

Anterior and posterior waves of cyclic *her1* gene expression are differentially regulated in the presomitic mesoderm of zebrafish

Martin Gajewski^{1,*}, Dirk Sieger^{1,*}, Burkhard Alt¹, Christian Leve¹, Stefan Hans², Christian Wolff³, Klaus B. Rohr² and Diethard Tautz^{1,†}

¹Universität zu Köln, Institut für Genetik, Weyertal 121, 50931 Köln, Germany

²Universität zu Köln, Institut für Entwicklungsbiologie, Gyrhofstr. 17, 50923 Köln, Germany

³Max-Planck Institut für Entwicklungsbiologie, Spemannstrasse 35, 72076 Tübingen, Germany

*These authors contributed equally to this work

†Author for correspondence (e-mail: tautz@uni-koeln.de)

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SUMMARY

Somite formation in vertebrates depends on a molecular oscillator in the presomitic mesoderm (PSM). In order to get a better insight into how oscillatory expression is achieved in the zebrafish *Danio rerio*, we have analysed the regulation of *her1* and *her7*, two bHLH genes that are co-expressed in the PSM. Using specific morpholino oligonucleotide mediated inhibition and intron probe in situ hybridisation, we find that *her7* is required for initiating the expression in the posterior PSM, while *her1* is required to propagate the cyclic expression in the intermediate and anterior PSM. Reporter gene constructs with the *her1* upstream sequence driving green fluorescent protein (GFP) expression show that separable regulatory regions can be identified that mediate expression in the posterior versus intermediate and anterior PSM. Our

results indicate that the cyclic expression is generated at the transcriptional level and that the resulting mRNAs have a very short half-life. A specific degradation signal for *her1* mRNA must be located in the 5'-UTR, as this region also destabilises the GFP mRNA such that it mimics the dynamic pattern of the endogenous *her1* mRNA. In contrast to the mRNA, GFP protein is stable and we find that all somitic cells express the protein, proving that *her1* mRNA is transiently expressed in all cells of the PSM.

Supplemental data available online

Key words: Somitogenesis, bHLH genes, mRNA stability, Enhancer analysis, Morpholino-oligonucleotide mediated knockdown

INTRODUCTION

Somites are transient segmental structures that are formed along the anterior-posterior axis of the vertebrate embryo (for a review, see Saga and Takeda, 2001; Maroto and Pourquié, 2001). They are generated from the mesenchymal presomitic mesoderm (PSM), which flanks the notochord on both sides. There are three major phases of somitogenesis. First, the pre-patterning of the unsegmented PSM and the establishment of the rostrocaudal polarity of the future somite (Stern and Keynes, 1987; Aoyama and Amasoto, 1988); second, the formation of the somitic border and third, the differentiation of the somites to generate the muscles and vertebrae of the trunk and tail (Tam and Trainor, 1994).

It has long been speculated that the pre-patterning of the somites is achieved by an oscillator mechanism in the PSM (Cooke and Zeeman, 1976; Meinhardt, 1986) (reviewed by Dale and Pourquié, 2000). The first evidence for this oscillator mechanism was provided by the identification of the *c-hairy1* gene (Palmerim et al., 1997), which is dynamically expressed in the PSM of chicken. Owing to its cyclic expression, which progresses from the posterior to the anterior PSM, the cells in

the chick embryo undergo several on and off phases of *c-hairy1* transcription before they become a somite. *c-hairy1* encodes a bHLH transcription factor, which is a homologue of the *Drosophila* pair-rule gene *hairy* (Ish-Horowitz et al., 1985). More recently, several *hairy* (*h*) and *Enhancer of split* (*E(spl)*) related genes have been identified, which also have a dynamic expression in the vertebrate PSM. This includes the *c-hairy2* and *c-Hey2* genes in chick (Jouve et al., 2000; Leimeister et al., 2000) as well as the *Hes1* and the *Hes7* genes in mouse (Jouve et al., 2000; Bessho et al., 2001a; Bessho et al., 2001b). In zebrafish, nine *h/E(spl)* related genes have been discovered so far [*her1-her6* (von Weizsäcker, 1994; Müller et al., 1996; Pasini et al., 2001), *her7*: AF240772; *her8a/b*: AY007990/AY007991 and *her9* (Leve et al., 2001)] but only two of them, *her1* and *her7*, show an oscillating expression in the PSM (Holley et al., 2000; Oates and Ho, 2002). The analysis of a deletion mutant for *her1* and *her7* as well as morpholino-oligonucleotide (MO) knockdown studies suggest that Her1 and Her7 protein function is required for the pre-patterning of the zebrafish PSM (Henry et al., 2002; Oates and Ho, 2002). The loss of Her1/Her7 protein leads to somites that show alternating weak and strong boundaries (Henry et al.,

2002). In addition, a disruption of rostrocaudal polarity within the somites has been observed (Henry et al., 2002; Oates and Ho, 2002).

The current data suggest that the *Delta-Notch* signalling pathway is the major trigger of cyclic gene expression in the vertebrate PSM (for reviews, see Maroto and Pourquié, 2001; Saga and Takeda, 2001). In zebrafish, mutants of *deltaD* (*after eight*) and *Notch1* (*deadly seven*), as well as MO-knockdown of *deltaC* abolish the cyclic expression of *her1* and *her7* (Dornseifer et al., 1997; van Eeden et al., 1998; Takke and Campos-Ortega, 1999; Holley et al., 2000; Holley et al., 2002; Oates and Ho, 2002). In these cases *her1* and *her7* usually only show an irregular expression in the anterior PSM and a weak, diffuse expression in the posterior part of the PSM and the tailbud. Furthermore, *her1* and *her7* appear to crossregulate each other, and both are required for the transcription of *deltaC* and *deltaD* (Holley et al., 2002; Oates and Ho, 2002; Henry et al., 2002).

We have focussed here on a better understanding of the differential roles of *her1* and *her7* in regulating the cyclic gene expression by analysing the effects of MO-knockdown on each other's expression. We have found different roles for Her1 and Her7 in regulating the anterior and posterior parts of cyclic *her* gene expression. Analysis of the *her1* promoter reveals that these anteroposterior differences are the result of separable regulatory elements.

MATERIALS AND METHODS

her1 and *her7* genomic sequence and *her-7* cDNA sequence

By screening a zebrafish genomic library (MoBiTec) with a *her1* cDNA probe, one clone containing the upstream region and the first exon as well as several clones containing different downstream parts were isolated. The presence of the *her7* gene on the upstream region clone was deduced from the genomic sequence and confirmed by an EST sequence (EST-ID: fb97b02) found in the zebrafish EST database (Sprague et al., 2001). The cDNA clone was obtained from RZPD (clone ID: MPMGp609E0421Q8). Sequences were determined on an ABI377XL sequencer (Perkin Elmer/Applied Biosystems) and submitted to GenBank under the accession numbers AF292032 (genomic sequence for *her1* and *her7*) and AF240772 (*her7* cDNA sequence).

Sequence comparisons and phylogeny

Amino acid sequences were aligned using the Pileup program of the GCG software package (Devereux et al., 1984; Senger et al., 1998). Similarity trees were generated using PAUP, calculations are based on Pileup alignments. Trees were displayed using Treeview (Page, 1996). The accession numbers of the compared genes are: *c-hairy1*: AF032966, *c-hairy2* (Jouve et al., 2000), *Drosophila hairy*: X15905, *her1*: X97329, *her4*: X97332, *her6*: X97333, *her7*: AF240772, *her9*: AF301264, mouse *Hes1*: NM008235, mouse *Hes7*: AB049065, *X-hairy1*: U36194, *X-hairy2A*: AF383159, human *HES1*: Q14469, human *HES7*: NM032580.

Whole-mount in situ hybridisation and histological methods

Fish were bred at 28.5°C in a 14 hour light/10 hour dark cycle. Embryos were collected by natural spawning and staged according to Kimmel et al. (Kimmel et al., 1995). For automated in situ hybridisations we followed the protocol of Leve et al. (Leve et al., 2001) using a programmable liquid handling system described by

Plickert et al. (Plickert et al., 1997). The hybridisation temperature for the *her1* intron probe had to be reduced to 50°C, because of its high AT content. Digoxigenin- or fluorescein-labelled RNA probes were prepared using RNA labelling kits (Roche). Staining was performed with BM purple (Roche) for single in situ hybridisations or, for double fluorescence in situ hybridisations, Vector[®] Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories) and the ELF[®]-97 mRNA In Situ Hybridisation Kit (Molecular Probes) were used according to the method of Jowett and Yan (Jowett and Yan, 1996). Whole-mount embryos were observed under a stereomicroscope (Leica) and digitally photographed (Axiocam, Zeiss). Flat-mounted embryos were observed with an Axioplan2 microscope (Zeiss). For observation of the Vector[®] Red staining or the ELF[®]-97 precipitate a rhodamine filter set or a DAPI filter set was used, respectively.

Reporter gene constructs and transgenic lines

A 10.9 kb *NcoI* fragment from the upstream region of the *her-1* gene was in-frame subcloned into the start methionine of the coding sequence of pEGFP (Clontech). After digestion with *PstI* and *EcoRI*, the promoter-reporter construct containing 8.6 kb upstream sequence and the EGFP reporter was inserted into pHSREM1 (acc. no. ATCC37642, kindly provided by D. Knipple) to yield construct I. Transgenic lines were produced by injection of a *PstI*-linearized fragment of this construct, or of PCR-amplified promoter deletions of it, into single cell embryos. For PCR-amplification the Expand High Fidelity PCR System (Roche) was used. For generation of constructs II-VI the same downstream primer (M13for: 5'-GTA AAA CGA CGG CCA GT-3') was used in combination with upstream primer II (5'-TAA ACT TTC CCC AGT CAG-3'), upstream primer III (5'-AAA GCC ACA TCA AAG CCC-3'), upstream primer IV (5'-TTA GCC ATG AAC GAT GCC-3'), upstream primer V (5'-AGC AAC TCC ATA AAA TCC-3'), upstream primer VI (5'-CTA TGA GAC AAC GAT GAG-3'), respectively. Between five and 15 transgenic lines were obtained in each case, but not all showed a sufficiently strong expression. Four, two and one line were eventually analysed for constructs I-III. There were only quantitative (expression level) but no qualitative differences between the lines. The DNA fragments were gel purified prior to injection using a gel extraction kit (Bio-Rad, Gibco BRL). The DNA concentration of the injected solutions was between 80-100 ng/μl in water containing 0.2% phenol red and 0.1 M KCl. Injections were carried out using FemtoJet[®] and a Micromanipulator (Eppendorf). To test for possible transgenic animals, DNA of 100 embryos was extracted as described previously (Meng et al., 1999). Positive PCR controls were the Wnt5a sense primer 5'-CAG TTC TCA CGT CTG CTA CTT GCA-3' and the Wnt5a antisense primer 5'-ACT TCC GGC GTG TTG GAG AAT TC-3'. For the transgene test, two GFP primers were used (GFPfor3: 5'-CGG CAA CTA CAA GAC CCG CG-3' and GFPprev3: 5'-GTC CTC GAT GTT GTG GCG GA-3'). The following PCR profile was carried out: 95°C for 2 minutes, then 35 cycles 95°C for 15 seconds, 55°C for 15 seconds and 72°C for 30 seconds followed by an elongation step of 72°C for 5 minutes after the 35 cycles.

Morpholino injections

Antisense morpholino-modified oligonucleotides (GeneTools) were designed against the first 25 nucleotides of the 5'-UTR and against the start of the ORF of both the *her1* cDNA (X97329) and the *her7* cDNA (AF240772). Sequence for *her1*-anti5' morpholino: 5'-AGT ATT GTA TTC CCG CTG ATC TGT C-3, sequence for the *her1*-antiATG morpholino: 5'-CAT GGC TGA AAA TCG GAA GAA GAC G-3', sequence for *her7*-anti5' morpholino: 5'-ATG CAG GTG GAG GTC TTT CAT CGA G-3', sequence for the *her7*-antiATG morpholino: 5'-CAT TGC ACG TGT ACT CCA ATA GTT G-3'. 0.5 mM of the *her7^{mo}*s and 1 mM of the *her1^{mo}*s were injected into single-cell stage embryos. The injection solution additionally contained 0.1 M KCl and 0.2% phenol red. Control injections were done with the morpholino-modified oligonucleotide recommended by GeneTools,

or with buffer. The death rate caused by injection of the different morpholinos was usually between 5 and 11%.

RESULTS

Genomic analysis of *her1* and *her7*

The genomic region of the *her1* gene was analysed by sequencing two overlapping genomic clones. The *her1* gene consists of 4 exons distributed over approximately 6 kb (Fig. 1). We found that *her7* is located in a head to head orientation approximately 11 kb upstream of *her1*. A *her7* cDNA (acc. no. AF240772) and *her1* were previously mapped at 121.5 cM on linkage group 5 (Kelly et al., 2000). All of the other known *her* genes are located on different chromosomes [see ZFIN: www.zfin.org (Sprague et al., 2001)].

Alignment and phylogenetic analysis of *her1* and *her7* shows that the gene products are only distantly related to each other and are also not closely related to the oscillating *c-Hairy1/2* genes from chicken or mouse *Hes1* (Fig. 2 and see supplemental data at <http://dev.biologists.org/supplemental>). The Her7 protein, like the Her1 protein, shows all structural features of a subfamily of basic Helix-Loop-Helix (bHLH) proteins that act as transcriptional repressors. This includes a conserved proline in the basic domain, two additional helices termed the orange domain (Dawson et al., 1995) and a WRPW motif (Fisher et al., 1996) at the C terminus. One feature by which the Her7 protein can be distinguished from the other

h/E(spl) proteins is a proline residue occurring C-terminally of the WRPW motif. The Her7 protein has this residue in common with the human and mouse Hes7 as well as with the *enhancer of split* related proteins ESR-4/5 from *Xenopus* (Jen et al., 1997; Bessho et al., 2001a).

her7 is co-expressed with *her1*

The spatiotemporal expression pattern of *her7* mimics that of *her1* (Müller et al., 1996; Oates and Ho, 2002) (see also supplemental data at <http://dev.biologists.org/supplemental>). To analyse whether *her1* and *her7* are expressed in the same cycle phase, we have used double fluorescent in situ hybridisation, a technique that avoids detection interference (Jowett and Yan, 1996). Superimposition of the signals shows that *her7* expression overlaps exactly that of *her1* in the tailbud and the posterior two stripes in the presomitic mesoderm (Fig. 3). In the anterior-most stripe, *her7* expression appears weaker than *her1* expression, which may be due to faster degradation. In a previous study, *her1* expression has been found to be more persistent at the posterior borders of each stripe than *her7* (Oates and Ho, 2002). This discrepancy could be due to the different techniques that were employed.

Effect of *her1* and *her7* on cyclic gene expression

Given the overlapping expression of *her1* and *her7*, we have investigated whether they might play similar roles in regulating cyclic gene expression. Antisense morpholino-modified oligonucleotides (MO) against *her1* and *her7* were used to

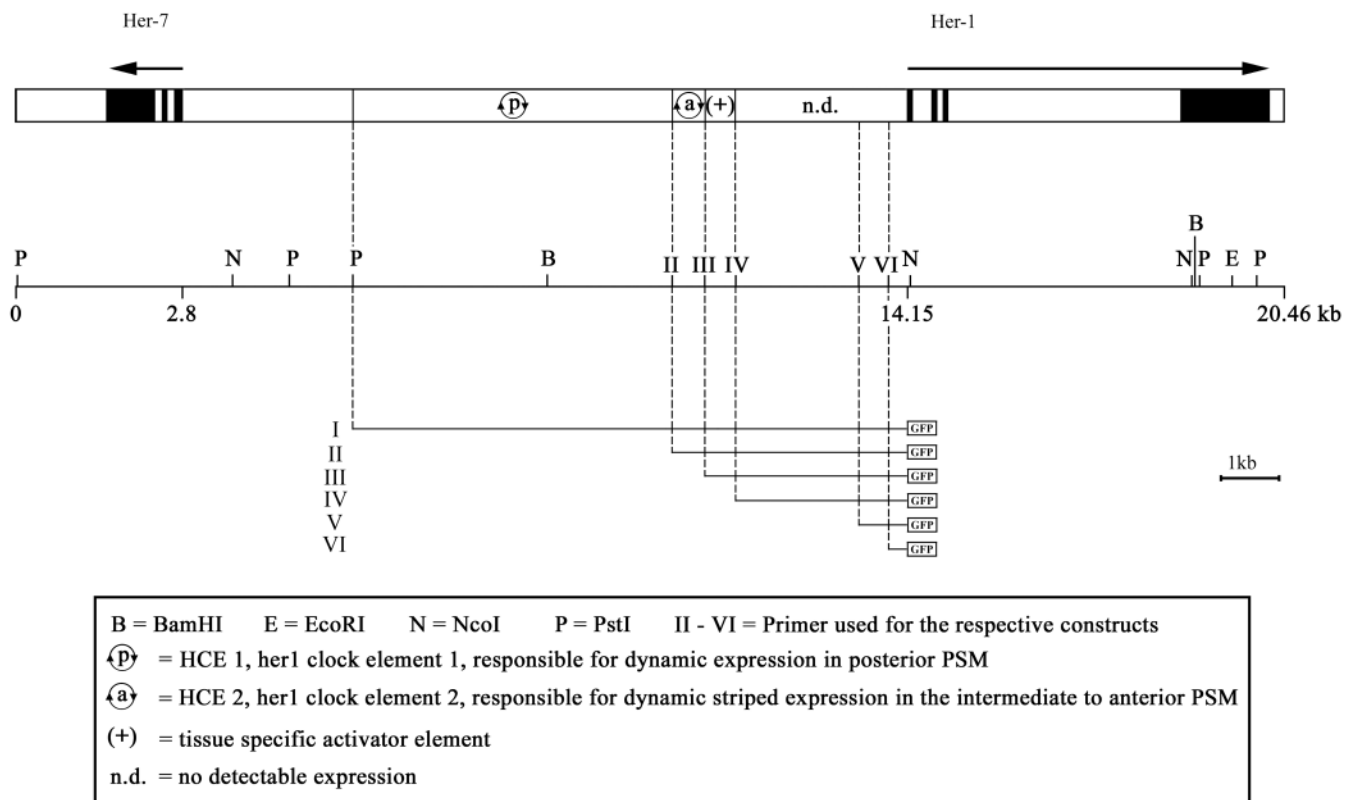


Fig. 1. Genomic organisation of *her1* and *her7* and cis regulatory regions identified in transgenic lines. The organisation and the exon-intron structure of *her1* and *her7* are shown on the top. Exons are indicated as black boxes. The arrows indicate the direction of transcription. *her1* consists of 4 exons and *her7* has 3 exons. The extent of the promoter-reporter gene constructs is shown below. Construct sizes: I, 8.6 kb; II, 3.3 kb; III, 2.8 kb; VI, 2.3 kb; V, 0.8 kb; VI, 0.3 kb.

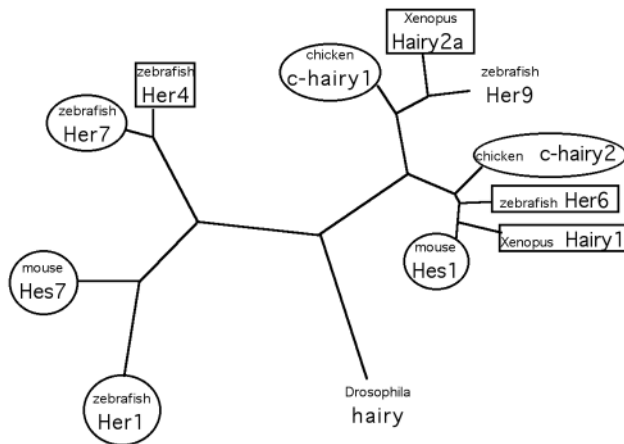


Fig. 2. Phylogram of different vertebrate *hairy/E(spl)*-like proteins and *Drosophila hairy*. Circles indicate genes that are cyclically expressed and boxes indicate genes that are expressed in the PSM, but not cyclically. It is evident that the phylogenetic similarities and expression similarities do not correspond.

specifically inhibit the function of these genes. All effects described below were observed with a high penetrance (>90%) in at least 75–175 embryos and in several independent experiments using two different MOs in each case, and were not observed in control injections (see Fig. 4 legend for details). We find that in both, the *her1^{mo}*- and the *her7^{mo}*-injected embryos (or morphants) their own stripe expression is equally strongly perturbed (Fig. 4H–L and O–S) and knockdown of both bHLH genes lead to a similar disruption of *deltaC* and *deltaD* expression patterns (Fig. 4M,N,T,U). Comparable effects have been shown previously with different MO variants (Holley et al., 2002; Oates and Ho, 2002). However, there are some important differences in details that we want to discuss in turn.

The analysis of MO-mediated disruption of a gene function on its own expression needs to take into account that the MO can stabilise the mRNA, even though the mRNA is not translated (Oates and Ho, 2002). We have therefore analysed the *her1* expression pattern both with an exon probe and an intron probe to differentiate between transcriptional regulation and mRNA stability effects. For riboprobe production we have used a part of the largest intron of *her1*, which yielded in wild-type embryos the same expression pattern as a *her1* exon probe, albeit much weaker (Fig. 4A–C, note the different staining times). The exon probe shows a broad and strong expression in the PSM of *her1* morphants (Fig. 4H) (Holley et al., 2002; Oates and Ho, 2002). The intron probe, however, reveals a different expression pattern and therefore confirms a suspected MO-mediated stabilisation effect. We found embryos with a distinct posterior U-shaped domain with a broad stripe at the anterior end (Fig. 4I) and embryos with an anterior stripe separated from the posterior domain (Fig. 4J). These two types of patterns occur in nearly equal frequencies (71/75 out of 146 embryos analysed), comparable to the pattern of the cyclic expression of the U-shaped domain in wild-type embryos. Thus it appears that only the two most anterior PSM stripes are not formed, while the posterior expression is less affected and apparently oscillates. This changes the previous

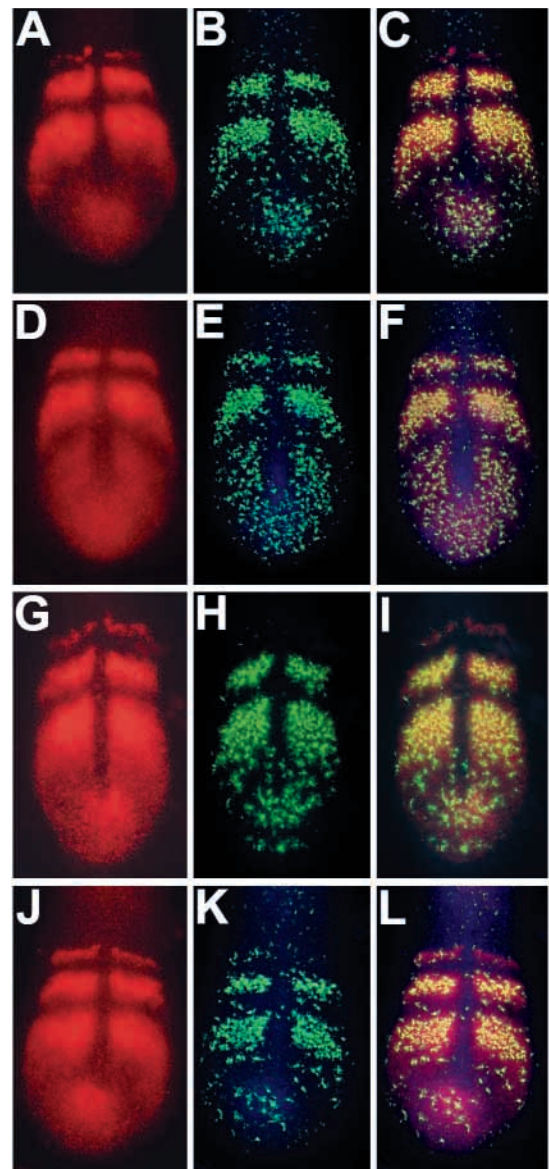


Fig. 3. Comparison of the expression pattern of *her1* and *her7* during one cyclic phase by double fluorescent in situ hybridisation. (A–C) 5-, (D–F) 5+, (G–I) 5++, (J–L) 6-somite stage. All in dorsal view, anterior to the top, flat-mounted embryos. (A,D,G,J) *her1* expression, (B,E,H,K) *her7* expression, (C,F,I,L) superimposition of the expression of both genes.

inference that *her1* acts as a repressor of itself (Holley et al., 2002; Oates and Ho, 2002), and suggests instead that it is required as an activator for its own two anterior-most PSM stripes. Monitoring the expression of *her7* in *her1* morphants shows that *her7* is strongly affected in the same way as *her1* (Fig. 4K,L), while in a previous study only mild effects on cyclical behaviour could be observed (Oates and Ho, 2002), which are probably caused by insufficient penetrance of the morpholino used. Thus, for Her1 we find a common role in activating the *her1* and *her7* stripe formation in intermediate and anterior PSM. In addition, Her1 is not involved in the *her1* and *her7* wave generation in the posterior PSM, neither as a repressor nor as an activator.

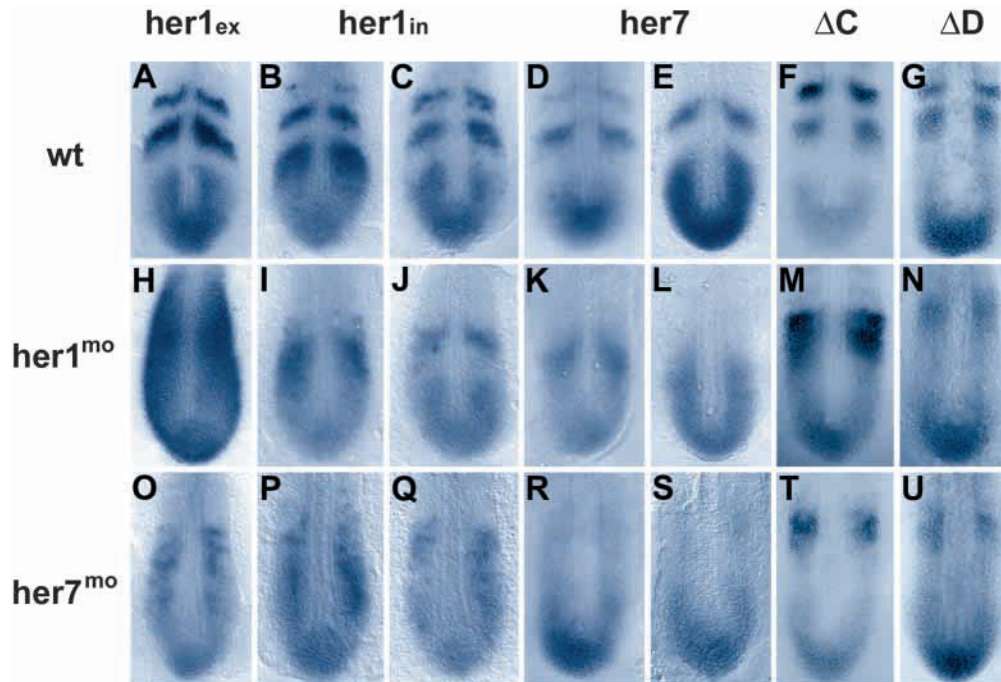


Fig. 4. Effects of morpholino injections on the pattern of genes expressed in the PSM. (A-G) The wild-type pattern, (H-N) embryos injected with *her1^{mo}* and (O-U) embryos injected with *her7^{mo}*. Two different morpholinos were used in each case (see Materials and Methods), which caused identical effects, respectively. The embryos are between the 8- and 12-somite stage. The columns represent the different probes that were used for in situ hybridisation. *her1_{ex}* refers to a cDNA (exon) probe, *her1_{in}* to the intron probe. *deltaC* and *deltaD* are abbreviated. Two columns are shown for the *her1_{in}* and *her7* probes, to show different stages of the dynamic expression. Note that the *her1_{in}* probe yields a much weaker signal and requires about 15-fold longer staining times than the exon probe (1 hour compared to approximately 15 hours). Moreover, it leads often to additional apparently random punctate expression in the PSM, which is of unknown significance. The embryos that are selected here have relatively few such additional punctate signals – some of the other embryos in the same preparations did show a higher number of these irregular signals. In total 185, 190, 88 and 84 *her1^{mo}*-injected embryos from independent experiments were hybridised with antisense riboprobes of *her1*, *her7*, *deltaC* and *deltaD*, respectively. The penetrance of the observed effects was 92%, 91%, 94% and 90%, respectively. In total 183, 163, 89 and 82 *her7^{mo}*-injected embryos from independent experiments were hybridised with antisense riboprobes of *her1*, *her7*, *deltaC* and *deltaD*, respectively. The penetrance of the observed effects was 96%, 92%, 91% and 91%, respectively. In total 230 *her1^{mo}*-injected embryos were hybridised with *her1* intron probe. The observed penetrance in 3 independent experiments was 92%, 96% and 93%, respectively. All in dorsal view, anterior to the top, flat-mounted embryos.

Embryos injected with the *her7^{mo}* also show disruption of *her1* and *her7* cycling, but with different effects on each and differences when compared to *her1* morphants. *her1* is expressed in a punctate pattern in the PSM of *her7* morphants and a more pronounced domain is observed in the tailbud (Fig. 4O,P,Q). *her7* also shows a pronounced tailbud expression in *her7* morphants, and at best a residual weak signal without punctate pattern in the intermediate PSM (Fig. 4R,S). Thus, it appears that the mRNA stabilisation effect is much weaker than that for *her1*. However, the perturbation of *her1* and *her7* wave generation in posterior PSM of the *her7* morphants, which is not seen in *her1* morphants, indicates a unique role for the Her7 protein in this process.

***her1* regulation by distinct promoter elements**

The above results indicate that at least *her1* regulation is governed by two distinct phases. This is supported by reporter gene constructs in transgenic lines. *her1* upstream fragments of various size were fused to GFP as reporter and the DNA was injected into early embryos. Stably transformed lines were established from these and analysed for their regulatory effects by in situ hybridisation to the GFP mRNA. Lines containing

an 8.6 kb upstream fragment (construct I, see Fig. 1) confer the full normal cyclic pattern (Fig. 5). Double hybridisation with the *her1* probe shows that it is essentially indistinguishable from the endogenous expression (Fig. 5G,H) indicating that all important elements for in phase cycling are present. However, the transgenic embryos of all 8.6 kb lines show an additional strong expression in the notochord, which is not seen for the *her1* gene itself, suggesting that construct I is missing a notochord-specific repressor. Furthermore, the expression in the most posterior domain is more persistent than for the *her1* RNA. It is unclear whether this is due to slight differences in the RNA stability, or to differences in the action of the enhancer included in our construct.

Lines containing 3.3 kb upstream sequence (construct II, see Fig. 1) show only a subset of the expression pattern. There is no expression in the posterior PSM and notochord expression is now absent. However, the stripes in the intermediate and anterior PSM are still generated and show cyclic expression (Fig. 6B,D,F). Taking the tip of the tailbud as reference (bar in Fig. 6B,D,F) one can see three different phases. Three stripes are visible in the first phase, with the most posterior stripe close to the tail bud (Fig. 6B). Two stripes are visible in the other

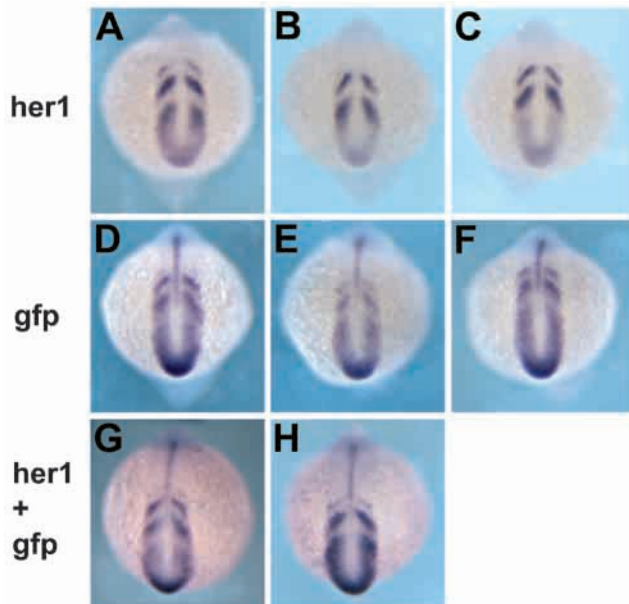


Fig. 5. GFP reporter gene mRNA expression and *her1* expression in transgenic embryos with the 8.6 kb construct. (A-C) *her1* probe, (D-F) GFP probe and (G-H) both probes mixed. Whole-mount embryos, all in dorsal view, anterior to the top. The embryos are between the 10 and 12 somite stage. Note the ectopic GFP expression in the notochord in the transgenic line, which indicates a missing repressor element.

phases, with the most posterior stripe either at an intermediate distance to the tailbud (Fig. 6D) or with a large distance to the tailbud (Fig. 6F). Thus, the most posterior stripe behaves in the same way as the anterior border of the dynamic U-shaped domain (compare Fig. 6A,C,E with B,D,F respectively). Formation of the stripes is obviously independent of wave generation in the posterior but must be linked, since budding of the most posterior stripe appears always at the very end of the peak of the oscillating U-shaped domain in the tailbud.

A further shortening of the upstream region to 2.8 kb (construct III in Fig. 1) shows a loss of dynamic stripe expression in the intermediate of the PSM and only a weakly expressed broad domain is visible (Fig. 6G). Lines containing shorter reporter gene constructs (IV-VI) with 2.3, 0.8 and 0.3 kb upstream sequences did not show any expression narrowing down the elements, which specifically drive *her1* expression, between 2.3 and 8.6 kb upstream of the transcription start.

Regulation of reporter gene constructs

To analyse whether the reporter gene constructs depend on *her1* and *her7* regulation in the same way as the endogenous genes, we have used MO knockdown of *her1* and *her7* in the background of the respective transgenic lines. For lines containing the 8.6 kb construct we find that the stripes in the anterior PSM are indeed disrupted by *her1*^{mo} and *her7*^{mo} injection, while the expression in the posterior PSM persists (Fig. 7B,C). A similar picture is seen in lines with the 3.3 kb construct. The stripe formation is clearly disrupted and a broad domain persists instead (Fig. 7E,F). These results confirm that the essential elements of the stripe regulation must be included in our constructs. In particular, the binding site(s) for Her7,

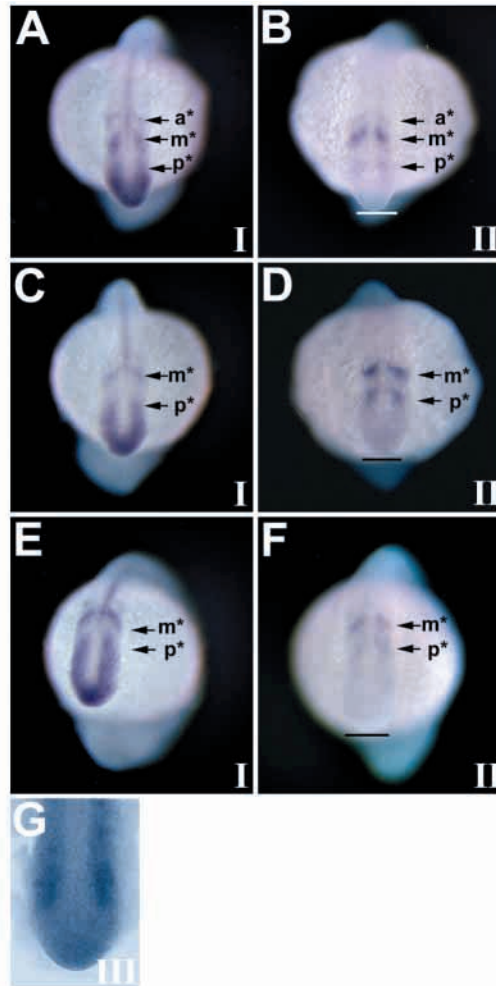


Fig. 6. GFP reporter gene mRNA expression in the different transgenic lines. The roman numerals in A-F correspond to the construct numbers in Fig. 1. In A-G a*, m* and p* mark the anterior, middle and posterior stripes of GFP mRNA expression, respectively. Whole-mount embryos all in dorsal view, anterior to the top. The embryos are between the 12- and 14-somite stage.

which regulate the stripes of *her1* expression, must be included in construct II (compare Fig. 7D with F).

However, we do not see evidence for residual cycling in construct I-containing lines and the anterior border of the remaining domain also appears to be anteriorly shifted in both lines, when compared to the endogenous pattern in the respective morphants. This may be explained by a slightly higher MO-mediated stability of the GFP mRNA.

Evidence for cyclic *her1* expression

Although the analysis of carefully staged embryos has provided a clear indication that *her1* expression is dynamic in the PSM (Holley et al., 2000), this can be demonstrated more directly in the transgenic lines by comparing the GFP mRNA expression with the GFP protein expression. Because the GFP protein is much more stable than the GFP mRNA, it persists in all cells in which the mRNA was at least transiently expressed. Accordingly, if the *her1* expression moves across the PSM in the same way as it was demonstrated for *hairy*-like

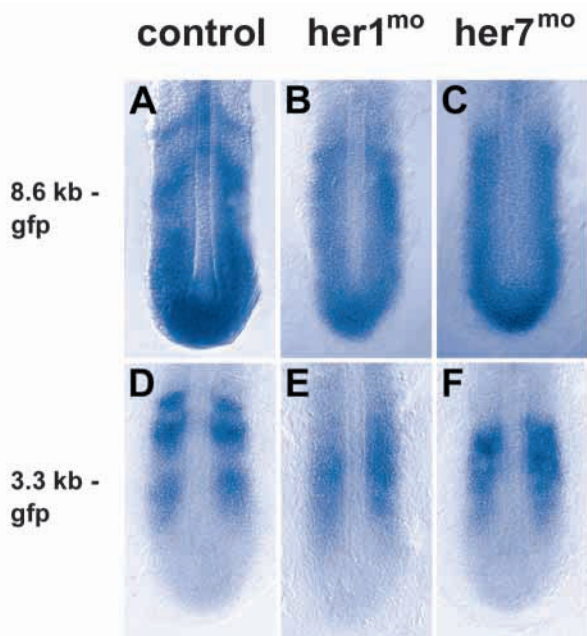


Fig. 7. Effects of morpholino injections on the GFP reporter mRNA expression in the PSM of the transgenic lines. Transgenic lines with (A-C) 8.6 kb of the upstream sequence and (D-F) 3.3 kb of the upstream sequence. (A,D) Uninjected control embryos, (B,E) *her1*^{mo}-injected embryos and (C,F) *her7*^{mo}-injected embryos. All in dorsal view, anterior to the top, flat-mounted embryos between the 10- and 12-somite stage.

genes in chicken (Palmeirim et al., 1997), then we would expect all somites to express GFP protein. The somites and the PSM show GFP fluorescence in the 8.6 kb line, with a fading of the signal towards the oldest somites (Fig. 8A,B). Since the GFP protein derived from the posterior U-shaped domain could cause this effect alone in the 8.6 kb line, we monitored GFP fluorescence in the 3.3 kb line, which lacks the posterior PSM expression. This line also shows continuous fluorescence, apart of the signal in the posterior part of the PSM (Fig. 8C,D). We can therefore conclude that the stripes in the intermediate and anterior PSM also move across all cells, confirming that the stripes seen in the RNA pattern are indeed solely due to a very short half-life of the mRNA.

DISCUSSION

Although cyclic expression of *hairy/E(spl)*-related genes is now well known to be an essential component of somite formation in fish, chicken and mouse, the generation of this dynamic expression is still not fully understood. It is, however, clear that the *Delta-Notch* signalling pathway is required. Effector genes of the *Delta-Notch* pathway, such as *Suppressor of Hairless*-related genes (Oka et al., 1995; Sieger et al., 2003), *hairy/E(spl)*-related genes (discussed above), or *lunatic fringe*-related genes (Dale et al., 2003) are involved in this process. However, there are interesting species-specific differences in the recruitment of genes for this process (Prince et al., 2001; Leve et al., 2001). This is also reflected in the phylogenetic relationships between the different oscillating *hairy/E(spl)*-

related genes. Zebrafish genes, such as *her9*, that are most similar to the respective chicken *hairy*-like genes are not expressed in the zebrafish PSM (Leve et al., 2001) while a possible orthologue of the zebrafish *her1* gene is not evident in mouse or chicken. Moreover, although *her1* and *her7* in zebrafish are closely linked, they are not the result of recent gene duplication, as each is more similar to other genes in zebrafish than they are to each other. An in depth analysis of the relationships of the *hairy/E(spl)*-related genes in the completely sequenced genomes of *Takifugu* and *Tetraodon* yielded 5 chicken *hairy*-like genes in pufferfish species and it was suggested that there are probably even more such genes in zebrafish (Gajewski and Voolstra, 2002). However, our own preliminary analysis of the zebrafish genome (assembly 06) does not support this, since we find only one copy of the chicken *hairy* homologues *her6* and *her9*. Interestingly, 3 copies of *her4* are present in the zebrafish genome and one of them is known to be expressed in the PSM (Takke et al., 1999). Since only two copies of *her4* exist in *Takifugu* (Gajewski and Voolstra, 2002) it seems that number and copy of the different *hairy/E(spl)*-related genes is highly variable even in teleost fish.

Different roles of *her1* and *her7*

The MO knockdown results suggest that *her1* and *her7* act in a common pathway, as they both affect the other's expression, as well as *deltaC* and *deltaD* expression. The effect on the latter two genes is almost indistinguishable between *her1* and *her7*. Knockdown of these bHLH proteins disrupts *deltaC* and *deltaD* expression (Fig. 4). Since overexpression of *her1* leads to a decreased transcript level of these *delta* genes (Takke and Campos-Ortega, 1999), the results are consistent with the proposed role of a Her-linked *Delta-Notch* feedback loop (Holley et al., 2002; Oates and Ho, 2002).

However, different mutual effects are observed for *her1* and *her7*, on each other, as well as on their own expression. Lack

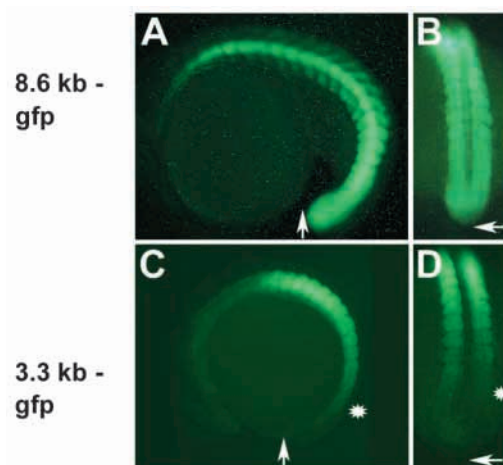


Fig. 8. GFP reporter gene protein expression in two transgenic lines monitored by fluorescence. Transgenic lines with (A,B) the 8.6 kb construct, (C-D) the 3.3 kb construct. Whole-mount embryos in lateral view, anterior to the left in A and C, and dorsal view, anterior to the top in B and D. Arrows mark the tip of the tailbud. Stars in C and D indicate the posterior border of the GFP-fluorescence in the intermediate PSM in the transgenic line with the 3.3 kb upstream region.

of Her1 protein results in a specific loss of the stripes in the intermediate and anterior PSM, while the dynamic expression in the tailbud and posterior PSM of both bHLH genes appears nearly unaffected. In contrast to previous suggestions (Holley et al., 2002; Oates and Ho, 2002), *her1* thus acts formally as an activator rather than a repressor, not only on its own but also on *her7* transcription. Since the Her1 protein possesses all features of bHLH repressors (see alignment in supplemental data at <http://dev.biologists.org/supplemental/>) and experimental evidence also supports this, another yet unidentified component must be postulated to act in intermediate and anterior PSM. Two working models seem most likely: Her1 might repress transcription of another repressor resulting in own-stripe activation, or, a modulator protein might switch the function of Her1.

The role of *her7* is more complex. Loss of Her7 protein leads to disruption of *her1* and *her7* cycling, indicating that Her7 is needed for the wave generation of both genes in the posterior PSM. Only residual tailbud expression of *her7* is visible in *her7* morphants and in intermediate and anterior PSM *her7* is very weakly expressed suggesting that stabilisation of this transcript plays a less important role than in the case of *her1* in *her1* morphants. *her1* also loses its dynamic expression upon Her7 knockdown, but is expressed throughout the PSM. This would lead to the conclusion that Her7 plays different roles in regulating the two bHLH genes in intermediate and anterior PSM, while Her1 regulates both genes in a similar manner. Formally, Her7 acts as an interstripe repressor on *her1*, but as an activator on itself. Again, Her7 displays all features known for bHLH repressors (see alignment in supplemental data at <http://dev.biologists.org/supplemental/>) suggesting that Her7-mediated activation is indirect and might involve an unknown component. Whether this component is the same as postulated for Her1-mediated activation remains to be investigated.

Separable promoter elements of *her1*

The results discussed above suggest that *her7* is specifically required to initiate the dynamic expression wave, while *her1* is required to carry it further on. This points towards a functional separation of the regulation in the posterior PSM from that in the intermediate and anterior PSM. The analysis of the promoter elements of *her1* confirms this.

Our results suggest that there are at least two distinct elements controlling the PSM expression of *her1*. One mediates a specific activation in the most posterior region of the PSM and the second mediates the expression in the intermediate and anterior PSM. Genetic analysis of various mutants (van Eeden et al., 1998; Holley et al., 2000), as well as additional experiments (Holley et al., 2002) suggest a three-phase model for the activation and action of *her1*. The first phase is activation through *deltaD* and *deltaC* in the most posterior part of the PSM. The second is the generation of the dynamical stripe pattern and the third is the stabilisation of the stripes during the early stages of somite boundary formation (Holley et al., 2002). Our transgenic lines provide support for at least the first two of these phases. A possible enhancer that is required for the activation of the cycles could be located in the region between -8.6 to -3.3 kb. This would explain the absence of the most posterior expression of GFP mRNA in transgenic embryos containing the 3.3 kb construct. We note, however, that this enhancer will still have to be better defined,

because the respective construct leads in addition to an ectopic expression in the notochord. The fact that the 3.3 kb construct specifically drives the dynamic expression of the stripes supports the notion that the second phase of expression is driven by a separate enhancer, which includes activating and repressing subelements. The presence of specific activator elements is suggested by the GFP mRNA expression pattern in the line containing the 2.8 kb construct, which shows only a broad domain, but no distinct stripes in the respective region (see Fig. 1). A distinct enhancer for the most anterior stripe, and thus evidence for the third phase of expression, was not detected in our experiments.

mRNA stability

Our promoter studies confirm the notion that the dynamic expression of the *her1* gene is caused by differential transcriptional regulation, rather than differential mRNA stability, a result that is in line with comparable experiments on *lunatic fringe* regulation in the mouse (Cole et al., 2002; Morales et al., 2002). However, it is clear that the *her1* mRNA must be very unstable, since it would otherwise accumulate in the PSM, like the stable GFP reporter protein. It is thus likely that there is a specific element in the mRNA that causes this instability. A specific 3'-UTR degradation signal has been identified for the gene *xhairy2a* in *Xenopus* (Davis et al., 2001). A sequence of 25 bases in the 3'-UTR of this gene seems to be necessary and sufficient for the rapid turnover. Similarities with this 25 base block are found in some other *hairy*-related mRNAs, like *c-hairy1* and human *Hes4*. However, we do not find this motif in the 3'- or 5'-UTRs of *her1* and *her7*. Interestingly, two copies of the motif occur in the 3'-UTR of *her9*, a gene that is closely related to the *c-hairy1* gene, but that is not expressed in the PSM of zebrafish (Leve et al., 2001).

The mRNA of our GFP reporter gene appears to be equally unstable as the endogenous *her1* mRNA. This reporter gene contains only the 5'-UTR of *her1*, suggesting that the destabilisation signal must be located there. Also the fact that the *her1* mRNA is stabilised by the *her1^{mo}* that binds in the 5' region of the mRNA would suggest that a destabilisation signal is linked to the 5'-UTR. MOs against *her7* 5' mRNA regions stabilise coinjected *her7* mRNA as well as a GFP hybrid mRNA that contained only the *her7* 5'-UTR (Oates and Ho, 2002) indicating that the destabilisation signal for *her7* could also be linked to the 5'-UTR. In contrast, the stabilisation effect of MOs on the endogenous *her7* mRNA is apparently only weak, at least when compared to *her1* (see Fig. 4).

Models of cyclic gene regulation

Holley et al. (Holley et al., 2002) and Oates and Ho (Oates and Ho, 2002) have proposed models that integrate the effects of Delta-Notch signalling in the regulation of *her1* and *her7* in the PSM. A core component of these models is that the Her proteins repress their own transcription as well as the transcription of the *delta* genes, leading to oscillatory gene expression. Our data suggest that *her1* and *her7* do not act as repressors, but formally as activators. However, since both proteins belong to a family of bHLH proteins, which are only known as repressors, we have to postulate additional components (see above).

Lewis (Lewis, 2003) has proposed a model in which autoinhibition of *her1* and *her7* coupled with transcriptional

delay could serve as the basis of an intracellular oscillation, which would be brought into phase by Delta-Notch signalling (Jiang et al., 2000). This model is also not fully in line with our findings, because it requires a direct repression effect of both genes on themselves and a fully equivalent role of both genes. However, the model seems sufficiently flexible to allow for indirect repression and for the possibility of slightly different roles of *her1* and *her7*.

It is clear that any model that takes only Delta-Notch signalling components into account cannot be complete. Holley et al. (Holley et al., 2002) have shown that *fused somites (fss)* and *beamter (bea)* are involved in gene regulation in the anterior PSM. Furthermore it is known that an FGF gradient is required for coordinating the segmentation process and the differentiation of cells in the anterior PSM (Dubrulle et al., 2001; Sawada et al., 2001). Finally, results from the mouse indicate that the wnt-signalling pathway could be an essential component that is linked to the *Delta-Notch* pathway genes (Aulehla et al., 2003).

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