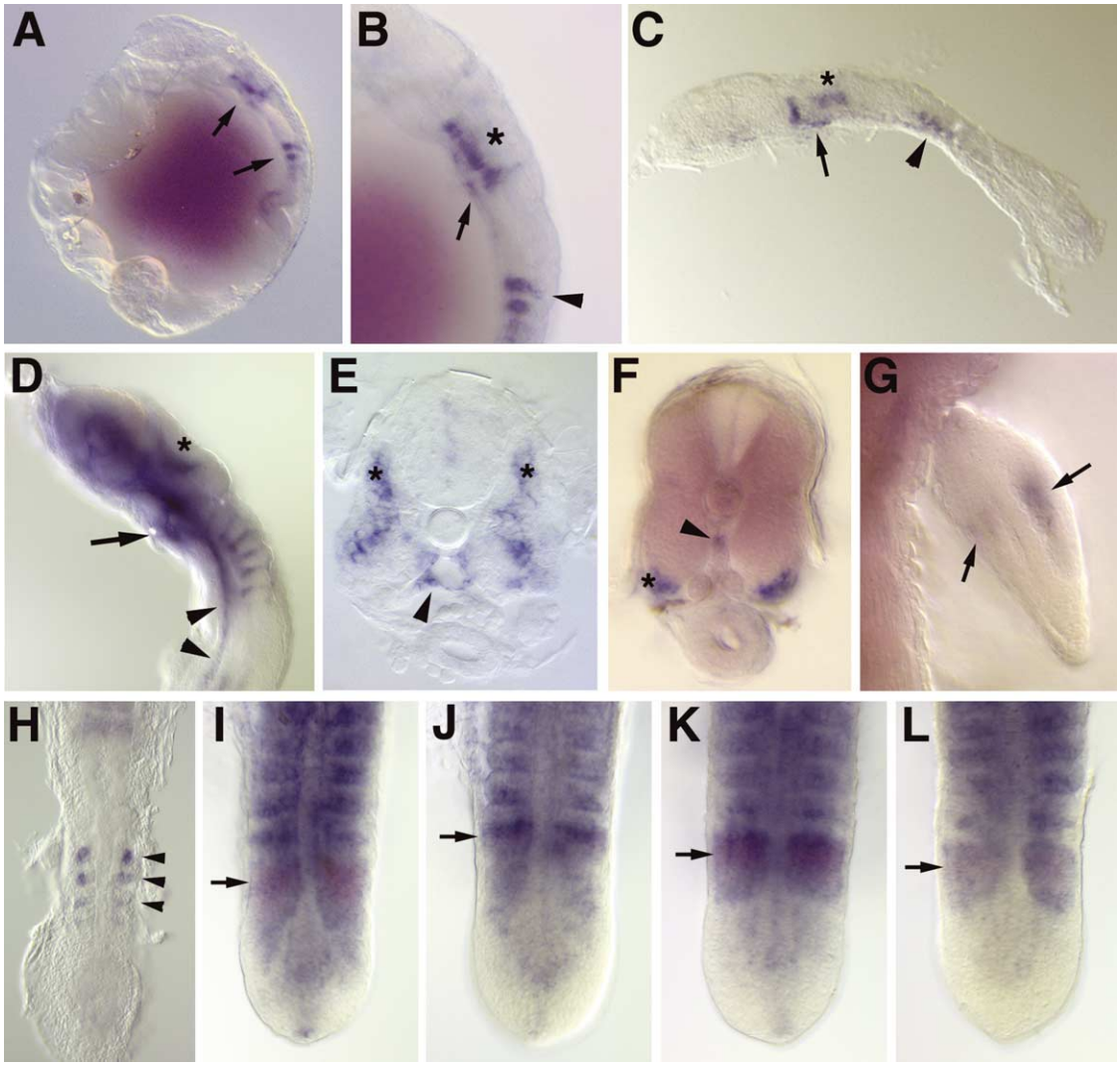

Fig. 3. Expression of *hey1* during medaka embryogenesis. Lateral views of *hey1* expression in embryos at the 2-(A,B) and 4-somite stage (C). Expression in the ventral hindbrain (asterisks in B and C) and forming somites (arrowheads in B and C) is indicated. (D), *hey1* expression in the head region of an embryo at the 12-somite stage. *hey1* shows elevated expression in the midbrain (asterisk), the presumptive heart (arrow) and dorsal aorta (arrowheads). (E) Transversal section at the trunk level through embryo shown in D. Expression is found in the somites (asterisks) and the dorsal aorta (arrowhead) that is located ventral to the notochord. (F), Cross section through the trunk of an embryo at stage 32 reveals *hey1* expression reduced to the ventral portion of the somites (asterisk) and the dorsal aorta (arrowhead). (G), *hey1* expression in the developing pectoral fin at stage 32. (H), Dorsal view of an embryo at the 4-somite stage showing *hey1* expression in the posterior part of the forming somites. (I–L), Expression of *hey1* (in blue) and *mesp* (in red; arrows) during different phases of somite formation in embryos at the 10-somite stage. Note different distributions of *hey1* transcripts in the intermediate PSM.

unsuccessful to identify *hey2*, whereas *hey1* and *heyL* could be amplified under identical conditions. *heyL* is expressed in the ventral neural tube in a very similar pattern in both zebrafish (Winkler et al., 2003) and medaka (data not shown). It will be interesting to see whether *hey2* is present in the medaka genome or, alternatively, has been lost in medaka and possibly other fish species. It is possible that medaka *hey1* has retained all regulatory elements important for expression in heart and aorta, while these were lost in the zebrafish *hey1* gene, but retained in *hey2*. This would imply that the medaka *hey1* gene fulfils functions comparable to those of *hey2* in other species. Analyzing *hey* gene expression in different fish species therefore provides an excellent experimental system to study the divergence of gene regulation and possibly even function during teleost evolution.

In order to exclude that the differences in expression observed between zebrafish and medaka genes were resulting from erroneous comparisons between paralogous genes, we analyzed the sequence databases available for zebrafish (including the third assembly Zv3 covering about 1500 Mb of the zebrafish genome), Fugu and other organisms using the medaka *lnfg*, *her7* and *hey1* nucleic acid and protein sequences as queries (see Section 2). Phylogenetic analysis of retrieved sequences with significant similarities confirmed that the medaka genes

analyzed in this work are the true orthologues of zebrafish *lnfg*, *her7* and *hey1*.

Taken together, our expression analysis of three somitogenesis genes in medaka revealed important differences not only between medaka and higher vertebrates, but also between two related fish species. Analyzing the mechanisms of cyclic gene expression in different vertebrates might contribute to elucidate the regulatory pathways that control oscillating gene expression and thus somite formation.

2. Experimental procedures

2.1. Maintenance of fish

Medaka embryos were produced from matings of wild-type fish of the Carbio strain that are kept as inbred lines in our laboratory for many generations. Embryos were raised at different temperatures (18–30 °C) until the desired stages were obtained. Embryos were staged according to Iwamatsu (1994).

2.2. Preparation of lfng, her7 and hey1 riboprobes

The zebrafish sequences for *lfng* and *her7* were used as query to screen the available medaka databases. Several EST sequences were identified that encode fragments of the putative medaka Lfng and Her7 proteins (*lfng*, accession number BJ011253; *her7*, AJ457226). The following primers were designed to PCR amplify partial medaka *lfng* and *her7* by using cDNA prepared from medaka embryos at 48 hpf as templates: *lfng*-001 (5′-GGACAGGACACCGTATGGAC-3′), *lfng*-002 (5′-GTCGTATTCAACCGCCATCT-3′), *her7*-001(5′-TGCAAGATCAGGATGAATCG-3′) and *her7*-002 (5′-GTCTTTGCTCGCTGCTCTCT-3′). Similarly, an EST encoding a partial sequence of the putative medaka Mesp was identified (accession number BJ012665). The primers used for amplification were *mesp*-001 (5′-GCCATGGAGATGTCCTTCTG-3′) and *mesp*-002 (5′-GCTCTCTGCTGACCTTGGAG-3′). For *myf5*, primers *myf*-up (5′-CTTCCCAAGGTGGAGATCCT-3′) and *myf*-down (5′-GCGTCAGTGGGTATAACAGC-3′) were designed based on an available EST sequence (accession number BJ020021). The amplified fragments were cloned into the pCRII-TOPO vector (Invitrogen) and used for antisense riboprobe preparation. For medaka *hey1*, a partial cDNA of 778 nucleotides was amplified using the degenerate primers *allhey5i* (5′-ACTGGTGCCTACCGCCTTYGAR-AARCARG-3′) and *code-blik3* (5′-GAAGGCGCC-GACCTCNGTNCCCCA-3′). Amplification conditions, cloning and sequencing of PCR products was done as described in Winkler et al., 2003. The *hey1* sequence was submitted to GenBank (accession number AJ621001).

2.3. Analysis of phylogenetic relationships between medaka and zebrafish genes

In order to investigate the phylogenetic relationships between medaka genes and sequences from zebrafish and other organisms, public sequence databases including the zebrafish whole genome sequencing trace database and the third assembly Zv3 covering about 1500 Mb of the zebrafish genome (http://www.sanger.ac.uk/Projects/D_rerio/; http://danio.mgh.harvard.edu/blast/blast.html; http://www.sanger.ac.uk/Projects/D_rerio/), the Fugu genome assembly release 3 (329 Mb; http://fugu.hgmp.mrc.ac.uk/blast/) and other databases accessible from the NCBI server (http://www.ncbi.nlm.nih.gov/blast/) were searched by blast analysis (Altschul et al., 1990) using the medaka nucleic and protein sequences as queries. Sequences with significant similarities were aligned using PileUp of the GCG Wisconsin package (Version 10.3, Accelrys Inc., San Diego, CA). Phylogenetic bootstrap analyses were performed using different methods (neighbor-joining, maximum parsimony, maximum likelihood) as implemented in PAUP* (Swofford DL, Sinauer Associates, Sunderland, Massachusetts). All relationships of orthology between medaka and zebrafish sequences were supported by the different methods with bootstrap values higher than 80.

2.4. RNA in situ hybridizations

One and two-color RNA whole mount in situ hybridization were performed as described (Winkler et al., 2003), except for embryos shown in Fig. 3I–L that were incubated in staining solution for two days at room temperature. Stained embryos were dissected from the yolk and flat-mounted in glycerol. Embryos were photographed as whole-mounts or after sectioning them manually with a razor blade.

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