Two zebrafish Notch-dependent *hairy/Enhancer-of-split-related* genes, *her6* and *her4*, are required to maintain the coordination of cyclic gene expression in the presomitic mesoderm

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Summary

Alterations of the Delta/Notch signalling pathway cause multiple morphogenetic abnormalities in somitogenesis, including defects in intersomitic boundary formation and failure in maintenance of somite regularity. Notch signalling has been implicated in establishing the anteroposterior polarity within maturing somites and in regulating the activity of a molecular segmentation clock operating in the presomitic mesoderm. The pleiotropy of Notch signalling obscures the roles of this pathway in different steps of somitogenesis. One possibility is that distinct Notch effectors mediate different aspects of Notch signalling. In this study, we focus on two zebrafish Notch-dependent hairy/Enhancer-of-split-related transcription factors, Her6 and Her4, which are expressed at the transition zone between presomitic mesoderm and the segmented somites. The results of overexpression/gain-of-function and of morpholino-mediated loss-of-function experiments show that Her6 and Her4 are Notch signalling effectors that feedback on the clock and take part in the maintenance of cyclic gene expression coordination among adjacent cells in the presomitic mesoderm.

Key words: Zebrafish, Somites, Notch, Segmentation clock, *her6*, *her4*

Introduction

Somitogenesis is coordinated by a 'clock' mechanism that ensures the correct spatiotemporal periodicity of somite generation (Maroto and Pourquié, 2001). Although the nature of the clock is still unknown, its existence is revealed by genes expressed in a cyclic fashion within the presomitic mesoderm (PSM). These genes [*hairy1*, *hairy2*, *Hey2* and *lunatic fringe* in the chicken (Palmeirim et al., 1997; McGrew et al., 1998; Jouve et al., 2000; Leimeister et al., 2000); *Hes1*, *Hes7*, *Hey2*, *Lfng* and *Axin2* in the mouse (Forsberg et al., 1998; Jouve et al., 2000; Leimeister et al., 2000; Bessho et al., 2001, Aulehla et al., 2003); *her1*, *her7* and *deltaC* in zebrafish (Holley et al., 2000; Jiang et al., 2000; Sawada et al., 2000; Oates and Ho, 2002)] are expressed in a dynamic pattern of stripes which sweep across the PSM in a caudal to rostral direction, progressively slow down and become finally restricted to the anterior (A) or posterior (P) compartment of each mature somite. The progression of these stripes reflects the periodic switching on and off of genes in PSM cells (Palmeirim et al., 1997; Jiang et al., 2000). Synchronisation of the expression cycles among neighbouring cells is crucial for generating a pattern of kinematic stripes with a periodicity corresponding to the time of formation of one somite.

With the exception of *Axin2*, all the cyclically expressed genes so far identified are components of the Delta/Notch pathway. Somitogenesis is defective in animals in which this pathway is disrupted by either activating or inactivating mutations (reviewed by Pourquié, 2000; Pourquié, 2001). Analysis of Notch pathway mutants is complicated by the pleiotropy of Notch signalling which is required at different steps of the somitogenetic process. The basic helix-loop-helix (bHLH) protein Mesp2 is required in a Notch-dependent fashion to establish the identity of anterior and posterior compartments of the maturing somites in mouse (Takahashi et al., 2000). A role in implementing boundary formation has been attributed to Notch and its modulator *lunatic fringe* in the chick (Sato et al., 2002). Notch signalling is also linked to the segmentation clock although the exact nature of this relationship is not yet understood. Several observations suggest that Notch signalling is a central component of the oscillatory mechanism: (1) the cycling expression of mouse *Hes1* depends on the Notch ligand *Dll1* (Jouve et al., 2000); (2) the activity of the Notch-dependent factors Her1, Her7, Hes1 and Hes7 is required for their own cyclic expression (Hirata et al., 2002; Holley et al., 2002; Oates and Ho, 2002; Bessho et al., 2003); (3) the oscillatory expression pattern of *lunatic fringe*, required for somite segmentation, is under the direct transcriptional control of Notch signalling and is lost in embryos lacking *Dll1* or *Hes7* (del Barco Barrantes et al., 1999; Bessho et al., 2001; Cole et al., 2002; Morales et al., 2002; Dale et al., 2003; Serth et al., 2003). Alternatively, it has been proposed that the main function of Notch signalling is to maintain the synchronisation of cyclic gene expression in the PSM (Jiang et al., 2000). This

hypothesis is based on the observation that in zebrafish that are mutant for Notch pathway components, the dynamic stripes of *deltaC* expression are replaced by a single broad stripe in which cells express *deltaC* at variable levels, giving rise to a 'salt and pepper' pattern. This phenotype can be explained by assuming that individual PSM cells still express *deltaC* in a cyclic fashion, but the spatiotemporal co-ordination of expression among adjacent cells is lost (Jiang et al., 2000).

Among the best characterised effectors of Notch signalling in vertebrates are the bHLH transcription factors of the Hairy/Enhancer-of-split [E(spl)] family. These are sequence-specific transcriptional repressors whose activity is largely dependent upon their interaction with co-repressors of the Groucho/TLE family (Davis and Turner, 2001). The role of Hairy/E(spl) proteins in somitogenesis is still unclear and investigations have until now focused on family members expressed in a cyclic fashion within the PSM (Takke and Campos-Ortega, 1999; Jouve et al., 2000; Bessho et al., 2001; Bessho et al., 2003; Henry et al., 2002; Oates and Ho, 2002). We have addressed the role of two Notch-dependent zebrafish *hairy/E(spl)*-related genes, *her6 and her4*, which are expressed at the transition zone between PSM and somites. We show that these two genes are necessary for normal paraxial mesoderm segmentation and that the activities of their protein products are required to maintain synchronisation of the cyclical expression of both *deltaC* and *her1*.

Materials and methods

Generation of constructs

The cloning of full length *her6* cDNA has been previously described (Pasini et al., 2001). The open reading frame of *her6* was PCR-amplified with the primers 5′-AA**GGATCCATGGAATTCGAAGATG**CCTGCCGATATCATGG (restriction sites for *Bam*HI, *Nco*I and *Eco*RI, Kozak consensus sequence and Met codon are in bold) and 5′-TTTT**CTCGAGCATATGCTA**CCAAGGCCG (restriction sites for *Nde*I and *Xho*I and stop codon in bold), which introduce restriction sites suitable for subcloning. The PCR product was digested with *Bam*HI and *Xho*I and subcloned into the pCS2+ vector, thus creating the vector pCS2+wt her6.

The construct pCS2+her6-tr was created by PCR-amplifying the *her6* cDNA with the primers 5′-AAGGATCCATGGAATTCGAAGATGCCTGCCGATATCATGG and 5′-TTTT**CTCGAGCATATGCTA**AACGGAGTCTGACGT (restriction sites for *Nde*I and *Xho*I and stop codon in bold). This latter primer introduces an in-frame stop codon immediately upstream of the codons encoding the C-terminal WRPW domain of Her6. The resulting fragment was digested with *Bam*HI and *Xho*I and subcloned into the corresponding sites of the pCS2+ vector.

To generate the construct pCS2+her6-VP16, a plasmid called pCS2+VP16 was first created by subcloning into the *Bam*HI and *Xba*I sites of pCS2+ the VP16 activation domain excised from pUC18-VP16. A 3′-truncated version of the her6 cDNA was amplified with the primers 5′-AAGGATCCATGGAATTCGAAGATGCCTGCCGATATCATG and 5-GCG**GGATCC**AACGGAGTCTGACGT (*Bam*HI site in bold), cut with *Bam*HI and subcloned in frame with the VP16 activation domain into the *Bam*HI site of pCS2+VP16.

PCR amplifications were performed with the high fidelity Pfu polymerase (Promega) and all PCR-generated constructs were sequenced to check for the absence of mutations. All constructs were in vitro translated with the TNT Sp6-coupled Reticulocyte Lysate System (Promega) to verify that protein products of the correct size were expressed. In addition, the transcriptional activities of the Her6 wild-type and mutant proteins were tested in a luciferase gene reporter assay carried out in HEK293 cells with the luciferase vector pHesLuc (Takebayashi et al., 1994). The luciferase activities were measured using the Luciferase Reporter Gene Assay Kit (Boehringer Mannheim) according to the manufacturer's instructions. The pCS2+wther4 construct was a gift from J.-A. Campos-Ortega.

In vitro mRNA synthesis

All constructs were linearised with *Not*I and capped mRNAs were synthesised with the Sp6 RNA polymerase. mRNAs were resuspended in RNAse-free water (Sigma) at a concentration of 1 mg/ml, aliquoted and stored at –80°C. Immediately prior to injection, synthetic mRNAs were diluted in RNAse-free water containing 1 μg/μl 2,000,000 M_r lysinated fluorescein dextran (Molecular Probes). Owing to its very high molecular weight, this polymer does not leak through the cytoplasmic bridges or into the yolk and is therefore suitable for lineage tracing in the zebrafish (Strehlow and Gilbert, 1993).

Morpholinos

The following morpholino-modified antisense oligonucleotides (GeneTools) were used in this study:

her6MO, 5′-TATCGGCAGGCATCTTCTCTGGGAA-3′;
her4MO, 5′-TTGATCCAGTGATTGTAGGAGTCAT-3′;
her9MO, 5′-GCTCGCTGGCATTCTTTGGCTTGTC-3′.

Stock solutions of morpholinos (1 mM) were made in RNAse-free water (Sigma) or in 1×Danieau's solution. Working dilutions were made in 1×Danieau's solution.

The efficiency and specificity of morpholinos were determined on the basis of their ability to block mRNA transcription in the TnT Coupled Reticulocyte Lysate System (Promega).

Embryo production and microinjection

Zebrafish embryos obtained by natural spawning were staged according to Kimmel et al. (Kimmel et al., 1995). For injections of mRNA, a volume of about 1-2 nl was injected with a Picospritzer into the cytoplasm of one cell at the two-cell stage. Morpholinos were injected into the yolk of one-cell to four-cell stage embryos. Embryos were allowed to develop to the desired stage, screened for developmental abnormalities under an Axiophot microscope and photographed alive or fixed overnight in 4% PFA and processed for whole-mount in situ hybridisation.

In situ hybridisation

Whole-mount in situ hybridisations were carried out as described by Oxtoby and Jowett (Oxtoby and Jowett, 1993). Two-colour whole-mount in situ hybridisation with digoxigenin- and fluorescein-labelled probes were performed according to the protocol of Hauptmann and Gerster (Hauptmann and Gerster, 1994). When lysinated fluorescein dextran was to added the injected mRNAs as a lineage tracer, it was detected with an anti-fluorescein antibody coupled to alkaline phosphatase, using either Fast Red or INT-BCIP (Roche) as chromogenic substrates.

Results

Expression of *her6* in the paraxial mesoderm

Weak expression of *her6* in the blastoderm margin becomes detectable by *in situ* hybridisation at about 40-50% epiboly and is maintained throughout the epiboly process (Fig. 1A-C), but no expression is observed in the lateral hypoblast. From the end of epiboly to five-somite stage (ss), *her6* is expressed in the tailbud and, starting at about 3 ss, in the posterior lateral plate mesoderm (Fig. 1D-F). Paraxial mesoderm expression becomes evident during somitogenesis, when *her6* transcripts mark the posterior region of segmented somites (Fig. 1E-H) (Pasini et al., 2001). Expression levels decrease during somite

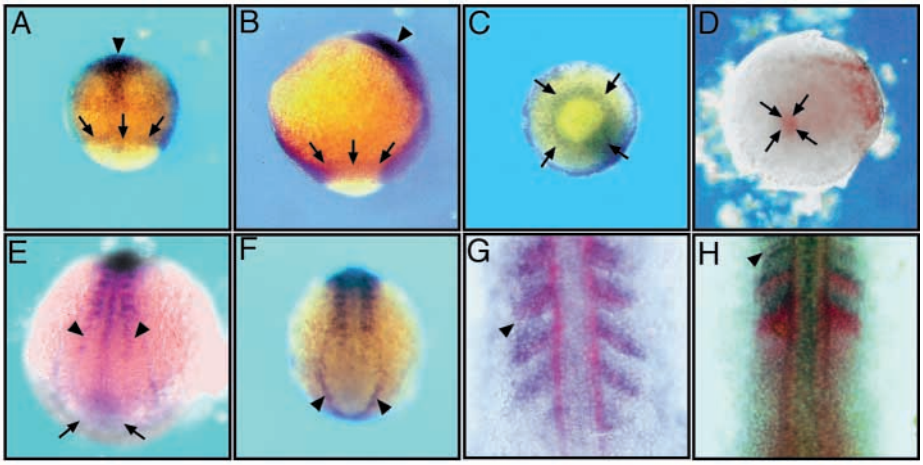

Fig. 1. (A-C) *her6* is weakly expressed by cells in the blastoderm margin (arrows) throughout epiboly. (A) Dorsal view of a 70% epiboly embryo. (B,C) Lateral (B) and vegetal (C) pole view of a 90% epiboly embryo. In A and B, anterior is towards the top; in B, dorsal is rightwards. In A and B, the arrowhead indicates *her6* expression in the anterior epiblast. (D-F) After the end of epiboly, *her6* is expressed in the tailbud until about the five-somite stage (arrows in D,E), in the posterior compartment of all somites and in two stripes in the anterior PSM. (D) Vegetal pole view of a two-somite stage embryo, dorsal is rightwards. (E,F) Dorsal views of a four-somite stage (E) and a 10-somite stage (F) embryos. Arrowheads indicate *her6* expression in the margins of the neural plate (E) and in the posterior lateral mesoderm (F). (G,H) *her6* expression identifies the posterior compartment of the forming somite (S0) and of the first prospective somite (S-1). *her6* expression (dark blue staining in G and H) overlaps with the expression of the prospective posterior compartment marker, *myod* (red staining in G), and intercalates with the expression of the prospective anterior compartment marker, *papc* (red staining in H).

maturation and by the 10 ss *her6* is downregulated in the older anterior somites, while persisting in posterior, newly formed somites. Two pairs of bilateral stripes of *her6* expression are present in the anteriormost PSM (Fig. 1G,H) (Pasini et al., 2001), whereas no expression is detected in the posterior PSM or, after 5 ss, in the tailbud. To define the location of the *her6*-expressing domains in the PSM, we exploited the two markers *myod* and *paraxial protocadherin* (*papc*; *pcdh8* – Zebrafish Information Network), which label respectively the posterior and the anterior compartments of the forming (S0) and first prospective (S-1) pair of somites (Weinberg et al., 1996; Yamamoto et al., 1998). The *her6* stripes in the PSM overlap with the *myod* stripes and intercalate with the *papc* stripes, indicating that expression of *her6* in the anterior PSM occurs in the posterior compartment of somites S0 and S-1 (Fig. 1G,H). A measurement of the distance separating the two PSM stripes of *her6* expression from each other and from the last formed intersomitic cleft failed to uncover any oscillatory pattern of *her6* expression in the anterior PSM (data not shown). Although we cannot rule out that this is due to a failure to detect very weak waves of *her6* expression in the posterior and intermediate PSM, another zebrafish homologue of cycling mammalian and avian genes, *lfng*, also does not show a clear oscillatory behaviour (Prince et al., 2001).

The expression of *her6* in the paraxial mesoderm is under the control of the Notch pathway

To investigate the dependence of *her6* expression upon the Notch pathway, we expressed in zebrafish embryos either a dominant-negative [Su(H)-DBM] or a constitutively active [Su(H)-Ank] form of the *Xenopus* Notch effector, Suppressor of Hairless 1 [X-Su(H)1]. Both mutant proteins disrupt paraxial mesoderm segmentation in frog and zebrafish and exert opposite effects on the expression of the two *hairy/E(spl)* related genes, *ESR-4* and *ESR-5* (Jen et al., 1999; Henry et al., 2001). In Su(H)-DBM-injected embryos, *her6* expression is suppressed or strongly downregulated in the anterior PSM and in the somites (Fig. 2A). By contrast, in embryos injected with Su(H)-Ank, *her6* is maintained in the anterior paraxial

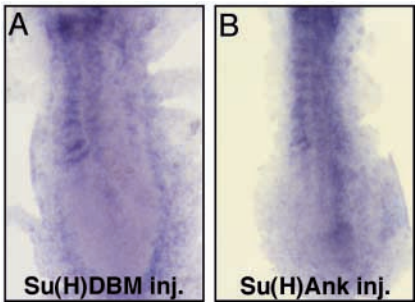

Fig. 2. The expression of *her6* within the paraxial mesoderm is under the control of the Notch pathway. Two-cell stage embryos were injected in one cell with a dominant-blocking (A) or a constitutively active (B) form of the Notch effector Suppressor-of-Hairless [Su(H)] and analysed at 6- to 10-somite stage. In both A and B, the injected side is on the right, the control non-injected side is on the left. Anterior is towards the top. *her6* is ectopically expressed throughout the PSM in embryos injected with the active form Su(H)Ank (B) and downregulated in both the anterior PSM and the segmented paraxial mesoderm in embryos expressing the dominant blocking Su(H)DBM (A).

mesoderm and ectopically expressed throughout the PSM, including its most caudal region (Fig. 2B). Thus, a spatially restricted activation of Notch signalling is required for segmental expression of *her6* in the paraxial mesoderm.

Alterations of Her6 activity and spatially restricted expression disrupt somitic AP polarity

To address the function of *her6* we generated constructs encoding: (1) wild-type Her6 protein (wt-her6); (2) truncated Her6 (her6tr) lacking the last four C-terminal amino acids WRPW, which is required for the interaction with Groucho co-repressors (Fisher et al., 1996) and (3) a fusion protein in which the C-terminal WRPW is replaced by the activation domain of the herpes simplex virus VP16 protein (her6VP16). The constructs were tested in an in vitro luciferase reporter assay. In the absence of bona fide consensus sequences for Her6

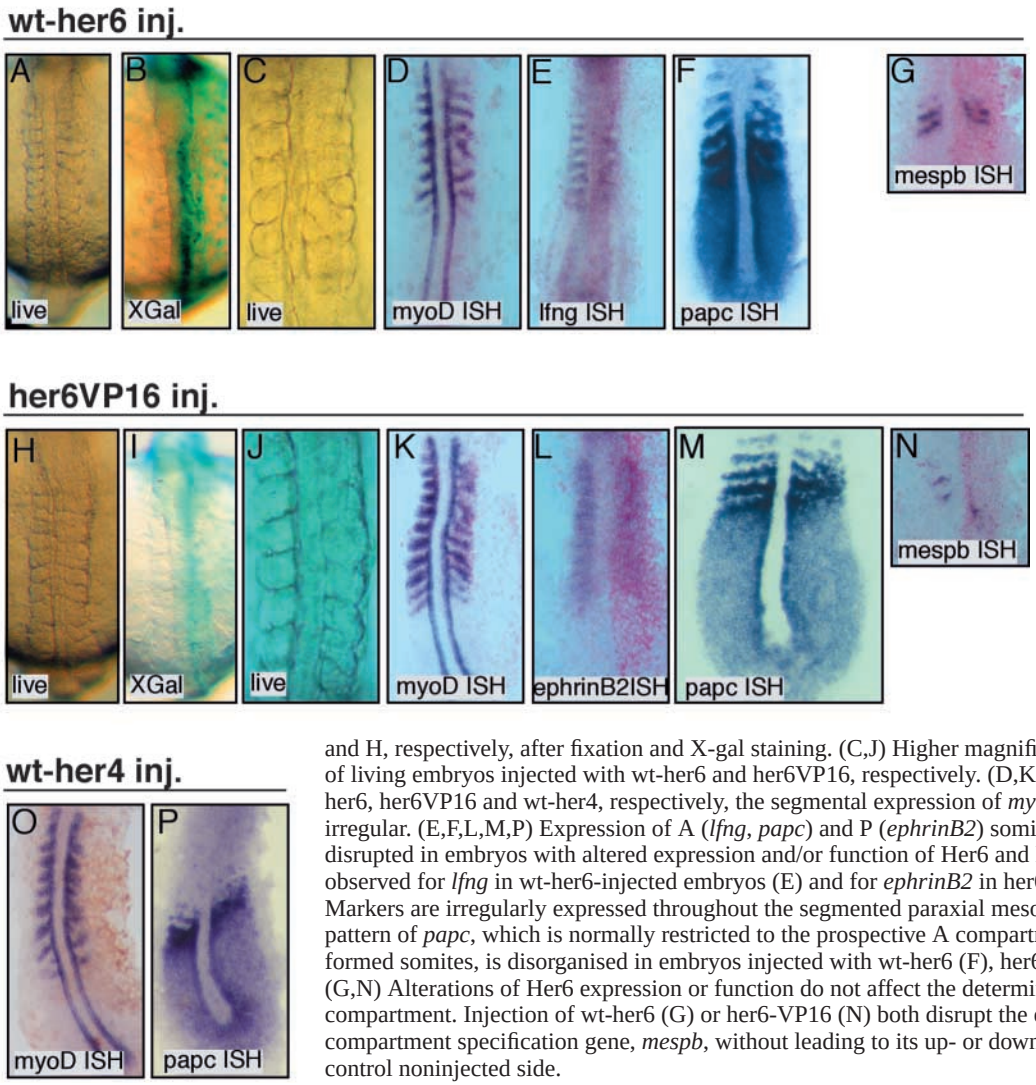

Fig. 3. Alterations of Her6 and Her4 expression disrupt paraxial mesoderm segmentation and AP somitic polarity without affecting the specification of A or P somitic compartments. Dorsal view of whole-mounted (A-C,H-J) or flat-mounted embryos at 7-15 ss. Anterior is towards the top. Two-cell stage embryos were injected in one cell only. In all cases, the injected side is on the right, the control non-injected side on the left. Red (D,E,G,K,L,N) or brown (O) staining marks the tracer co-injected with the various mRNAs. (A-C,H-J) Embryos injected with wt-her6 or her6VP16 mRNAs, have abnormal somites with irregular boundaries. Living embryos injected with wt-her6 (A) or her6VP16 (H) and *lacZ* mRNA as a lineage tracer. (B,I) The same embryos as in A and H, respectively, after fixation and X-gal staining. (C,J) Higher magnification views of the anterior somites of living embryos injected with wt-her6 and her6VP16, respectively. (D,K,O) In embryos injected with wt-her6, her6VP16 and wt-her4, respectively, the segmental expression of *myod* in the paraxial mesoderm is irregular. (E,F,L,M,P) Expression of A (*lfng*, *papc*) and P (*ephrinB2*) somite compartment markers is disrupted in embryos with altered expression and/or function of Her6 and Her4. Identical abnormalities are observed for *lfng* in wt-her6-injected embryos (E) and for *ephrinB2* in her6VP16-injected embryos (L). Markers are irregularly expressed throughout the segmented paraxial mesoderm. The segmental expression pattern of *papc*, which is normally restricted to the prospective A compartment of the forming and newly formed somites, is disorganised in embryos injected with wt-her6 (F), her6-VP16 (M) or wt-her4 (P). (G,N) Alterations of Her6 expression or function do not affect the determination of the A somitic compartment. Injection of wt-her6 (G) or her6-VP16 (N) both disrupt the expression pattern of the A compartment specification gene, *mespb*, without leading to its up- or downregulation compared with the control noninjected side.

binding, we used a plasmid carrying the luciferase gene under the control of the *Hes1* promoter, which contains several N-box consensus sequences for the binding of HES1, the closest mammalian homologue of Her6 (Jouve et al., 2000; Pasini et al., 2001). Expression of wtHer6, Her6VP16 or Her6tr led to a sixfold decrease, a tenfold increase and no change in baseline luciferase activity, respectively (data not shown), demonstrating that Her6 has C-terminal-dependent transcriptional repressor activity and that fusion to an exogenous transcriptional activator generates an antimorph mutant, capable of activating genes normally repressed by Her6.

RNAs coding for wild-type and the two mutant forms of Her6 were injected into cleavage-stage zebrafish embryos, which were allowed to develop until 10-15 ss and visually screened for mesoderm segmentation abnormalities. Embryos were considered affected if at least three ipsilateral somites are irregular in shape and/or size along their AP axis and if at least two consecutive intersomitic boundaries are absent, incomplete or irregular. Embryos severely defective in epiboly, gastrulation or convergent extension were discarded and the concentrations of injected mRNAs were adjusted to minimise such defects. Defects in paraxial mesoderm segmentation occurred in a high proportion (53%; 298/559) of embryos injected with wt-her6 mRNA (40-50 pg/embryo) and less frequently (25%; 55/225) in embryos injected with the same amount of her6tr mRNA. Injection of 40-50 pg her6VP16 mRNA resulted in a high percentage of embryos with impaired gastrulation but after reducing the amounts of injected mRNA to 10 pg/embryo specific somitic defects were observed (37%; 118/321). By contrast, segmentation defects were infrequent (20%; 33/159) in embryos injected with 40-50 pg of mRNA coding for Her9, a zebrafish Her protein highly homologous to Her6 but not expressed in the paraxial mesoderm (Leve et al., 2001) and rare in embryos injected with *lacZ* mRNA (7%; 10/147).

In wt-her6- and her6VP16-injected embryos, the paraxial mesoderm was always segmented, but intersomitic clefts were incomplete or irregular and somites split or fused (Fig. 3A-C,H-J). Differentiation of somitic derivatives appears normal, as shown by hybridisation with *myod*. The segmental restriction of gene expression is impaired, with both markers for the A (*lfng*, *papc*) and P (*myod*, *ephrinb2*) somitic compartments expressed in broad, irregular domains (Fig. 3D-

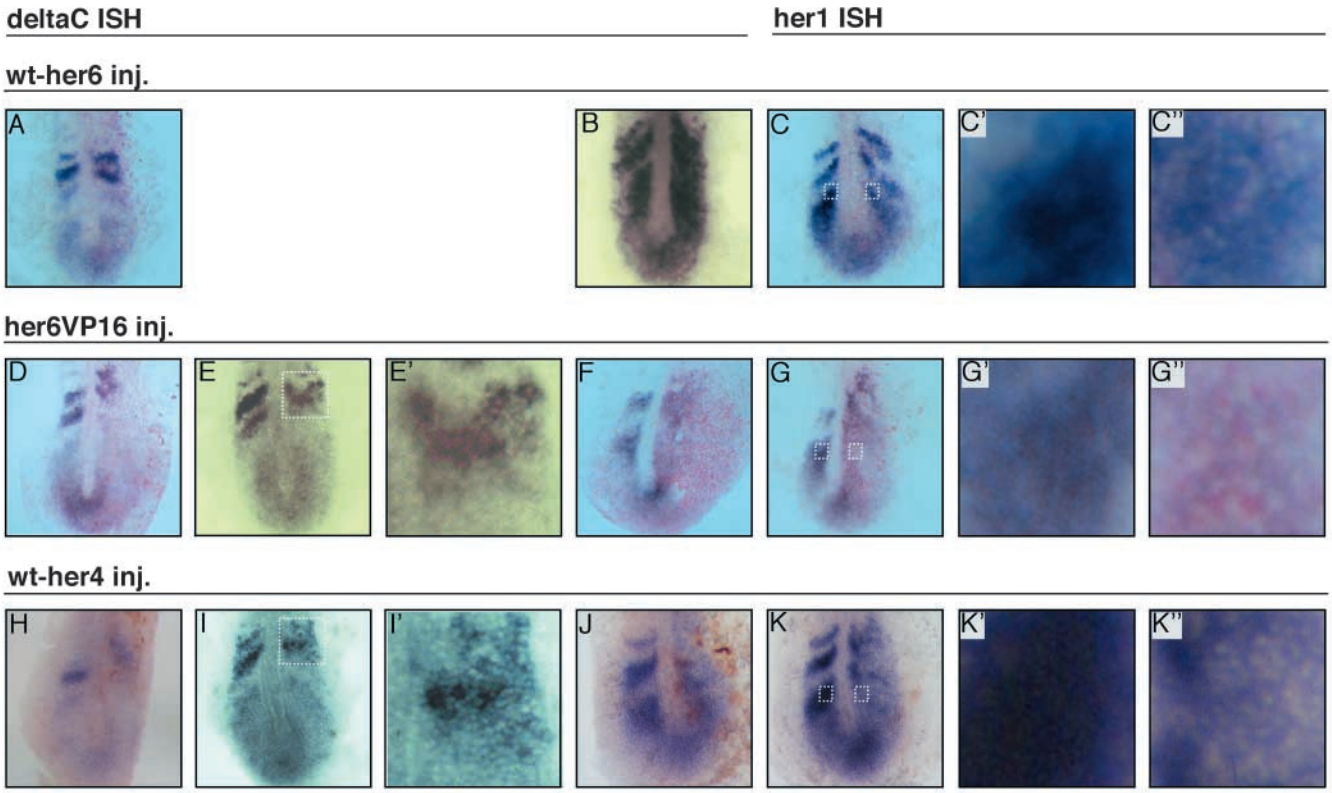

Fig. 4. Alterations of Her6 and Her4 expression and/or activity disrupt the coordinate cyclic expression of *deltaC* and *her1*. Flat-mounted embryos at 10-12 ss. Two-cell stage embryos were injected in one cell only. In all cases, the injected side is on the right. Red (A,C,D,F,G) or brown (H,J,K) staining labels the injected side. The pattern of sharp *deltaC* stripes is disrupted in the anterior PSM of embryos injected with wt-her6 (A), her6-VP16 (D,E) or wt-her4 (H,I). In the anterior PSM of her6-VP16- (E′) and wt-her4- (I′) injected embryos, a random mixture of cells expressing *deltaC* at different levels is found. (E′,I′) Higher magnifications of the boxed regions in E and I, respectively. The sharp stripes of *her1* expression are replaced by a random mixture of *her1*-positive and *her1*-negative cells throughout the PSM of embryos injected with wt-her6 (B-C″), her6-VP16 (F-G″) or wt-her4 (J-K″). (C′,C″,G′,G″,K′,K″) Magnified views of the boxed areas in C, G and K, respectively, to allow a better comparison between injected and noninjected side of the embryos.

F,K-M). In the anterior PSM, segmental expression of *mespb*, which confers anterior somitic compartment identity (Sawada et al., 2000), is disrupted but not downregulated in wt-her6-injected embryos (Fig. 3G) nor expanded in her6VP16-injected embryos (Fig. 3N). Her6 therefore does not appear to play a role in the Notch-dependent establishment of A and P compartment identity. Embryos in which the spatially restricted expression of *her6* or the transcriptional activity of its protein product are altered thus resemble the Notch pathway mutants *beamter* (*bea*), *deadly seven* (*des*), *after eight* (*aei*) and *mind bomb* (*mib*), with abnormal AP somite polarity and irregular intersomitic boundaries (Durbin et al., 2000; Jiang et al., 2000; Gray et al., 2001), rather than the *fused somites* (*fss*) mutant or *mespb*-injected embryos, in which the A and P somite compartments, respectively, do not form and intersomitic boundaries are absent (Durbin et al., 2000; Sawada et al., 2000, Nikaido et al., 2002).

Ectopic expression of Her6 or Her6VP16 disrupts the expression of cycling genes in the PSM

As injections of mRNAs coding for Her6 or Her6VP16 result in irregular intersomitic boundary formation and loss of somitic AP polarity, without affecting the specification of compartment identity, we asked whether Her6 could be involved in earlier Notch-dependent steps of somitogenesis, by regulating the expression of the cycling genes *deltaC* and *her1* in the PSM. *deltaC* is not significantly down- or upregulated in the PSM of embryos injected with wt-her6 or her6VP16 but its expression pattern is severely disrupted and a correct pattern of bilateral sharp bands fails to establish (Fig. 4A,D-E′). Defects in *deltaC* expression pattern are stronger in her6VP16-injected embryos, in which a single broad and irregular stripe is observed in the anteriormost PSM (Fig. 4D-E′), than in wt-her6-injected embryos which have distorted or fuzzy anterior stripes (Fig. 4A). The injection of wt-her6 or her6VP16 mRNAs also affects the expression pattern of *her1* whose stripes of expression become blurred throughout the PSM (Fig. 4B,C,F,G).

Ectopic expression of another Her-type factor, Her4, elicits defects similar to those due to alterations of Her6 function

Redundancy and cooperation between zebrafish Her genes co-expressed during somitogenesis have been reported (Henry et al., 2002; Oates and Ho, 2002). Besides *her6*, another zebrafish Notch-dependent *her* gene, *her4*, is expressed in the tailbud and in the anterior PSM in two bilateral stripes corresponding to S0 and S-1 (Takke et al., 1999; Takke and Campos-Ortega,

1999). We compared the effects of Her4 and Her6 or Her6VP16 overexpression on somitogenesis.

Injection of 40-50 pg/embryo of RNA encoding wild-type Her4 resulted in a higher percentage of embryos with specific paraxial mesoderm segmentation defects (74%; 107/145) than injection of the same amount of wt-her6 (48%; 55/114). However, the morphological somite abnormalities and the expression patterns of the AP somitic polarity markers *myod* and *papc* were not different in embryos injected with wt-her4, wt-her6 or her6VP16 (Fig. 3D,F,K,M,O,P). As with injections of wt-her6 or her6VP16 mRNAs, overexpression of Her4 also led to disruptions of the periodical pattern of expression of the *deltaC* and *her1* genes, without affecting their expression levels in the PSM (Fig. 4H-K).

The similarity of the defects observed after disrupting the spatially restricted expression pattern and activity of Her6 and Her4 suggests that the two factors may act in the same step of the somitogenetic process. It is notable that, in embryos injected with her6VP16 or wt-her4, and to lesser extent in those injected with wt-her6, the domains of expression of *deltaC* and *her1* often consist of a mixture of cells with different levels of expression (Fig. 4C-C″,E′,G-G″,I′,K-K″) reminiscent of that observed in Notch pathway mutants (Jiang et al., 2000).

Antisense morpholino oligonucleotides against *her6* and *her4* disrupt posterior somitogenesis

To test whether Her6 and Her4 are required for somitogenesis, separately or in combination, we designed antisense morpholino oligonucleotides (MO) to specifically block translation of their mRNAs. In the absence of antibodies against the Her6 and Her4 proteins, the efficiency and specificity of the two MOs were tested in an in vitro translation assay, where 1 μM her4MO was sufficient to block the translation of *her4* mRNA without affecting that of *her6* mRNA, whereas 1 μM her6MO led to a substantial decrease of Her6 synthesis while leaving the levels of Her4 unchanged (Fig. 5A). Embryos were then injected with various amounts of either her6MO or her4MO and stained with an anti-myosin heavy chain antibody to highlight the morphology of somites and intersomitic boundaries. Injections of more than 6 ng/embryo of either her6MO or her4MO result in embryos with arrested epiboly (data not show). We could not determine whether this phenotype reflects nonspecific toxicity or an early role of *her6* and *her4*. However, in all the subsequent experiments, a maximum of 6 ngMO/embryo was injected and embryos showing early defects were discarded. Both Her6 and Her4 knockdowns result in abnormal CNS and endodermal development, and embryos injected with 6 ng of her6MO or her4MO often have a bent notochord. In her6MO-injected embryos the first somites form normally, but during somitogenesis the newly-formed somites and intersomitic boundaries become progressively more irregular (Fig. 5C-C‴,D,D′). This phenotype is much less severe in embryos injected with her4MO, in which only the last 5 to 7 somites are affected (Fig. 5E,F,F′), and is only rarely observed in embryos injected with a MO against *her9* (data not shown). As shown in Table 1, the penetrance of the intersomitic boundaries disruption phenotype depends on three factors: (1) it is dose-dependent; (2) at equal doses it is higher in her6MO-injected embryos than her4MO-injected embryos; and (3) within the same batch of her6MO- or her4MO-injected embryos, it increases with time. When 3 ng each of her6MO and her4MO are injected together, both the penetrance and the severity of the phenotype increase cooperatively (Fig. 5G-G″ and Table 1). In all the injected embryos, the orderly stacking of myosin fibrils was disrupted even in somites delimited by normal boundaries, possibly reflecting a role for Her6 and Her4 in refining the internal somitic organisation. This phenotype became aggravated in the more posterior somites, where myofibrils often cross the defective boundaries (Fig. 5C″,C‴,F′) and was particularly severe in her6MO+her4MO-injected embryos, with fibrils spanning the length of several somites (Fig. 5G′,G″). Counting the number of normal somites in injected embryos at 48 hour stage shows that the onset of boundary disruption occurs on average between somites 20 and 22 in embryos injected with her6MO, between somites 23 and 25 in embryos injected with her4MO and around somite 11 in embryos coinjected with her6+her4MO (Table 1).

Table 1. Quantification of the effect of morpholino-mediated depletion of Her6 and/or Her4

MO Treatment	Percentage of embryos with visible intersomitic boundary abnormalities (*n*)		Somite number where boundary abnormalities start
	Time		
	20 hours	48 hours	
her6 (3 ng, *n*=44)	14 (6)	75 (33)	22±0.5
her6 (6 ng, *n*=46)	74 (34)	93 (43)	20±0.5
her4 (3 ng, *n*= 48)	2 (1)	29 (14)	25±0.6
her4 (6 ng, *n*=45)	27 (12)	71 (32)	23±0.5
her6 + her4 (3 + 3 ng, *n*=30)	76 (23)	100 (30)	11±1

n indicates the total number of embryos analysed for each condition. Each batch of injected embryos was analysed at two different time points during development (20 hours and 48 hours). At 48 hours, the embryos were processed for immunohystochemistry with an anti-myosin heavy chain antibody and the number of normally formed somites was counted. A somite was considered normal if both the anterior and the posterior boundaries were complete. Numbers represent the mean±s.e.m.

The segmental expression of *her6* is independent of Her6 function

Direct transcriptional autorepression of the mammalian *Hes1* and *Hes7* genes via binding of their protein products to multiple E- and N-box elements in the *Hes1* and *Hes7* promoters has been demonstrated in vitro (Takebayashi et al., 1993; Bessho et al., 2003) and provides the basis for their oscillatory pattern of expression in vitro and in vivo (Hirata et al., 2002; Bessho et al., 2003). It has been suggested that an analogous mechanism is responsible for the pattern of dynamic stripes of *her1* and *her7* expression in the PSM (Holley et al., 2002; Oates and Ho, 2002). We asked whether a negative autoregulation phenomenon could account for the segmental expression of *her6* in the anterior PSM. We found that injection of 6 ng/embryo of her6MO leads to a stronger *her6* signal, without disrupting its segmental pattern, in the somites and anterior PSM (Fig. 5H-H″,I-I″). The *her6* signal is also increased in the lateral mesoderm, the notochord, the ventral neural tube and, remarkably, in the epidermal ectoderm and at the boundary between epidermal and neural ectoderm, two territories that do not express *her6* at detectable levels in control embryos. No

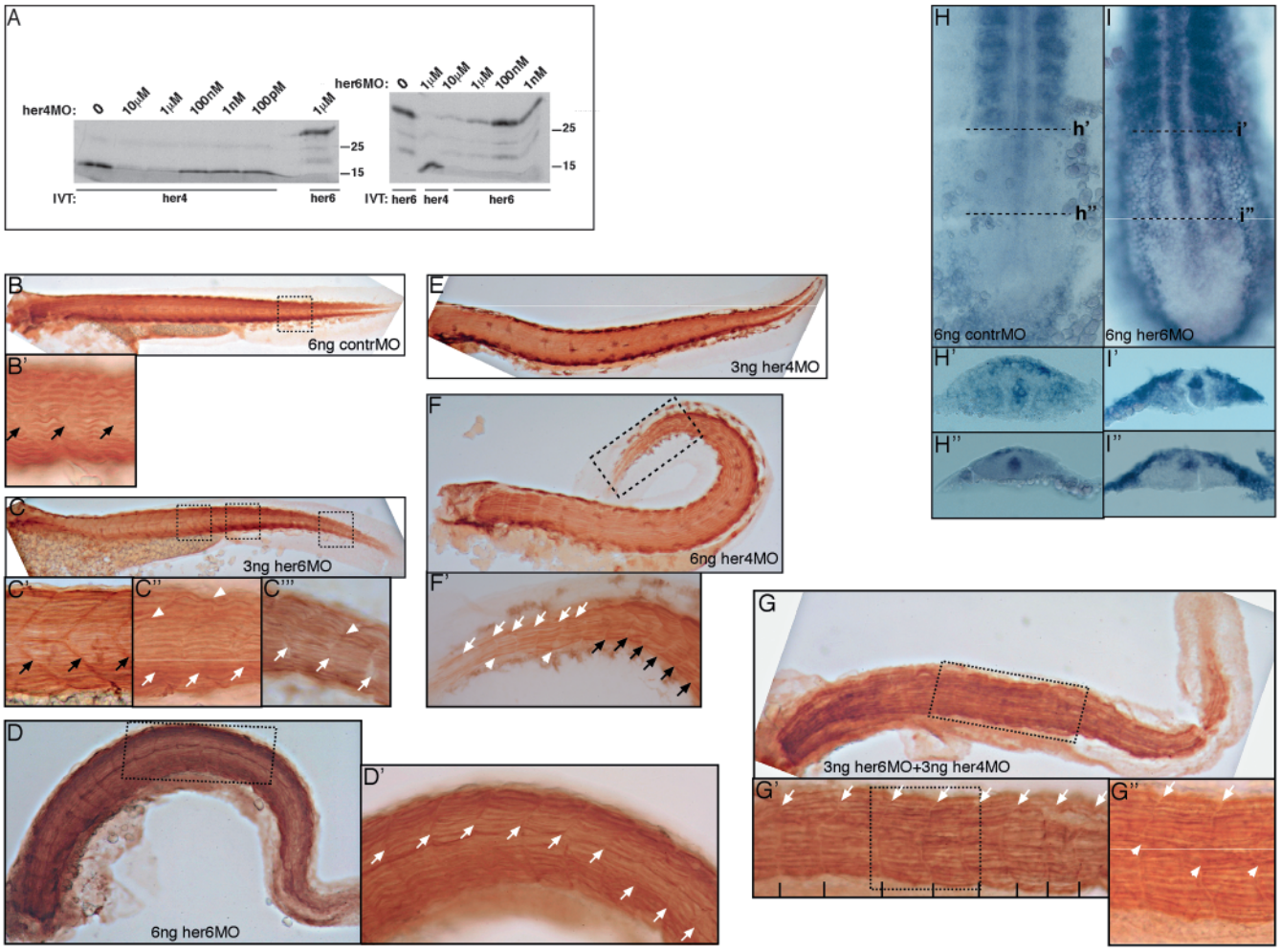

Fig. 5. (A) her4MO (1 μM) is sufficient to suppress Her4 synthesis in a radioactive in vitro translation assay without blocking her6 mRNA translation (left panel). her6MO (1 μM) reduces Her6 synthesis without affecting Her4 (right panel). (B-G′) Somitogenesis is disrupted in Her6, Her4- and Her6+Her4-depleted embryos. Forty-eight hour embryos stained with anti-myosin heavy chain antibody. Anterior is towards the left. (B-G) Low-magnification images to compare the general morphology of control and experimental embryos. Details of somite and intersomitic boundary morphology are shown in the corresponding high-magnification images. Black arrows indicate complete intersomitic boundaries, white arrows indicate irregular and/or defective intersomitic boundaries, white arrowheads mark myosin fibrils crossing intersomitic boundaries. (B) Control embryo. (B′) High magnification of the boxed area in B. (C-C‴) In 3 ng her6MO-injected embryos, somitogenesis is initiated normally but somites and intersomitic boundaries become progressively more irregular. (C′,C″,C‴) High magnification of the anterior, intermediate and posterior boxed areas in C, respectively. (E) her4MO-injected (3 ng) embryos develop normally and only the tail tip is affected. (D,F) The effect of her6MO and her4MO on somitogenesis is dose dependent. her6MO- (D) and her4MO- (F) (6 ng) injected embryos have a bent body axis and the onset of somitic abnormalities is shifted rostrally. D′,F′ are magnified views of the boxed areas in D and F. In D′, somites as anterior as number 16 have ill-defined boundaries. In F′, the last normal intersomitic boundary occurs at somite 25. (G,G′) Somitogenesis defects occur earlier in embryos co-injected with 3 ng each her6MO and her4MO than in embryos injected with 6 ng of either her6MO or her4MO. (G′) High magnification of the boxed area in G: intersomitic boundaries are ill-defined (white arrows) and irregularly spaced (black ruler at the bottom of the picture). (G″) A high-magnification view of the boxed area in (G′): large myosin fibrils span several somites. The expression pattern of *her6* in the PSM is not dependent on the function of the Her6 protein. 10 ss control (H) or her6MO-injected (I) embryos probed for the expression of *her6*. The levels of *her6* transcript are higher in the somites, anterior PSM, notochord/ventral neural tube and ectoderm of her6MO-injected embryos (I) than in the corresponding tissues of control embryos (H). The segmental expression of *her6* in the somites and the anterior PSM is not affected. (H′,H″,I′,I′) sections of the embryos in H and I cut along the broken lines marked by the corresponding small letters. The *her6* signal is stronger in the anterior PSM of her6MO-injected embryos than in control ones, but no accumulation of *her6* transcript is detected in the intermediate/posterior PSM.

expression of *her6* is detected in the intermediate or posterior PSM of her6MO-injected embryos. In the absence of sequence information about the promoter region of *her6*, our data are insufficient to prove the existence of a direct *her6* autoinibitory loop. The stronger *her6* signal in her6MO-injected embryos could be due to a morpholino-induced increased stability of *her6* mRNA (Oates and Ho, 2002). However, three conclusions can be drawn from this experiment. First, the segmental pattern of expression of *her6* in the somites and in the anterior PSM does not depend on the activity of the Her6 protein. Second,

her6 is expressed at very low levels in the epidermal ectoderm overlying the PSM. Third, the finding that *her6* is not detected in the intermediate/posterior PSM of her6MO-injected embryos argues against the existence of waves of low-level *her6* expression in the PSM.

A reduction of Her6 function disrupts *notch1a/des* expression in the anterior PSM

To determine if a decrease in Her6 activity affects the expression of other Notch pathway components in the somites and anteriormost PSM, embryos injected with 6 ng her6MO were probed for *notch1a/des* (Bierkamp and Campos-Ortega, 1993), *notch1b*, *notch5*, *notch6* (Westin and Lardelli, 1997) or *lfng* (Prince et al., 2001) expression at the 10-15 ss. We analysed the embryos at a stage preceding the onset of morphologically recognisable her6MO-induced phenotype in order to identify early alterations of expression patterns which presage, and could be the cause of, somitogenesis defects, rather than late disruptions which could be secondary to abnormal somite morphology. As shown in Fig. 6, of all the genes analysed only *notch1a/des* shows a clear disruption of its expression pattern. The PSM of control embryos can be subdivided into three territories with varying levels of *notch1a/des* expression: strong in the tailbud and posterior PSM, intermediate in the intermediate PSM and low in the anterior PSM, at the level of somite 0 (Fig. 6A,C). In her6MO-injected embryos, this progressive downregulation is impaired: *notch1a/des* is expressed at equally strong levels throughout the PSM and its expression domain is not delimited by a sharp boundary between somites 0 and 1 (Fig. 6B,D). The expression patterns of *notch1b*, *notch5* and *notch6* are not affected (Fig. 6G-L). The pattern of *lfng* expression is not altered, but its levels are increased in her6MO-injected embryos (Fig. 6E,F). Thus, the activity of Her6 is required to progressively downregulate *notch1a/des* within the intermediate and anterior PSM and to refine the boundary of its expression domain at the transition between PSM and somites.

Depletion of Her6 or Her6 and Her4 leads to a progressive disruption of the coordinated cycling expression of *deltaC* and *her1*

To explore whether the progressive disruption of somitogenesis correlates with a gradual breakdown of the segmentation clock as in Notch mutants or embryos depleted of Her7 or Her7 and Her1 (van Eeden et al., 1998; Jiang et al., 2000; Oates and Ho, 2002), *deltaC* and *her1* expression was analysed at different time points on batches of her6MO- and her6MO+her4MO-injected embryos. Regardless of the dose and type of injected MO, embryos at early stages of somitogenesis show normal sharp stripes of *deltaC* and *her1* expression. However, in embryos analysed at progressively later time points, this periodicity of expression is gradually lost (Fig. 7). The time at which abnormalities of *deltaC* and *her1* expression pattern appear depends on the dose and type of injected MO and correlates with the onset of somitic defects (compare Table 1 with Fig. 7). In embryos injected with her6MO+her4MO, the homogeneous *deltaC* stripes in the anterior PSM are progressively replaced by a broad and irregular band within which cells expressing *deltaC* at strong and weak levels are intermixed (Fig. 7M-S′,T,T′). A similar mixture of cells expressing different levels of *deltaC* is observed in the posterior PSM (Fig. 7S″).

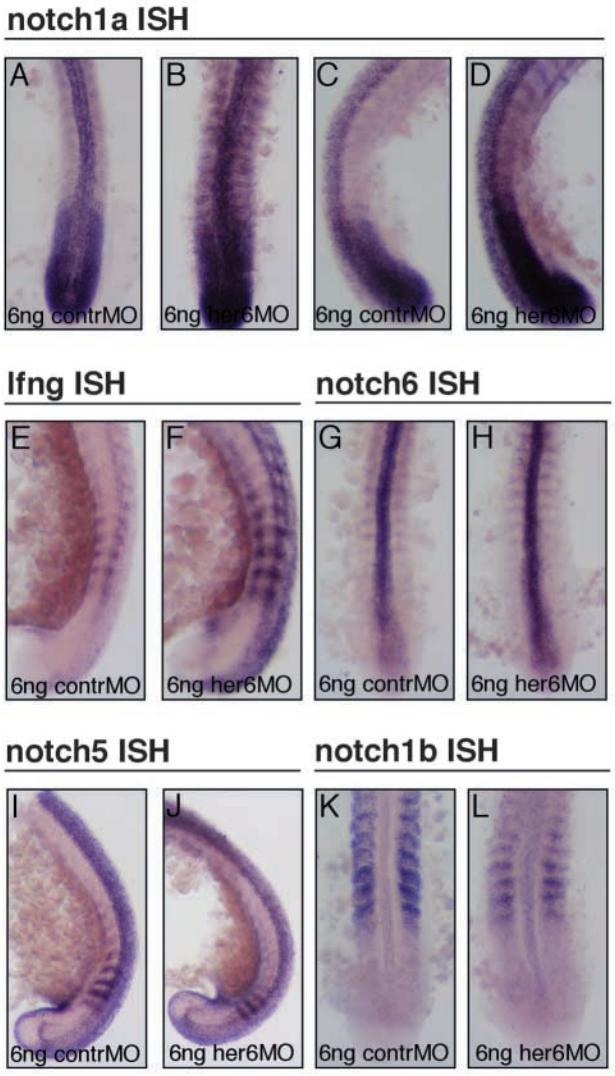

Fig. 6. Control (A,C,E,G,I,K) or her6MO-injected (B,D,F,H,J,L) embryos probed for the expression of the Notch pathway genes *notch1a/des* (A-D), *lfng* (E,F), *notch6* (G,H), *notch5* (I,J) and *notch1b* (K,L) at 14- to 18 ss. Staining was developed for exactly the same amount of time on control MO- and her6MO-injected embryos, to allow for a precise comparison of the gene expression levels. Levels of *notch1a/des* expression are high in the posterior PSM, decrease towards the anterior PSM and become very low in the somites (A,C). The transition between PSM and somites is defined by a sharp drop in *notch1a/des* expression. In 6 ng her6MO-injected embryos (B,D), this downregulation is impaired and expression of *notch1a/des* persists in the newly formed somites. No abnormalities are observed in the expression pattern of *notch1b*, *notch5*, *notch6* (H,J,L). Expression of *lfng* is upregulated, but not disrupted, in the neural tube and newly formed somites (F).

Discussion

her6 is a target of Notch signalling in the paraxial mesoderm

We started our investigation of the role played during somitogenesis by the zebrafish *hairy/E(Spl)-related* gene, *her6*, by analysing the pattern of its expression in the paraxial

mesoderm and its dependence upon the Notch signalling pathway. During most of somitogenesis, expression of *her6* is confined to two stripes in the anterior PSM and to the posterior compartment of the mature somites. Expression in the tailbud is only observed early during somitogenesis and no expression is detected in the intermediate PSM at any stage. Although the two *her6* stripes in the anterior PSM show some variability in their strength, their distances from one another, from the *myod* stripes in the PSM or from the last formed intersomitic cleft do not vary among embryos with the same number of somites. In addition to this, and in contrast to the cycling zebrafish *hairy/E(Spl)-related* genes *her1* and *her7* (Holley et al., 2002; Oates and Ho, 2002), the formation and maintenance of the *her6* stripes do not depend on the activity of Her6 protein. Thus, *her6* does not show an oscillatory behaviour and, despite its high degree of homology to the cycling genes mouse *Hes1* and chicken *hairy2* (Jouve et al., 2000), its expression pattern in the PSM resembles that of the non-cycling frog gene *x-hairy2* (Davis et al., 2001).

Differences in the PSM expression pattern of Notch pathway components between zebrafish on one hand and mouse and chicken on the other have already been noticed. Zebrafish *lfng*, in contrast to its mouse and chicken homologues, does not cycle in the PSM (Prince et al., 2001), whereas Delta genes cycle in zebrafish but not in mouse or chicken (Jiang et al., 2000). One possible explanation for these discrepancies is that different vertebrate classes exploit different cycling components of Notch pathway to fulfil the same functions during somitogenesis. Alternatively, it is possible that *Hes1* and chick *hairy2* exert different functions in the PSM and in the segmented somites and that in zebrafish such functions have been shared among distinct *hairy/E(Spl)-related* genes, of which some – *her1* and *her7* – cycle within the PSM (Holley et al., 2002; Oates and Ho, 2002), while others, such as *her6*, are expressed in a static fashion within the anterior PSM and the somites.

Our data show that the pattern of expression of *her6* is dependent on the integrity of the Notch signalling pathway and on its spatially restricted activation: a block of the Notch signal by dominant-negative Su(H) results in a loss of *her6* expression in somites and the anterior PSM, while ubiquitous and sustained activation of Notch signalling by constitutively active Su(H) leads to ectopic expression of *her6* throughout the PSM. Four zebrafish Notch genes with spatially restricted expression patterns have been identified to date (Bierkamp and Campos-Ortega, 1993; Westin and Lardelli, 1997). Ubiquitous expression of constitutively active Notch1a, NIC, which leads to increased and ectopic expression of *her1* and *her4* (Takke et al., 1999; Takke and Campos-Ortega, 1999), fails to induce an ectopic expression of *her6* in the posterior PSM (A. P., Y.-J. J. and D. G. W., unpublished). However, the expression pattern of *her6* in the anterior PSM and the segmented somites is remarkably similar to that of *notch5* (Westin and Lardelli, 1997). Thus, it is possible that Her6 is a specific effector of the Notch5-mediated signal.

The function of Her6 and its relation to the segmentation clock

Her6 is unlikely to be involved in the determination of somitic compartment identity

The pattern of *her6* expression and its dependence upon Notch signalling suggest that this gene could be an output of the segmentation clock, required to determine the identity of the posterior somitic compartment, thus providing a link between the mechanism setting the tempo for the generation of the somites and their internal patterning. We addressed this hypothesis by injecting zebrafish embryos with RNAs coding for wild-type or an antimorph form of the Her6 protein. Paraxial mesoderm forms normally in all the injected embryos but its segmentation is imperfect. Internal somitic AP patterning required for the formation and positioning of intersomitic clefts is disrupted in embryos in which the function of Her6 or its expression profile are altered. However, in contrast to what is expected if Her6 were required for determining the P compartment identity by repressing A compartment identity markers, both A and P compartment markers are expressed throughout the segmented paraxial mesoderm. Similarly, *mespb*, which codes for a bHLH protein determining the identity of A compartment (Sawada et al., 2000), is not downregulated in wt-her6-injected embryos nor up-regulated in her6VP16-injected ones. Thus, we can conclude that *her6* does not play a major role in the determination of the P somitic compartment.

Another possibility is that Her6 represents an output of the clock directly involved in establishing and/or maintaining the intersomitic boundaries. An inductive interaction dependent on the spatial restriction of dynamic *lunatic fringe* expression and Notch pathway activation and responsible for the morphological implementation of intersomitic boundaries has been recently described in chicken embryos (Sato et al., 2002). Notch targets of the Hairy/E(Spl) family are likely to be among the effectors of such an activity. If a comparable phenomenon exists in zebrafish, Her6 could be a candidate for regulating the expression of boundary-forming cell surface molecules. However, in this report we focus our attention on a different aspect of Her6 function, its effect on the activity and synchronisation of the segmentation clock.

Her6 is required (together with Her4) to maintain the coordinate cyclic expression of *delta*C and *her1*

Altering the restricted expression pattern of Her6 or its transcriptional repressor function disrupts the dynamic expression of *deltaC* and *her1* in the PSM. To explore the possibility that Her6 acts as an effector of Notch signalling which feeds back on the clock to control the cyclic expression of genes in the PSM, we knocked down its activity by injection of morpholino oligonucleotides. A reduction of Her6 function led to disruption of the posterior paraxial mesoderm segmentation in a dose-dependent manner, the abnormalities arising earlier during somitogenesis in embryos injected with higher doses of her6MO. In her6MO-injected embryos, the onset of morphological defects correlates with the first signs of a progressive breakdown of coordinated cycling expression of *deltaC* and *her1*. The appearance of both the morphological and the molecular defects is cooperatively accelerated when the functions of *her6* and of a second *her* gene expressed in the anterior PSM, *her4*, are simultaneously reduced by coinjection of morpholinos against *her6* and *her4*.

The exact relationship between Notch signalling and the somite segmentation clock is still unclear. In particular, it is debated whether Notch signalling is a central component of the clock, which is necessary for the establishment and/or maintenance of its oscillatory behaviour, or whether it acts only


control
A
10ss
B
14ss
C
14ss
D
14ss
D'
D''
E
18ss
F
18ss
G
20ss
H
20ss
J
24hrs
K
26ss
I
14ss
I'
L
18ss
3ng her6MO+3ng her4MO
M
8ss
N
8ss
O
10ss
P
10ss
Q
10ss
R
10ss
R'
S
14ss
S'
S''
T
14ss
T'
U
8ss
V
10ss
V'
W
14ss
W'
X
14ss
6ng her6MO
Y
14ss
Z
20ss
α
26ss
γ
18ss
δ
20ss
3ng her6MO
β
26ss

Fig. 7. Control (A-L) or morpholino-injected (M-δ) embryos hybridised with probes against *deltaC* (A-K,M-T′,Y-β) or *her1* (I-L; U-X; γ,δ) at different time points. (A-L) In control embryos, stripes of *deltaC* and *her1* are maintained throughout somitogenesis. (D′,D″) Higher magnifications of the boxed areas in the anterior and posterior PSM, respectively, of the embryo in D. (I′) High magnification of the boxed area in I. (M-X) In embryos injected with 3 ng her6MO+3 ng her4MO, the periodicity of *deltaC* and *her1* expression breaks down between 8 and 10 ss and is lost by 14 ss. *deltaC* stripes become blurred between 8 and 10 ss (M-O). Between 10 and 14 ss, the anterior stripes fuse (P,Q), lose their homogeneous character (compare R′, a magnification of the boxed area in R, with D′) and are replaced by an irregular domain expressing *deltaC* at different levels (S,S′,T,T′). (S′,T′) Higher magnifications of the boxed areas in the anterior PSM of S and T. (S″) Higher magnification of the posterior boxed area in S, showing that an irregular *deltaC* pattern is also found in the posterior PSM (compare S″ with D″). *her1* stripes become blurred around 10 ss (compare V,V′ with I,I′) and by 14 ss a mixture of cells expressing *her1* at different levels is found throughout the PSM (W-X). (Y-α) In embryos injected with 6 ng her6MO the periodicity of *deltaC* expression is maintained at 14 ss (Y), breaks down around 20 ss (Z) and is lost by 26 ss (α). (γ,δ) disruption of her1 starts around 18 ss and is complete by 26 ss. (β) In an embryo injected with 3 ng her6MO, only weak *deltaC* abnormalities are visible at 26 ss.

as a coupling system, which is required to synchronise the oscillation among individual, autonomously cycling PSM cells (Jiang et al., 2000; Holley et al., 2002; Aulehla et al., 2003). Alternatively, Notch signalling could exert a dual action, being responsible for both the initiation and maintenance of the clock oscillations and for keeping their synchrony (Oates and Ho, 2002). In this case, it will be interesting to understand whether the two functions can be separated and attributed to distinct components of the Notch cascade or whether they represent two aspects of the same molecular interaction(s). In addition, 'Notch signalling' has often been used as a general byword for the sum of signalling events triggered by the different Notch receptors expressed in the PSM and only few attempts have been made at identifying the respective contribution of the different Notch genes and their different effectors during somitogenesis (Henry et al., 2002; Oates and Ho, 2002). In zebrafish, at least four *notch* genes (*notch1a*, *notch1b*, *notch5* and *notch6*) and four *her* genes (*her1*, *her4*, *her6* and *her7*) are expressed in the PSM with spatially restricted expression patterns (Bierkamp and Campos-Ortega, 1993; Westin and Lardelli, 1997; Takke et al., 1999; Holley et al., 2000; Pasini et al., 2001; Oates and Ho, 2002), suggesting that distinct Notch receptors could regulate the transcription of specific Her nuclear effectors to mediate distinct aspects of a general Notch signalling function. *her6* and *her4* are only expressed in the tailbud and as two pairs of static stripes in the anterior PSM (Takke et al., 1999; Pasini et al., 2001), and are therefore unlikely to represent central elements of the clock oscillatory mechanism. Accordingly, in both Her6- and Her6+Her4-depleted embryos, the coordinated periodic pattern of *deltaC* and *her1* stripes is correctly initiated. However, this pattern fails to be maintained throughout somitogenesis and is gradually replaced by an irregular mixture of cells expressing *deltaC* and *her1* at variable levels. This phenotype resembles the one described in the Notch pathway mutants *bea*, *deltaD/aei*, *notch1a/des* and *mib* or in Her7-depleted embryos and interpreted as the result of a progressive loss of synchronisation among PSM cells cycling between a *deltaC*-positive and a *deltaC*-negative status (Jiang et al., 2000; Oates and Ho, 2002). Therefore, Her6 and Her4 represent components of the Notch-dependent machinery which feed back on the clock but are only required to maintain the synchronisation among cycling PSM cells. In Her6-, Her4- or Her6+Her4-depleted embryos, the onset of morphological abnormalities and *deltaC* pattern disruption is delayed compared with the *notch1a/des* and *deltaD/aei* mutants. This is consistent with the hypothesis that *her6* and *her4* only account for part of the Notch-dependent response, and that in the absence of their protein products the synchronisation of gene expression oscillation in the PSM is maintained over a longer period of time than when a more central component is withdrawn. However, our finding that the onset of morphological abnormalities and of *deltaC* pattern disruption is shifted caudally in embryos injected with decreasing amounts of her6MO or her4MO shows that a partial depletion of Her6 or Her4 allows the coordination of *deltaC* dynamic expression to be maintained over an even longer time and thus highlights the importance of Her6 and Her4 protein dosage. Therefore, an alternative explanation of the relatively weak phenotype elicited by 6 ng/embryo injections of her6MO and her4MO is that these lead to a downregulation rather than a complete suppression of *her6* and *her4* translation and the residual amounts of proteins synthesised are sufficient to maintain clock synchronisation over a certain number of cycles. If this was the case, injecting MO doses higher that 6 ng/embryo would shift anteriorly the onset of somitogenesis defects. This hypothesis could not be tested, since we found that high her6MO or her4MO doses lead to an early arrest of epiboly.

According to a recent report (Gajewski et al., 2003), a single *her6* gene and three *her4*-related genes are present in the zebrafish genome. No data are at present available regarding the expression profile of the two previously unknown *her4* pseudoalleles but, should they be expressed in the PSM, it is possible that they escape targeting by her4MO and provide protein products capable of partially rescuing clock synchronisation, thus providing an alternative explanation for the weak effect of her4MO.

The mechanism by which Her6 and Her4 exert their function is likely to involve the transcriptional repression of clock components. Although no bona fide target of Her6 and/or Her4 transcription repressor activity is yet known, our data show that the progressive downregulation of *notch1a/des* within the anteriormost PSM and the establishment of the boundary of its expression domain at the transition between PSM and somites are impaired by a decrease in the function of Her6. *notch1a/des* regulates the expression of the cycling gene *her1* (Takke and Campos-Ortega, 1999). The Her1 protein negatively regulates the transcription of *her1* itself and of the Notch ligand *deltaC*. (Holley et al., 2002; Oates and Ho, 2002). Regardless of whether such an autoregulatory loop constitutes the core oscillator responsible for clock activity (Oates and Ho, 2002) or is exclusively required as a synchronising mechanism (Jiang et al., 2000), its localised disruption within the anterior PSM of Her6-depleted embryos can account for the somitogenesis defects observed.

Our data show that, starting from about 14-somite stage, a

'salt and pepper' pattern of *deltaC* expression is present not only in the anterior PSM, but also in the tailbud of her6+her4MO-injected embryos. As expression of both *her6* and *her4* is essentially restricted to the anterior PSM, it may seem surprising that a loss of function of their protein products results in a loss of clock synchronisation throughout the PSM. However, oscillator synchronicity is by definition a non cell-autonomous phenomenon, which requires a fast and accurate intercellular coupling element. Notch signalling-based synchronising activities are likely to rely on intercellular feedback loops analogous to those proposed to explain the generation of oscillations (Oates and Ho, 2002; Dale et al., 2003). In such models, local disruptions can be corrected by community effects as long as they affect a number of cells below a given threshold, beyond which they override the whole system. It is therefore conceivable that a localised failure of the ability of some cells to cycle in synchrony with their neighbours is amplified and gradually spread to the entire PSM by the same Delta/Notch intercellular signalling that is normally responsible for maintaining synchrony. An alternative explanation is that the early and transient expression of *her6* and *her4* in the involuting marginal zone and in the tailbud (Fig. 1A-E) (Takke et al., 1999) provides enough transcription factor products sufficient and necessary for maintaining the clock synchronisation in the posterior PSM throughout several cycles of *deltaC* expression. In this case, Her6 and Her4 would be independently required within the anterior and the posterior PSM and morpholino-mediated block of their transient early synthesis would be enough to affect later synchrony of *deltaC* expression in the posterior PSM.

Co-operation between Her6 and Her4

Morpholino-mediated depletion experiments indicate that Her6 and Her4 are partially redundant in maintaining the coordination of *deltaC* dynamic expression. This synergy could underlie a direct cooperation between the Her6 and Her4 transcription factors. Indeed, hairy/E(spl)-related proteins are known to homo- and/or heterodimerise (reviewed by Davis and Turner, 2001) and it has been suggested that this phenomenon is the basis of a combinatorial network among cyclically expressed Hairy/E(spl)-related factors in the chicken PSM (Leimeister et al., 2000). A similar network comprising Her6, Her4 and other cyclically or non cyclically expressed bHLH and hairy/E(spl)-related molecules could function within the anterior PSM and be responsible for coordinating the synchronisation of the dynamic waves of expression of cycling genes. Total loss or partial reduction in the function of one or more of the network components will result in a more or less rapid disruption of the cycling coordination among PSM cells, and this will in turn lead to intersomitic defects arising at different points along the rostrocaudal axis.

Conclusions

The zebrafish transcription factor Her6 is an output of the Notch signalling pathway that feeds back by regulating the expression of *notch1a* in the anterior PSM. Together with another Notch-dependent hairy/E(Spl) factor, Her4, Her6 is required for maintaining the synchronisation of cyclic gene expression among adjacent cells within the PSM. Future studies of their transcriptional regulation as well as identification of their molecular partners and transcriptional targets will clarify the relationship between Notch signalling and the segmentation clock.

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