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Accelerating the Tempo of the Segmentation Clock by Reducing the Number of Introns in the Hes7 Gene

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SUMMARY

Periodic somite segmentation is controlled by the cyclic gene Hes7, whose oscillatory expression depends upon negative feedback with a delayed timing. The mechanism that regulates the pace of segmentation remains to be determined, but mathematical modeling has predicted that negative feedback with shorter delays would give rise to dampened but more rapid oscillations. Here, we show that reducing the number of introns within the Hes7 gene shortens the delay and results in a more rapid tempo of both Hes7 oscillation and somite segmentation, increasing the number of somites and vertebrae in the cervical and upper thoracic region. These results suggest that the number of introns is important for the appropriate tempo of oscillatory expression and that Hes7 is a key regulator of the pace of the segmentation clock.

INTRODUCTION

During somitogenesis, Hes7 expression oscillates owing to delayed negative feedback in the presomitic mesoderm (PSM), and disruption of this oscillation blocks the oscillatory expression of many other genes, such as Lng and Dusp4, leading to somite fusion (Pourquie, 2011; Oates et al., 2012; Eckalbar et al., 2012; Kageyama et al., 2012; Bessho et al., 2001; Niwa et al., 2007; Sparrow et al., 2012). This suggests that Hes7 plays an important role in the segmentation clock, although it remains to be determined whether Hes7 is the fundamental pacemaker or whether it acts downstream of another oscillator. The clock period can be altered by inhibition of Wnt signaling, inhibition of Notch signaling coupling, or mutations of Nrarp in mice or hes6 in zebrafish (Gibb et al., 2009; Herrgen et al., 2010; Schröter and Oates, 2010; Kim et al., 2011). However, the expression of β-catenin, a Wnt signaling effector, does not cycle (Aulehla et al., 2008), and Nrarp and hes6 are not essential for periodic segmentation (Wright et al., 2009; Schröter and Oates, 2010; Kim et al., 2011). Thus, oscillators that are essential for segmentation are not included in the list of genes that could affect the pace of segmentation. In addition, all mutations that have been reported to date result solely in disruption or slower tempos of the segmentation clock (Oates et al., 2012; Kageyama et al., 2012; Bessho et al., 2001; Schröter and Oates, 2010; Kim et al., 2011), and such defects are also observed under other conditions, such as lower temperatures, that slow or inhibit embryogenesis in general (Jiang et al., 2000).

Mathematical modeling suggests that negative feedback with appropriate transcriptional/translational delays, which include the time required for transcription, splicing, translation, and transport of messenger RNA (mRNA) and protein products, underlies the oscillatory expression of the segmentation clock genes (Lewis, 2003; Monk, 2003; Jensen et al., 2003; Hirata et al., 2004; Zeiser et al., 2008). The sum of such delays regulates the stability and period of the oscillation, and shorter delays would both accelerate the tempo of the oscillation and dampen or abolish it (Lewis, 2003; Monk, 2003; Jensen et al., 2003). If Hes7 is the fundamental pacemaker, manipulations that increase the frequency of Hes7 oscillation should lead to a faster tempo (i.e., a shorter period) of somite segmentation. One way to shorten the delays and accelerate Hes7 oscillation is to delete the introns, because transcription and splicing of intron sequences increase the time necessary for mRNA production. The Hes7 gene has three introns, and deletion of all three introns reduces the delay by 19 min and completely abolishes oscillatory expression, leading to steady Hes7 expression and fusion of all somites (Takashima et al., 2011). Mathematical modeling suggests that such a short delay would abolish the oscillatory expression, and that a more moderate delay would give rise to more rapid but dampened oscillations (Takashima et al., 2011). We hypothesized that deletion of one or two introns from the Hes7 gene would lead to such a moderate delay, leading to shorter periodicity of somite segmentation, and proceeded to test that possibility.

RESULTS AND DISCUSSION

Increasing the Number of Cervical and Upper Thoracic Vertebrae by Reducing the Number of Introns in the Hes7 Gene

Mathematical modeling suggests that negative feedback with an appropriate delayed timing is essential for sustained oscillatory
expression, and that reduction of the delay by 19 min would abolish the oscillatory expression (Figure 1Ac). However, a more moderate delay (for example, 5 min shorter than the wild-type [WT]) would give rise to more rapid but dampened oscillations (Figure 1Ab, 8.9% shorter period than the WT shown in Figure 1Aa). We previously showed that deletion of all three introns within the Hes7 gene reduces the delay by 19 min and completely abolishes oscillatory expression (Takashima et al., 2011). Furthermore, we found that introduction of a Hes7 transgene lacking introns (pH7-Hes7-0; Figure S1) into WT mice caused severe segmentation defects in a dominant fashion even though two alleles of the WT Hes7 gene were present (Takashima et al., 2011). By contrast, introduction of the Hes7 transgene containing the three introns, which rescued the segmentation defects in Hes7 null mice, did not cause any defects in the WT background (pH7-Hes7-123; Figures S1, S2B, and S2C; Takashima et al., 2011). Taking advantage of this feature, we generated F0 transgenic mice carrying Hes7 transgenes lacking either one or two introns into the WT background and examined their vertebral segmentation. Hes7 transgenes containing either the first or third intron alone exhibited better segmentation than those carrying a transgene containing the second intron alone (Figures S2G–S2I). Interestingly, mice carrying a transgene containing the first or third intron alone had eight or nine cervical vertebrae (Figures S2G'–S2I'), whereas the WT and pH7-Hes7-123 mice had seven (Figures S2A'–S2C'). We performed subsequent experiments using the transgene containing the third intron alone (pH7-Hes7-3) because this transgene seemed to give rise to slightly better segmentations in the upper thoracic region.

We established two independent lines carrying pH7-Hes7-3 (Figure 1B) and measured Hes7 protein expression in the PSM. Line 1 expressed a high level of Hes7 protein in the PSM, whereas the level of expression by line 2 was similar to that of the WT endogenous level (Figure 1C). All line 1 mice (n = 11) exhibited eight or nine cervical vertebrae irrespective of the Hes7 background (+/+), (+/-), or (-/-), and line 2 (n = 4) exhibited seven (Figures 1Db, 1Dc, and S3). Furthermore, although the second thoracic (T2) vertebra in WT mice has a longer spinous process, this feature was instead associated with the T3 vertebra in some of the line 1 mice and all of the line 2 mice (Figures 1Dc, S3C, and S3D). These results indicate that one or two additional vertebrae formed in the cervical and upper thoracic region of the mutant compared with the control mice. When two additional vertebrae formed in line 1 of pH7-Hes7-3 mice, duplication of the first cervical vertebra (C1) and extra formation of C9 or T3 seemed...
to occur (Figures 1Db and 1Dc). Before cervical somites form, four pairs of occipital somites form. Furthermore, it has been reported that Her/Hes oscillation starts before the somite segmentation takes place in the chick (two cycles before somite formation) and zebrafish (five cycles before somite formation; Jouve et al., 2000; Riedel-Kruse et al., 2007). Thus, although it is unknown how many pulses of Hes7 oscillation occur before somite formation in mouse embryos, it is possible that at least six pulses of Hes7 oscillation occur before the first cervical somite forms. If this is the case, every six to eight somites, one extra somite could be formed in line 1. However, the vertebrae in the more-caudal region were fused in all of the pH7-Hes7-3 mice (Figure S2 I). These results suggest that the segmentation clock is set at a faster tempo during the cervical and upper thoracic segmentations but is halted during the more-caudal segmentation in mice carrying pH7-Hes7-3.

We next compared the delay in gene expression from the genes lacking all introns, containing only the third intron, and containing all three introns. We used a ubiquitinated luciferase reporter under the control of the Hes1 promoter as previously described (Takashima et al., 2011). This analysis showed that the gene containing the third intron alone expressed the reporter protein ~5 min earlier than the control gene (containing three introns) and ~13 min later than the gene containing no intron (Figure S4), which was within the expected range of in vivo splicing kinetics (Audibert et al., 2002; Singh and Padgett, 2009). Mathematical modeling predicted that such a delay caused by the control of the Hes1 promoter as previously described (Takashima et al., 2011).

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Increase of the Number and Decrease of the Size of Somites in the Anterior Region of pH7-Hes7-3 Mice

We compared the numbers of somites in pH7-Hes7-3 and control embryos at approximately embryonic day 8.5 (E8.5). At this stage, the control embryos contained 6.6 ± 0.5 somites, whereas their littermates carrying pH7-Hes7-3 had 7.4 ± 1.3 somites, indicating that the latter contained on average ~0.8 more somites than the control at this stage. However, the somite number varied between embryos even in WT mice (commonly differing by three or four somites; Tam, 1981), and thus it was difficult to determine whether the observed difference was due to normal variability or a faster tempo of the segmentation clock. We therefore counted the number of somites that formed in the anterior region at E10.5 by using the forelimb as a landmark. In situ hybridization of Uncx4.1 revealed that there were
The results suggest that the anterior border of the same region of pH7-Hes7-3 embryos (Figures 2 Ab, 2Ac, respectively, in control mice (Alexander et al., 2009), and the control and pH7-Hes7-3 embryos (Figures 2 B and 2C, Hoxb9 expression correspond to the 10th and 13th somites, respectively, in control mice (Alexander et al., 2009), they corresponded on average to the 10.9th and 14.7th somites, respectively, in the mutant (Figures 2B, 2B’, 2C, and 2C’). These results suggest that the anterior border of Hoxb6 and Hoxb9 expression was shifted caudally by one or two additional somites in pH7-Hes7-3 embryos, supporting the idea of a faster tempo of the segmentation clock. This result also indicates that the link between the segmentation clock and Hox gene activation (Zákány et al., 2001) can be easily dissociated, as observed in zebrafish hes6 mutation (Schröter and Oates, 2010).

If the segmentation clock were set at a greater frequency, the somite size would be smaller, in contrast to the larger somites observed when the clock cycles at a slower frequency (Schröter and Oates, 2010). Therefore, we compared the lengths of the three most recently formed somites between control and pH7-Hes7-3 embryos at the same stages. We found that they were reduced by 10%-20% in pH7-Hes7-3 embryos compared with the control at various stages (Figure 3). These data suggest that the tempo of somite segmentation is increased in mice carrying pH7-Hes7-3.

**Accelerated Tempo of the Segmentation Clock of pH7-Hes7-3 Mice**

To show decisively that the tempo of Hes7 oscillation and somite segmentation is accelerated in pH7-Hes7-3 embryos, we performed time-lapse imaging of whole-embryo cultures at E8.5. We examined Hes7 oscillations by using the Hes7 reporter pHeS7-UbLuc (Takashima et al., 2011; Niwa et al., 2011). In control embryos, Hes7 expression oscillated with an average period of 126.6 ± 2.0 min (Figures 4A, 4C, and 4D; Movie S1). Furthermore, somite segmentation occurred with a similar periodicity (Figure 4A; Movie S1). In contrast, in pH7-Hes7-3 embryos, Hes7 expression oscillated with an average period of 115.4 ± 1.1 min (8.8% shorter than the control), and somite segmentation also occurred with the same periodicity (Figures 4B–4D; Movie S2). These data indicate that removal of the two introns leads to a more rapid tempo of Hes7 oscillation and somite segmentation.

It has been mathematically predicted that altering the delay in negative feedback of clock genes should alter the oscillation period. Indeed, altering the intron length of an artificial oscillator gene results in alteration of the period (Swinburne et al., 2008). However, Stauber et al. (2012) recently reported that elongation of the intron length of another oscillator gene, Lfng, had no effect on the segmentation period. Thus, they were not able to alter the period by changing the delays in negative feedback of the natural clock genes. This could be because such an elongation was insufficient to cause a significantly longer delay. Alternatively, Lfng may not be a pace-making clock gene, because its oscillation is regulated by Hes7 (Bessho et al., 2001). Stauber et al. (2012) also attempted to elongate the intron length of the essential oscillator gene Hes7, but this abolished its expression, and therefore they were not able to change the period. Here, we found that reducing the number of introns in the Hes7 gene led to a faster tempo of the segmentation clock, which suggests that Hes7 is a pace-making clock gene for somite segmentation. Our study also shows that the number of introns is very important for the period and stability of oscillatory expression. Complete lack of introns abolishes oscillatory expression (Takashima et al., 2011), whereas removal of two introns leads to a more frequent but dampened oscillation. In

10.1 ± 0.1 Uncx4.1-expressing somites anterior to the posterior border of the forelimb in the control (Figures 2Aa and 2A’), whereas there were 11.4 ± 0.1 Uncx4.1-expressing somites in the same region of pH7-Hes7-3 embryos (Figures 2Ab, 2Ac, and 2A’). These results indicate that on average 1.3 additional somites form in the anterior region of the mutant. In agreement with the vertebral fusion defects in the caudal region, somites were fused caudally to the forelimb in the mutant (Figures 2Ab and 2Ac). These data suggest that the segmentation clock is set at a faster tempo but is subsequently halted during the formation of more-caudal somites.

We next examined the anterior border of Hox expression. The relative positions of the anterior borders of Hoxb6 and Hoxb9 expression to the forelimb bud were very similar between the control and pH7-Hes7-3 embryos (Figures 2B and 2C, arrowheads). However, whereas the anterior borders of Hoxb6 and Hoxb9 expression correspond to the 10th and 13th somites, respectively, in control mice (Alexander et al., 2009), they corresponded on average to the 10.9th and 14.7th somites, respectively, in the mutant (Figures 2B, 2B’, 2C, and 2C’). These results suggest that the anterior border of Hoxb6 and Hoxb9
contrast, the presence of two introns led to mostly normal segmentation, although there were some minor defects in caudal regions. Interestingly, another mouse oscillator gene, Hes5 (Takebayashi et al., 1995; Dunwoodie et al., 2002). Furthermore, the essential zebrafish oscillator genes her1 and her7 contain three and two introns, respectively (Gajewski et al., 2003). These data suggest that at least two introns are required for oscillatory expression of the segmentation clock genes, and point to the significant role of introns in the timing of gene expression.

EXPERIMENTAL PROCEDURES

Mathematical Simulation
Hes7 oscillations were simulated with the following equations as previously described (Hirata et al., 2004):

\[
\frac{dp(t)}{dt} = am(t - T_p) - bp(t)
\]

\[
\frac{dm(t)}{dt} = f(p(t - T_m)) - cm(t)
\]

where \( p(t) \) and \( m(t) \) are the quantities of functional Hes7 protein and Hes7 mRNA per cell at time \( t \), respectively; \( f(p) \) is the rate of initiation of transcription, which depends on the amount of the protein, \( p \), present at the time of initiation; \( a \) is the rate constant for translation; and \( b \) and \( c \) are the degradation rate constants for Hes7 protein and Hes7 mRNA, respectively, which are related to the half-lives of the molecules:

\[
b = \frac{\ln 2}{T_p} \quad c = \frac{\ln 2}{T_m}
\]

Because transcription is inhibited by a dimer of Hes7 protein, we assume

\[
f(p) = \frac{k}{1 + \left( \frac{p}{p_{\text{crit}}} \right)^2}
\]

where \( k \) is the number of molecules of Hes7 mRNA synthesized per unit time in the absence of inhibition, and \( p_{\text{crit}} \) is the amount of protein that gives half-maximal inhibition. We set \( a = 4.5 \) protein molecules per mRNA molecule per min, \( p_{\text{crit}} = 40 \) molecules per cell, \( k = 33 \) mRNA molecules per cell per min, \( T_p = 20 \) min, \( T_m = 8 \) min, and \( T_{m} = 29 \) min. Under these conditions, oscillatory expression continues (Figure 1Aa). In contrast, when \( T_{m} = 10 \) min (19 min shorter), oscillations are abolished (Figure 1Ac). When \( T_{m} = 24 \) min (5 min shorter), oscillations occur at a faster tempo but soon dampen (Figure 1Ab).

Transgenic Mice

The Hes7 transgene with three introns (pH7-Hes7-123) consisted of the genomic fragment of the Hes7 promoter region (5,393 bp upstream fragment from the first codon), a hemagglutinin (HA) tag fragment at the amino terminus, and a genomic sequence from the second codon to 76 bp downstream of the putative polyadenylation signal. To remove one or two introns, the intron-exon regions were replaced with Hes7 cDNA fragments. For the intronless Hes7 transgene (pH7-Hes7-0), the whole Hes7 coding and intron regions were replaced with Hes7 cDNA. Transgenic mice were generated by injecting linearized constructs without any vector sequence into the pronucleus of fertilized eggs. Mice carrying the Hes7 reporter pH7-UbLuc-In(−) were previously described (Takashima et al., 2011).
Genotyping was performed by PCR using the following primers: WT mice: 5'-AGAAAGGGCGAGGAGAATGGGCGAGCCAC-3' and 5'-GTTCGAAGCAGGAGGCTTGCTGGGATGG-3'  
Hes7 null mice: 5'-AGAAAGGGCGAGGAGAATGGGCGAGCCAC-3' and 5'-TTGGCTCAGCCCCGGGATCCACTAGTTGC-3'  
pH7- Hes7-3 mice: 5'-CTGACAGATTCCTATGTC-3'  
5'-CCCAAGGCTCTTCTTCTG-3'  
Western Blotting

The PSM parts of four embryos were mixed with 15 μl of lysis buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM MgCl2, 0.5% NP-40, 1x proteinase inhibitor cocktail, 1 mM phenylmethanesulfonylfluoride, 250 U/ml Benzonase) and incubated on ice for 30 min. After addition of 1.5 μl of 10% SDS, the samples were boiled and the protein concentrations were measured. The protein solution was boiled in sample buffer and then run on 12.5% SDS-PAGE. After the protein was transferred from the gel to polyvinylidene fluoride membrane (Millipore), the membrane was immersed in buffer containing 5% skim milk, anti-Hes7 antibody (1/300; Bessho et al., 2003), and other equipment as described previously (Masamizu et al., 2006).  

Bone and Cartilage Staining

Bone and cartilage of neonates were stained with alizarin red and alcian blue, respectively, as described previously (Bessho et al., 2001).  

REFERENCES


