her1 and the notch pathway function within the oscillator mechanism that regulates zebrafish somitogenesis

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SUMMARY

Somite formation is thought to be regulated by an unknown oscillator mechanism that causes the cells of the presomitic mesoderm to activate and then repress the transcription of specific genes in a cyclical fashion. These oscillations create stripes/waves of gene expression that repeatedly pass through the presomitic mesoderm in a posterior-to-anterior direction. In both the mouse and the zebrafish, it has been shown that the notch pathway is required to create the stripes/waves of gene expression. However, it is not clear if the notch pathway comprises part of the oscillator mechanism or if the notch pathway simply coordinates the activity of the oscillator among neighboring cells. In the zebrafish, oscillations in the expression of a hairy-related transcription factor, her1 and the notch ligand deltaC precede somite formation. Our study focuses on how the oscillations in the expression of these two genes is affected in the mutants aei/deltaD and des/notch1, in ‘morpholino knockdowns’ of deltaC and her1 and in double ‘mutant’ combinations. This analysis indicates that these oscillations in gene expression are created by a genetic circuit comprised of the notch pathway and the notch target gene her1. We also show that a later function of the notch pathway can create a segmental pattern even in the absence of prior oscillations in her1 and deltaC expression.


Key words: Zebrafish, deadly seven, notch1, her1, Somite, Segmentation, Oscillator, Morpholino

INTRODUCTION

Somites are segments within the vertebrate embryo that are the precursors of the vertebral and muscle of the trunk and tail. These segments form sequentially from anterior to posterior, concomitant with the posterior extension of the embryo. Embryological studies first suggested the existence of a prepattern within the morphologically unsegmented presomitic mesoderm (PSM) that presages segment border formation (Aoyama and Asamoto, 1988). Subsequently, this prepattern was linked to an oscillator mechanism (Cooke, 1998; Cooke and Zeeman, 1975; Meinhardt, 1982; Meinhardt, 1986; Palmeirim et al., 1997) that causes cells of the PSM to go through repeated cycles of expression and repression of homologues of the notch pathway target gene hairy (Palmeirim et al., 1997; Holley et al., 2000; Jouve et al., 2000; Sawada et al., 2000) and homologues of the notch pathway genes lunatic fringe (lfng) (Forsberg et al., 1998; McGrew et al., 1998; Aulehla and Johnson, 1999) and delta (Jiang et al., 2000). These studies show that the cells within the PSM undergo multiple cycles of expression and repression of these genes, in a manner that is coordinated intercellularly to create stripes of mRNA expression that travel through the cells of the PSM in a posterior-to-anterior direction. This oscillation halts in the anterior PSM as the pattern is stabilized and interpreted to give rise to regular somite borders. While earlier studies had demonstrated the involvement of the notch signaling pathway in somitogenesis (Conlon et al., 1995; Dornseifer et al., 1997; Evrard et al., 1998; Hrabě Angelis et al., 1997; Jen et al., 1997; Jen et al., 1999; Kusumi et al., 1998; McGrew et al., 1998; Oka et al., 1995; Takke and Campos-Ortega, 1999; Wong et al., 1997; Zhang and Gridley, 1998), the requirement for the notch pathway in creating the oscillations in gene expression was not appreciated until more recently (del Barco Barrantes et al., 1997; Holley et al., 2000). Nevertheless, both the specific function(s) of the notch pathway in this process and the identity of the oscillator remain unknown.

after eight (aei; dld – Zebrafish Information Network), deadly seven (des), fused somites (fss), beamter (bea) and white tailmindbomb (wit) are the five genes that are necessary for normal somite formation isolated in our zebrafish genetic screen (van Eeden et al., 1996; Jiang et al., 1996). We have shown previously that aei codes for the notch ligand deltaD (Dornseifer et al., 1997; Holley et al., 2000). Moreover, we have shown that aei/deltaD is required to create the oscillating pattern of her1, but that its mRNA expression does not oscillate (Holley et al., 2000). However, none of the genes shown to be necessary to produce the oscillating pattern of mRNA expression actually oscillate themselves. Thus, it is not clear if these genes aei/deltaD in the zebrafish and Delta-like1 (Dll1)
in the mouse] constitute core components of the oscillator or if they simply are necessary to produce the oscillator readout. Furthermore, the analysis of the oscillating genes hairy and fng in the chick, and Hes1 and Lfng in the mouse suggest that neither of these genes functions within the oscillator mechanism (Palmeirim et al., 1997; McGrew et al., 1998; Forsberg et al., 1998; Aulehla and Johnson, 1999; Jouve et al., 2000). Thus, it is likewise not clear if any of the known oscillating genes are central components of the oscillator.

We show that des encodes for notch1 (Bierkamp and Campos-Ortega, 1993). Like aeltdeltaD, descnotch1 expression does not oscillate, but its protein is required for the oscillation of both her1 and deltaC expression. Using ‘morpholino’ oligonucleotides (mo), we performed a series of gene ‘knockdown’ experiments to ascertain the functions of the oscillating genes her1 and deltaC during somitogenesis. We find that both genes are required to create the oscillating pattern of her1 and deltaC expression. Further analysis of double-mutant and double-‘knockdown’ embryos indicates that the epistatic relationship between the notch pathway and her1 changes along the anterior-posterior axis of the PSM. This demonstrates that these notch pathway genes have at least two functions during somitogenesis and that these genes operate within a notch pathway→her1→notch pathway regulatory circuit (Takke and Campos-Ortega, 1999). Because this circuit is comprised of genes that are necessary to create the oscillations in gene expression, these data suggest a model in which both the notch pathway and her1 comprise part of the oscillator that regulates zebrafish somitogenesis.

**MATERIALS AND METHODS**

**Fish work**

Fish were raised as described elsewhere (Haffter et al., 1996). Embryos were derived from natural crosses at 28°C.

**Mapping**

Radiation hybrid mapping was performed as previously described (Geisler et al., 1999). For mapping of des, PCR reactions for specific SSLPs were performed as for the radiation hybrid mapping but at half the volume per reaction.

**Allele sequencing**

For each allele of des, PCR products derived from three independent reverse transcriptase (RT) reactions were sequenced using the ABI system and analyzed using the Lasergene software package. Total RNA was isolated from mutant embryos using TriStar reagent (Angewandte Gententechnologie Systeme GmbH) according to the kit protocol. RT-PCR was performed using the SuperScript kit (GIBCO BRL). From each RT reaction, the notch1 mRNA was amplified in nine overlapping 1 kb fragments. Current allele designations relate to the originals (van Eeden et al., 1996) as follows: desAXO1B, desC201; desH35B, desH35B, desP37A, desP37; desM145B, desM145.

**Morpholino injections**

Morpholinos (Gene-Tools, http://www.gene-tools.com) were injected at the one-cell stage at a concentration of 50 μM (deltaCm1), 5'-agacctacgctttgcctgtgtcgtc-3', 50 μM (deltaCm2), 5'-agacatctcgctttgcctgtgtcgtc-3', 250 μM (deltaCm3), 5'-cgatagcagactgtgagagtagtcc-3'; 100 μM (deltaCm4), 5'-aaacagctatcattagtcgtcccat-3'; and 100 μM (notch1m1), 5'-ttcaacgaaagctgtggcagcgtctca-3', 1 mM (her1m1), 5'-cgacctacgtggatggtactca-3', 1 mM (her1m2), 5'-cgattgacacttactgg-actaatca-3') and 100 μM (her1m2, 5'-tggtcaaatcagaaagctgtggcagcgtctca-3') in 1×Danieau (Nasevicius and Ekker, 2000).

**In situ hybridization**

In situ hybridization experiments were performed as previously described (Holley et al., 2000).

**RESULTS**

**deadly seven is notch1**

Examination of both the morphology of the somitic mesoderm and gene expression within the PSM has failed to identify any clear difference between des and aeltdeltaD, suggesting that des also encodes for a notch pathway gene. Genetic linkage between des and notch1 was found using a zebrafish microsatellite map to position des and an anchored radiation hybrid map to position notch1 (Fig. 1C) (Geisler et al., 1999; Knapik et al., 1996; Knapik et al., 1998; Postlethwait et al., 1998; Shimoda et al., 1999).

We then sequenced the notch1-coding region from four alleles of des and found premature stop codons in two alleles and amino acid substitutions in the other two alleles (Fig. 1D).

![Fig. 1. des is notch1. Morphological phenotypes of (A) wild-type and (B) desP37A embryos at about the 15-somite stage. des embryos form the first seven to nine somites but not the posterior somites. Anterior is leftwards. (C) des was mapped to linkage group 21 between z20701 and z7925, while notch1 was mapped between z27387 and z7925 (z15810). Genetic distance from the top of linkage group 21 (left) is given in cm. (D) Four independent alleles of des were sequenced. desAXO1B has a 7 bp insertion (5'-TGTTGCCG-3') between bases 2738 and 2739, creating a frame-shift and premature stop, seven codons to the C terminus. desH35B has a T to A transition at base 4552, converting a Cys to a stop. desP37A has a T to A transition at base 186, creating a Leu to Gln substitution within the hydrophobic domain of the signal peptide (SP). desM145B has a G to A transition at base 6683, causing a Val to Met substitution. There are no obvious differences between these alleles in the severity of the somite phenotype. Nucleotide and amino acid sequences refer to the published wild-type sequences (Bierkamp and Campos-Ortega, 1993). TM, transmembrane domain.](image-url)
indicates that the differences between the data in F and G are 25.25 pixels (3.1 cells) with a 95% C.I. from 18.7 to 31.7 pixels.

deltaC

Consecutive stripes decreases as the somite cycle progresses. The difference between the means in F and G is broken lines. The mean values for F and G were compared using a two-sample t-test. The difference between the means in F and G is not statistically significant (Fig. 2F-I). These studies indicate that the distance between the anterior borders of consecutive deltaC stripes decreases with the progression of the somite cycle: at the early 12-somite stage, this distance averages about 11 cells (Fig. 2F), while 20-25 minutes later at the late 12-somite stage, this distance averages seven to eight cells (Fig. 2G). This is the behavior that we have seen with her1 expression, and, accordingly, double in situ analysis of her1 and deltaC expression indicates that their oscillating expression patterns in the PSM superimpose (not shown).

Neither des/notch1 nor aei/deltaD expression oscillates

des/notch1 mRNA is expressed uniformly throughout the PSM (Fig. 2A) (Bierkamp and Campos-Ortega, 1993). Thus, des/Notch1 expression does not oscillate, but its protein is necessary for the oscillation of both her1 and deltaC expression (Fig. 3FL) (van Eeden et al., 1998; Holley et al., 2000; Jiang et al., 2000). While our previous analysis suggested that deltaD expression did not oscillate, several recent papers state that deltaD expression oscillates, although there are no data in the literature to support this conclusion. In light of these discrepancies, we compared the expression of deltaD with the oscillating expression of deltaC (Jiang et al., 2000) using the same protocol that we had previously used to show that her1 expression oscillates (Holley et al., 2000). We staged embryos morphologically at the early and late 12 somite stage and performed double in situ analysis. Embryos at both the early and late 12 somite stages were probed for either myoD and deltaC expression (Fig. 2B,C) or myoD and deltaD expression (Fig. 2D,E). These embryos were flat mounted and digitally photographed. Using Adobe Photoshop, we measured the distances between the anterior of the deltaC or deltaD stripe immediately posterior to the 13th MyoD stripe (‘13’ in Fig. 2B-E) and the next posterior stripe (‘14’ in Fig. 2B-E). This converts the in situ data into numerical data (distance in pixels) that can be analyzed statistically (Fig. 2F-I). These studies indicate that the distance between the anterior borders of consecutive deltaC stripes decreases with the progression of the somite cycle: at the early 12-somite stage, this distance averages about 11 cells (Fig. 2F), while 20-25 minutes later at the late 12-somite stage, this distance averages seven to eight cells (Fig. 2G). This is the behavior that we have seen with her1 expression, and, accordingly, double in situ analysis of her1 and deltaC expression indicates that their oscillating expression patterns in the PSM superimpose (not shown). Our previous timelapse analysis revealed no anterior cell compaction or cell migration within the PSM before somite formation, indicating that the stripes of expression are moving through the cells of the PSM. Similar to her1 expression, the anterior most deltaC stripe is immediately posterior to the 13th myoD stripe (Fig. 2C) and (Holley et al., 2000). As myoD is expressed in the posterior half-somite, this deltaC stripe must correspond to the anlage of the next anterior half-somite. Subsequently however, deltaC expression is found in the posterior half of each somite (Jiang et al., 2000), meaning that deltaC expression reverses polarity during the course of morphological somite formation.

In contrast to deltaC, we found no evidence that aei/deltaD expression oscillates. The more posterior deltaD stripe was never seen in the early 12-somite stage embryos but was formed in the late 12-somite stage embryos (Fig. 2D,E). This indicates that each deltaD stripe forms relatively late in each somite cycle. Additionally, the distance between the two deltaD stripes does not vary significantly (the data points in Fig. 2I are tightly clustered) indicating that, once formed, the stripes do not move. In the posterior tailbud, deltaD expression exhibits much less variation in expression than that observed in her1 and deltaC expression, and no clear distinction in this posterior expression can be made when comparing early and late 12-somite stage embryos (not shown). We therefore think that the slight posterior variation in deltaD expression is random and not due to the activity of the oscillator. In summary, while deltaD expression undergoes cyclical changes each somite cycle, the formation of the deltaD stripes is a process that is specific to the anterior PSM and is distinct from the oscillating expression of her1 and deltaC. This distinction is also seen in the anterior PSM of fss embryos, where deltaD expression persists, but her1 and deltaC expression is lost (van Eeden et al., 1998; Holley et al., 2000; Jiang et al., 2000).

The oscillations in gene expression are dependent upon both her1 and deltaC

Because we do not have zebrafish mutants that correspond to either of the oscillating genes, her1 and deltaC, we have used a reverse genetic approach to ascertain the function of these genes in generating the oscillating pattern. Morpholino oligonucleotides specifically inhibit the translation of their target mRNAs (Nasevicius and Ekker, 2000), and here we show that injection of morpholinos specific to either deltaD or notch1, can recapitulate the phenotype of aei and des, respectively, with over 90% penetrance (Fig. 3A,B,F,L).
Fig. 3. Injection of morpholinos specific to notch1, deltaD, her1 or deltaC perturbs somite formation. Embryos injected with (A) notch1 mo (four experiments; n=201; 97% affected) or (B) deltaD mo (four experiments; n=127; 99% affected) form the anterior seven to nine somites but fail to make regular posterior segments. (C,D) Dorsal views of her1 mo1 (4 experiments n=200; 91% affected) or deltaC mo1 (6 experiments; n=545; 78% affected) injected embryos, respectively. In contrast to aei/deltaD and des/notch1, her1 and deltaC are necessary for the formation of both the anterior and posterior somites. Arrowheads in D indicate the misplaced somite borders. (E-P) The expression patterns of her1 and deltaC seen in wild-type, bea and morpholino-injected embryos. These embryos are between the 8 and 12 somite stages. Anterior is upwards. (E,K) Wild-type expression patterns of her1 and deltaC, respectively. Injection of Notch1mo causes defects in her1 expression (F) (two experiments; n=133; 100% affected) and deltaC expression (L) (two experiments; n=38; 100% affected) identical to that observed in des embryos. Injection of deltaD mo recapitulates the pattern of gene expression that is observed in aei/deltaD embryos (not shown). Injection of deltaC mo1 disrupts her1 expression (G) (four experiments; n=199; 98% affected) and deltaC expression (M) (6 experiments; n=168; 100% affected). Injection of a second deltaC morpholino, deltaC mo2, that does not overlap the sequence of deltaC mo1, produces the same defect in the expression of both her1 (H) (four experiments; n=145; 100% affected) and deltaC (N) (four experiments; n=187; 100% affected). Conversely, a control morpholino identical to deltaC mo1, except for four nucleotide substitutions, deltaC moC, has no effect on the expression of either her1 (I) (three experiments; n=62; 0% affected) or deltaC (O) (3 experiments; n=56; 0% affected). (J,P) Expression of her1 and deltaC, respectively, in beaM98B embryos. beaM98B embryos were collected from a mating of homozygous beaM98B adults. deltaD expression is the same in all of the mutants and knockdown embryos, with the exception of ~15% of fss embryos, as previously noted (not shown) (Holley et al., 2000). For F-H and L-N, percentages are in reference to n, the number of pre-sorted morphologically affected embryos examined. The specificity of the individual morpholinos is illustrated by the fact that: (1) both the deltaD mo and notch1 mo phenocopy their known mutant phenotypes; and (2) deltaC mo1 and deltaC mo2 produce the identical phenotype, while deltaC moC produces no phenotype.

Injection of morpholinos specific to either her1 or deltaC leads to irregular somite border formation (Fig. 3C,D), and examination of gene expression indicates that both genes are necessary to generate the oscillating pattern of her1 and deltaC expression (Fig. 3G,H,M,N; Fig. 4B,D). The expression patterns that are observed in deltaC mo embryos are somewhat similar to the patterns observed in the existing mutants (Fig. 3). However, the expression patterns seen in her1 mo embryos are unique. In her1 mo embryos, her1 is expressed throughout the PSM and shows no variation in levels of expression between neighboring cells (Fig. 4B; see http://www.eb.tuebingen.mpg.de/papers/holley_dev_2002.html). This pattern reveals no evidence of oscillations in gene expression, indicating that Her1 protein is required to generate the oscillations in expression of her1 mRNA. deltaC is expressed weakly in the posterior and intermediate PSM of her1 mo embryos and more strongly in the anterior PSM. Again, there is no heterogeneity in the levels of expression of deltaC among neighboring cells in these embryos, except for the refinement seen in the anteriormost PSM (Fig. 4D, Fig. 5i; see http://www.eb.tuebingen.mpg.de/papers/holley_dev_2002.html). Therefore, her1 function also is necessary to generate the oscillations of deltaC expression.

Multiple requirements for notch signaling during somitogenesis

Gain-of-function experiments have suggested the existence of a notch pathway→her1→notch pathway regulatory loop within the zebrafish PSM (Takke and Campos-Ortega, 1999). Our loss-of-function analysis of mutant and morpholino-injected embryos shows that the notch pathway (aei/deltaD, des/notch1 and deltaC) acts upstream of her1 to promote her1 expression, and that her1 feeds back on the notch pathway to regulate deltaC expression. An additional series of epistasis experiments independently demonstrate the existence of this regulatory loop by showing that the notch pathway functions both upstream and downstream of her1 in the anterior PSM.

The first set of epistasis experiments uses the fss phenotype as a reference. fss is unique among the known zebrafish genes in that it functions not in creating the oscillating pattern but in maintaining this pattern in the anterior PSM. In fss embryos, one or two her1 (and deltaC) stripes are present, but the anteriormost stripe is always missing (Fig. 5C) (van Eeden et al., 1998; Holley et al., 2000). Analysis of fss/des/notch1 and fss/aei/deltaD double mutants indicated that the ‘salt and pepper’ expression of her1 in the anterior PSM of aei/deltaD and des/notch1 embryos is dependent entirely upon fss function (Fig. 5B-D) (van Eeden et al., 1998; Holley et al., 2000). This indicates that fss activity is required in the anterior PSM in the absence of des/notch1 and aei/deltaD. Thus, in the anterior PSM, fss functions downstream of des/notch1 and aei/deltaD. Ectopic expression of her1 in the anterior PSM is also observed in deltaC mo embryos, her1 mo embryos and bea embryos (Fig.
Fig. 4. Loss of her1 function eliminates all evidence of the oscillations in gene expression. These embryos are between the 8 and 12 somite stages. In all panels, anterior is upwards. (A) The wild-type her1 expression pattern is observed in all embryos injected with a control morpholino, her1moC, that is identical to her1mo1, except for four nucleotide substitutions (four experiments; n=182; 0% affected). (B,B’) injection of her1mo into wild-type embryos leads to a de-repression of her1 expression (three experiments; n=76; 100% affected). (B”,B’’’) injection of a second her1 morpholino, her1mo2, which does not overlap the sequence of her1mo1, produces the identical defect in her1 expression (three experiments; n=128; 100% affected). Notice that there is no heterogeneity in the levels of expression between neighboring cells. (C) Wild-type expression pattern of deltaC is seen in embryos injected with her1moC (three experiments; n=162; 0% affected). (D,D’) in embryos injected with her1mo1, deltaC expression is reduced throughout the posterior and intermediate PSM (three experiments; n=77; 100% affected). In the anterior PSM, deltaC is expressed in a smooth domain that undergoes a refinement in the anteriormost PSM. This refinement appears to originate from the anterior and creates stripes of deltaC expression that can be later seen in the somitic mesoderm. (D”,D’’) injection of her1mo produces the identical defect (three experiments; n=122; 99% affected). (E-E’’) The refinement of deltaC expression is lost in her1mo1;aeildeltaDAR33 embryos. Additionally, the stripes of deltaC expression in the somitic mesoderm are lost.

3G,H,J; Fig. 4B). We have found that this anterior expression is lost in fss:deltaCmo embryos and fss:bea embryos but not fss:her1mo embryos (Fig. 5E-G). Therefore, while fss functions downstream of both deltaC and bea, her1 is the only gene found so far that functions downstream of fss in the anterior PSM.

The second set of epistasis experiments makes use of a unique feature of the deltaC expression pattern in her1mo embryos: the strong domain of deltaC expression in the anterior PSM is refined, resulting in stripes of deltaC expression that persist in the somitic mesoderm (Fig. 4D; Fig. 5I). These stripes resemble the stripes of deltaC expression seen in wild-type embryos (Fig. 3K, Fig. 4C, Fig. 5H). We have used this refinement of deltaC expression in her1mo embryos as an assay to test for additional functions for fss and the notch pathway in the anteriormost PSM, downstream of her1.

her1 is epistatic to fss with regard to deltaC expression in the anterior PSM [i.e. deltaC, like her1, is expressed in the anterior PSM of her1mo:fss embryos (Fig. 5J) but not fss embryos (Jiang et al., 2000)]. However, the refining of the deltaC expression domain observed in her1mo embryos is lost (compare Fig. 4D and Fig. 5I with Fig. 5J). Thus, while her1 acts downstream of fss with regard to the maintenance of deltaC expression in the anterior PSM, fss functions downstream of her1 with regard to the later refining of deltaC expression in the anteriormost PSM. Analysis of double mutants between her1mo and either aeildeltaD, desNotch1, deltaCmo or bea, indicate that each of these latter genes functions downstream of her1 in the anteriormost PSM to create the refining pattern of deltaC (Fig. 5K-N). In these double mutant embryos, this refining pattern is converted into a weak ‘salt and pepper’ pattern, and the stripes of deltaC expression in the somitic mesoderm are eliminated (Fig. 4E; Fig. 5J-M).

DISCUSSION

her1 and the notch pathway may function within the oscillator

Both aeildeltaD and desNotch1 are necessary to promote the expression of the oscillating genes her1 and deltaC. Meanwhile, her1 regulates deltaC expression and functions, directly or indirectly, in a negative feedback loop to repress its own transcription. Thus, the notch pathway functions upstream of her1 to promote the transcription of her1 mRNA, and her1 functions upstream of the Notch pathway to create the oscillating pattern of deltaC transcription. This identifies a rudimentary genetic loop (notch pathway→her1→notch pathway) that functions within the PSM [Fig. 6A (I)]. Further, we show that fss functions downstream of the notch pathway but upstream of her1 in the anterior PSM [Fig. 6A (II)], and that the notch pathway and fss function downstream of her1 slightly later in the anteriormost PSM [Fig. 6A (III)]. Therefore, the regulatory circuit consisting of her1 and the notch pathway exists throughout the PSM. Because this genetic circuit comprises genes that are required to create the oscillations in gene expression, these findings suggest that her1
and the notch pathway have cyclical functions at the center of the somitogenesis oscillator.

The genetic analysis of her1 and the notch pathway suggest a model in which these genes somehow generate the oscillations in gene expression. The initiation of the oscillations may be coupled to the commitment to become paraxial mesoderm. The expression of each of these genes (her1, deltaC, aeideltaD and des/notch1) is initiated at the tip of the tailbud as cells subduct to form the paraxial mesoderm (Bierkamp and Campos-Ortega, 1993; Müller et al., 1996; Dorneisier et al., 1997; Jiang et al., 2000; Kanki and Ho, 1996). The subsequent activities of these proteins could then initiate the interactions that create the oscillations in gene expression (Fig. 6B), deltaC, aeideltaD and des/notch1 signaling would activate the transcription of her1 and deltaC. The subsequent increase in Her1 protein would then act to block the transcription of her1. As the hairy proteins typically function as transcriptional repressors (Fisher et al., 1996; Paroush et al., 1994), an increase in Her1 should result in a decrease in the expression of hairy proteins, and a gradual degradation of this protein would produce a gradual decrease in the repressive activity of Her1. Therefore, the predominant change in gene expression could be driven by the gradual loss of a repressive activity generated during the previous somite cycle. The positive regulation via notch could also display a cyclical variation, but ultimately the re-initiation of her1 and deltaC transcription would not occur until the level of Her1 drops below a specific threshold. In essence, this model suggests that the anterior progression of a stripe of gene expression is, at least in part, driven by the degradation of an existing, repressive activity (Her1), as opposed to the de novo synthesis of an activating component.

The loss-of-function phenotype of these genes now can be explained within the context of this model. After the expression of each of these genes is initiated at the posterior of the tailbud, the resulting proteins would initiate the oscillations. If Her1 is absent, then her1 expression is never downregulated in the PSM (Fig. 4B). If aeideltaD or des/notch1 function is lost, then Her1 derived from the initial burst of her1 expression in the tailbud will repress the expression of its own mRNA, and the loss of notch signaling would then lead to a failure to re-initiate her1 transcription (Fig. 5B). The phenotypes seen in the anterior PSM in the notch pathway mutants (the ‘salt and pepper’ pattern) are likely to be the result of an anterior-specific activity.

This model is in agreement with misexpression studies in Xenopus, suggesting that periodic changes in notch signaling activity occur in the PSM (Jen et al., 1999). Our model also can explain the observation that the anterior progression of a wave of chick hairy expression is unperturbed when the PSM is physically separated into anterior and posterior halves (Palmeirim et al., 1997). The ‘gradient’ of the repressive activity of chick hairy could provide an instructive memory within the cells of the PSM, and the remaining cell-cell...
contacts would provide the required Notch-Delta signaling interactions needed to re-initiate chick hairy expression. This type of regulation would not require the oscillating signal to always be propagated from the posterior by an intercellular relay.

**notch-dependent or notch-independent oscillations?**

In wild-type embryos, neighboring cells oscillate together (they turn on her1 expression together and turn off her1 expression together). This coordination creates the stripes of her1 expression. It has been proposed that the function of the notch pathway during somitogenesis is to synchronize, not to generate, the oscillations of gene expression (Jiang et al., 2000). According to this model, perturbation of notch pathway signaling will cause the cells to lose coordination, and the cells will continue to oscillate independently of their neighbors. These de-synchronized oscillations would not create stripes of gene expression. Instead, a ‘salt and pepper’ pattern is created in which there is random heterogeneity in levels of gene expression among neighboring cells.

The phenotype of the her1m0 embryos supports the model in which her1 and the notch pathway create the oscillations in gene expression and is inconsistent with the de-synchronization model. her1 is expressed throughout the PSM in her1m0 embryos and there is no significant variation in this expression between sibling embryos, i.e. there is no evidence of coordinated oscillations. Moreover, her1m0 embryos show no variation in the levels of her1 expression among neighboring cells, i.e. there is no evidence of de-synchronized oscillations (Fig. 4B; see http://www.eb.tuebingen.mpg.de/papers/holley_dev_2002.html). The expression of deltaC in her1m0 embryos is more similar to the expression pattern seen in the notch pathway mutants: deltaC is expressed weakly in the posterior PSM and in a strong domain always found in the anterior PSM. However, this anterior expression domain of deltaC in her1m0 embryos is uniform and not in a ‘salt and pepper’ pattern, i.e. there is no evidence of asynchronous oscillations (Fig. 4D, Fig. 5I). These phenotypes indicate that the oscillations in her1 and deltaC expression do not occur if her1 function is absent.

The de-synchronization model originally suggested that the ‘salt and pepper’ patterns of her1 and deltaC expression seen in aedeltaD embryos are indicative of continued but de-synchronized cellular oscillations in gene expression (Jiang et al., 2000). However, this model does not account for the absence of stripes of gene expression within the posterior PSM of aedeltaD embryos because the ‘salt and pepper’ pattern is restricted to the anterior PSM. aedeltaD embryos do not have cells within the posterior PSM that express her1 at levels equivalent to the high levels of expression seen within the...
posterior stripes in wild-type sibling embryos (compare Fig. 5A with 5B). Therefore, there is nothing to indicate that these cells in the posterior and intermediate PSM are oscillating in the absence of aeildeltaD function (Holley et al., 2000). Furthermore, the de-synchronization model does not explain why there is an abrupt or coordinated onset of the ‘salt and pepper’ pattern within the middle of the domain in which the oscillations normally are observed. If the oscillations in her1 and deltaC expression persisted in aeildeltaD embryos, then virtually all of these embryos should exhibit a strong ‘salt and pepper’ pattern gradually arising within the more posterior PSM, as observed for her1 expression in bea embryos (Fig. 3J).

In fss embryos, the oscillations in gene expression occur, but the anteriormost stripe is always missing, indicating that fss is not required to generate the oscillating pattern but is required to maintain this pattern in the anterior PSM (Fig. 5C) (van Eeden et al., 1998; Holley et al., 2000; Jiang et al., 2000). Here, the de-synchronization model would make a simple prediction: removal of notch pathway activity in the fss background via fss:aeildeltaD and fss:des/notch1 double mutant combinations should create a de-synchronized version of the oscillating pattern observed in fss embryos, i.e. a ‘salt and pepper’ pattern instead of stripes. However, only weak posterior expression is observed in these embryos and there is no variation in levels of expression among neighboring cells (Fig. 5D; see http://www.eb.tuebingen.mpg.de/papers/holley_dev_2002.html) (van Eeden et al., 1998; Holley et al., 2000). The cells turn on her1 expression posteriorly and together, gradually lose their expression as they mature and become relatively more anterior. Thus, the loss of Notch pathway function results in an elimination, not de-synchronization, of oscillations in gene expression.

These analyses indicate that all evidence of the oscillations in her1 and deltaC expression is absent in backgrounds in which either her1 or aeildeltaD function is missing. This suggests that the generation of the oscillations and the coordination of the oscillations between cells are one and the same, and that the two processes cannot be separated. Nevertheless, one cannot exclude the possibility that the oscillations persist in these mutants in some way that is not observed and that the oscillations in gene expression are a subset of a more general, unseen oscillation.

The anterior presomitic mesoderm

A ‘salt and pepper’ expression pattern could be created by a number of patterning processes gone awry and is not indicative inherently of oscillations. In fact, non-oscillating genes such as deltaD and mesp-b also can exhibit a patchy ‘salt and pepper’ pattern in the anterior PSM of the notch pathway mutants (not shown) (Durbin et al., 2000; Sawada et al., 2000). More importantly, we know that the anterior PSM is distinct from the posterior PSM, and the analysis of fss:aeildeltaD and fss:des/notch1 embryos indicates that the strong anterior ‘salt and pepper’ expression domain of her1 and deltaC in aeildeltaD and des/notch1 embryos is dependent entirely upon fss (van Eeden et al., 1998; Holley et al., 2000) and, therefore, is dependent upon an activity specific to the anterior PSM (Holley et al., 2000). This explains why the ‘salt and pepper’ pattern is found only in the anterior PSM of aeildeltaD embryos, and also led us to propose previously that this anterior expression was induced de novo by a separate, anterior ‘wave-front activity’. The wave-front would move from anterior to posterior along the body axis as the embryo extends posteriorly. This wave-front activity requires the function of the fss gene that normally functions in the anterior PSM to maintain or stabilize the oscillating pattern emanating from the posterior tailbud. In the absence of oscillations, this wave-front activity can induce or facilitate the expression of the oscillating genes in the anterior PSM, leading to the abrupt onset of the ‘salt and pepper’ pattern in the anterior PSM of the aeildeltaD embryos (Holley et al., 2000). Recent studies performed in the chick suggest that the wave-front could correlate with a drop in the level of FGF signaling, which is highest in the posterior PSM (Dubrulle et al., 2001).

The analysis of deltaC expression in her1f^no embryos uncovers an additional Notch-dependent patterning activity in the anterior PSM. This activity can create a segmental pattern of gene expression in the absence of any evidence of oscillations in her1 and deltaC expression: a smooth domain of deltaC expression is refined anteriorly to create stripes of expression that persist in the somitic mesoderm. This refinement requires the activity of fss, aeildeltaD, des/notch1, deltaC and bea, indicating that each of these genes has an additional function in the anterior-most PSM, downstream of her1. This is consistent with the fact that aeildeltaD, deltaC and des/notch1 are each transcribed within the PSM and later in the somitic mesoderm. In fact, this refining pattern is likely to be revealed only within the her1f^no embryos because her1 is the only one of these cloned genes whose expression is restricted to the PSM (Bierkamp and Campos-Ortega, 1993; Dorneiseler et al., 1997; Jiang et al., 2000; Muller et al., 1998). Ultimately, this indicates that the phenotypes observed in aeildeltaD and des/notch1 embryos are composites of defects that occur both upstream and downstream of her1 (oscillator) function. It has been shown that notch pathway signaling is involved in establishing the anteroposterior pattern within each somite (Conlon et al., 1995; Oka et al., 1995; Evrard et al., 1998; Hrabé Angelis et al., 1997; Kusumi et al., 1998; Wong et al., 1997; Zhang and Gridley, 1998; Takahashi et al., 2000). The late activity of the notch pathway described here probably represents this same anteroposterior patterning function. What is remarkable is that this late function can create a segmental pattern in the absence of prior oscillations in her1 and deltaC expression.

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