The elongation rate of RNA polymerase II in zebrafish and its significance in the somite segmentation clock

Anja Hanisch1,*, Maxine V. Holder1,*, Suma Choorapoikaiyil2,3, Martin Gajewski2,4, Ertuğrul M. Özbudak5,‡ and Julian Lewis1,‡

SUMMARY

A gene expression oscillator called the segmentation clock controls somite segmentation in the vertebrate embryo. In zebrafish, the oscillatory transcriptional repressor genes her1 and her7 are crucial for genesis of the oscillations, which are thought to arise from negative autoregulation of these genes. The period of oscillation is predicted to depend on delays in the negative-feedback loop, including, most importantly, the transcriptional delay – the time taken to make each molecule of her1 or her7 mRNA. her1 and her7 operate in parallel. Loss of both gene functions, or mutation of her1 combined with knockdown of Hes6, which we show to be a binding partner of Her7, disrupts segmentation drastically. However, mutants in which only her1 or her7 is functional show only mild segmentation defects and their oscillations have almost identical periods. This is unexpected because the intact embryo. This rate is unexpectedly rapid, at 4.8 kb/minute at 28.5°C, implicating that, for both genes, the time taken for transcript elongation is insignificant compared with other sources of delay, explaining why the mutants have similar clock periods. Our computational model shows how loss of her1 or her7 can allow oscillations to continue with unchanged period but with reduced amplitude and impaired synchrony, as manifested in the in situ hybridisation patterns of the single mutants.

KEY WORDS: Somite, Segmentation clock, Zebrafish, her1, her7, RNA polymerase II

INTRODUCTION

The pace of embryonic development and differentiation depends on the delays involved in switching gene activities on or off. These delays are generally difficult to measure in the intact organism. In some situations, however, genes switch on and off repeatedly in a regular, oscillatory fashion. The period of oscillation – the length of a tick of the molecular clock – is then a precisely defined measurable quantity. Such oscillations are typically driven by delayed negative feedback in the control of gene expression, and the length of the period reflects the length of the delays in the feedback loop. To understand how such a gene expression clock works, we need to know how and where the delays occur. Examination of this question not only leads to an understanding of the oscillator, but also provides insight into the nature and magnitude of gene switching delays in general.

The segmentation clock is a prime example of this type of system. This gene expression oscillator governs demarcation of the boundaries between the embryonic rudiments of the vertebrate body axis, the somites, which are generated sequentially in head-to-tail order from the precomitent mesoderm (PSM) at the tail end of the embryo (Palmeirim et al., 1997; Pourquié, 2011). Each somite consists of the cohort of cells that emerge from the PSM in the course of one oscillation cycle. Thus, the cyclic operation of the segmentation clock is recorded, writ large, in the spatially periodic structure of the vertebral column.

The set of oscillatory genes varies between species (Krol et al., 2011), but in all species includes member(s) of the hairy/E(spl) family as the one common element. In zebrafish, two linked members of this family, her1 and her7 (Gajewski et al., 2000), have been identified as central to the genesis of oscillations. When both are deleted (Henry et al., 2002) or blocked by morpholinos (Gajewski et al., 2003; Henry et al., 2002; Oates and Ho, 2002) all signs of oscillation are lost and segment boundary formation is disrupted all along the body axis. Although other members of the Her gene family, including her2, her4, her12 and her15 (Krol et al., 2011; Shankaran et al., 2007; Sieger et al., 2004), also display oscillatory expression in the zebrafish PSM, none of these has so far been shown to be essential for operation of the clock in the same way as her1 and her7.

her1 and her7 encode bHLH inhibitory transcriptional regulators that negatively regulate their own expression and that of each other (Giudicelli et al., 2007; Hoffer et al., 2002; Oates and Ho, 2002). Mathematical modelling has demonstrated that direct autoinhibition of her1 and her7 by their own products could be the mechanism that gives rise to oscillating gene expression (Fig. 1), provided that certain conditions are satisfied (Lewis, 2003). In particular, the transcriptional and translational delays are critical – that is, the time taken to make each molecule of her1 or her7 mRNA, from initiation of transcription to delivery into the cytoplasm, and the time taken from initiation of translation of each molecule of Her1 or Her7 protein to its arrival at its binding site in the nucleus. For sustained oscillations, the lifetimes Tm and Tp of the mRNA and protein molecules must be short compared with the sum of these delays. If the conditions for oscillation are met, the period of oscillation, T, to a good approximation is given by:

\[ T = 2(T_m + T_p + \tau_m + \tau_p) \]
This formula is derived for the idealised case of a single ‘her1/7’ autoinhibitory gene or, equivalently, of a pair of genes, her1 and her7, that have the same delays, lifetimes and regulation. Computer modelling shows that if her1 and her7 have somewhat different delays and lifetimes but are co-regulated, oscillations will occur with a period that is a compromise between that for a pure her1 oscillator and that for a pure her7 oscillator. Mutants in which her1 remains intact but her7 is functionally null, or vice versa, have recently become available, and in this paper we use them to test this prediction. Because the her1 and her7 genes are very different in length (Fig. 2A), we anticipated that they should have different transcriptional delays, leading to different periods of oscillation. To our surprise, we found that the difference of period is actually very small. To resolve this paradox, we measured the elongation rate of RNA polymerase II (RNA Pol II), for the first time in vivo in a vertebrate. The value, as measured in the PSM cells of the zebrafish, is 4.8 kb/minute at 28.5°C. This unexpectedly high rate means that the time taken to transcribe the two genes is so short as to be insignificant in comparison with other sources of delay, such as the time required for splicing. These findings reconcile our theory with the experimental observations and remove an important objection to the proposition that her1 and her7 are pacemakers of the segmentation clock.

MATERIALS AND METHODS

Ethics statement

Animal experiments were approved by the CRUK London Research Institute Ethical Review Committee (ref. JLE1706) and the UK Home Office (Project Licence 80/2081 held by J.L.).

Fish stocks, mutant and transgenic fish lines

Adult zebrafish (Danio rerio) were kept on a regular light-dark cycle (14 hours on/10 hours off) at 27°C. Embryos were maintained at 28.5°C. The her1hu2124 and her7hu2625 mutant lines (Busch-Nentwich et al., 2010) (S. Choorapoikayil, PhD Thesis, University of Cologne, 2008; C. Schröter, PhD Thesis, Technische Universität Dresden, 2010) were generated at the Hubrecht Laboratory as part of the ZF-MODELS project. b567 was a gift from Sharon Amacher (Henry et al., 2002).

The b21 DNA construct used for the generation of TgBAC_BX537304(her1:dEGFP)c1 transgenic fish was created by standard recombination techniques (Liu et al., 2003) using CH211-283H6 (http://bacpac.chori.org/) as the host BAC. This BAC contains the complete her1/7 locus plus adjacent sequences. The her7 second intron was split into two halves by insertion of intronic DNA from human dystrophin (DMD) intron 74-75 (excluding the first 300 and last 324 bases, total length 21.3 kb). dEGFP (Clontech) was inserted in place of the translated region of her1, preserving her1 5’ and 3’ UTRs. The BAC construct included an I

Fig. 1. The proposed core gene circuit of the zebrafish segmentation clock. Oscillations arise from delayed negative-feedback regulation of the her1 and her7 genes by their own protein products.

Fig. 2. Differences in her1 and her7 gene length might be expected to lead to differences in transcriptional delay. (A) The genomic locus of zebrafish her1 and her7. Black arrows indicate direction of transcription. (B-D) The her1/7 feedback loops in wild-type (B), her1–/– (C) and her7–/– (D) embryos.

Scel site to facilitate integration. The transgenic fish were generated by injection of the DNA (with I-Scel meganuclease) into fertilised eggs as described previously (Ozbudak and Lewis, 2008). We confirmed by PCR that the entire modified her7 locus, including the inserted intronic sequence, was present in the transgenic fish (supplementary material Fig. S2). The transgene has now been transmitted in simple Mendelian fashion through many generations and is clearly present only at a single locus in the genome, as is also evident from the number of nuclear dots marking sites of transcription of the transgene in fluorescent in situ hybridisation (FISH) specimens.

Probes and in situ hybridisation

We used RNA probes for published genes as follows: her1 (Takke and Campos-Ortega, 1999), her7 (Henry et al., 2002; Oates and Ho, 2002), deltaC (Jiang et al., 2000) and cb1045 (xirp2a – Zebrafish Information Network) (Riedel-Kruse et al., 2007). To detect the different parts of the artificial intron in the b21 transgenic fish, we used two intronic probes: probe A11 (899 nt) is complementary to the initial part of the artificial intron in the her7–/– transgene, from nt 551 of the intron to nt 1450; probe A12 (998 nt) recognises a middle segment of that intron, from nt 10,953 of the intron to nt 11,951.

For non-fluorescent ISH we used standard NBT/BCIP protocols (Oxtoby and Jowett, 1993). For FISH, specimens were hybridised with fluorescein-labelled probes and, in the case of double FISH, simultaneously with digoxigenin-labelled probes according to standard protocols. Bound fluorescein-labelled probe was detected using peroxidase-conjugated anti-fluorescein sheep antibody (Perkin Elmer; 1/125) and the TSA Plus Fluorescein system (Perkin Elmer). For double FISH, peroxidase activity
was then destroyed by a dehydration series in methanol and incubation for 30 minutes in 100% methanol with 1% H$_2$O$_2$, followed by rehydration. Then, embryos were incubated with peroxidase-conjugated anti-

digoxigenin mouse antibody (Jackson ImmunoResearch Laboratories; 1/1000) and peroxidase activity was detected using the TSA Plus Cyanine 3 system (Perkin Elmer). Specimens were counterstained with DAPI, mounted in SlowFade Gold (Invitrogen), and imaged with a Zeiss LSM700 or LSM510 confocal microscope.

Immunoprecipitation and western blotting

Full-length cDNAs of her1, her7, hes6 and mib were cloned in-frame 3’ with 6X-Myc tag or 1X-FLAG tag coding sequences, with an additional linker sequence encoding amino acids GAGAGA interposed between the 5’ tag and the start codon. The pCS2+MT vector (http://sitemaker.umich.edu/dlttimer.vectors/home) was used as backbone. All constructs were sequenced by cloning.

HEK293 cells were co-transfected with different combinations of plasmids coding for full-length N-terminally tagged Her1, Her7, Hes6 and Mib using TransIT-LT1 reagent (Mirus Bio). Forty-eight hours after transfection, cells were harvested in lysis buffer (50 mM Hepes pH 7.4, 175 mM NaCl, 0.5% Triton X-100, 0.03 µg/µl DNase and RNase). For immunoprecipitation, cell extracts were incubated with protein G Sepharose beads (Sigma) and anti-Myc 9E10 monoclonal antibody (CRUK LRI Cell Services). For western blots, we used either rabbit anti-FLAG or the anti-Myc 9E10 antibody and the ECL+Plus western blotting detection system (GE Healthcare).

Calcine staining and vertebra counts

Living zebrafish aged 21-28 days were immersed in calcine solution [Sigma-Aldrich, 21030-IQ; 0.2% (w/v) in aquarium water] for 10 minutes, rinsed through several containers of clean aquarium water, and then left to swim in clean aquarium water for at least a further 30 minutes, during which time they lost calcine from their soft tissues leaving only their skeletons stained. To count vertebrae, fish were anaesthetised in tricaine solution and observed under fluorescence illumination using a Leica Fluoro III stereomicroscope with FITC filter set. We counted the first Weberian vertebra as vertebra 1 and the urostyle as the last vertebra (Bird and Mabee, 2003).

Mathematical modelling

We used Mathematica (Wolfram Research) for computer modelling of her1/her7 segmentation clock dynamics. We extended our previous two-cell model (Lewis, 2003) to simulate an array of many cells with noisy oscillatory dynamics, with parameters roughly as in Giudicelli et al. (Giudicelli et al., 2007) and with Notch signalling assumed to work as in Ozbudak and Lewis (Ozbudak and Lewis, 2008) to keep neighbouring cells synchronised. Noise arises in the model from the stochastic nature of the association/dissociation reaction between the regulatory proteins and the regulatory DNA, modelled explicitly as a probabilistic (random) process (Lewis, 2003). The model is adjusted to take account of the evidence that Her7 functions as a heterodimer with Hes6; this involves a slightly changed assumption about the stoichiometry of gene regulation by Her1 and Her7, with Her7 binding to the regulatory DNA as a pair of heterodimers with Hes6 (i.e. as a Her7_Hes62 complex). For details of the model, the Mathematica program and output (including Movies 1 and 2), see supplementary material Model 1.

RESULTS

In her1$^{-/-}$ and her7$^{-/-}$ mutants, oscillations of gene expression in the PSM are disturbed but somite defects are mild

Morpholino experiments (Gajewski et al., 2003; Henry et al., 2002; Holley et al., 2002; Oates and Ho, 2002) have indicated that loss of function of her1 or her7 leads to segmentation defects, affecting anterior somites for her1 and posterior somites for her7, but in each case allowing some regions of normal segmentation. However, morpholinos can have misleading effects. We have therefore re-examined these results using loss-of-function mutants.

The mutant alleles her1hu2124 and her7hu2526 have point mutations that create stop codons in the HLH dimerisation domain (for her1hu2124, S46>Stop; for her7hu2526, K37>Stop). Both are therefore expected to be functionally null. Homozygotes of each type are viable but have reduced fertility. To compare homozygous embryos with wild-type and heterozygous siblings, we examined progeny of homozygote × heterozygote or heterozygote × heterozygote crosses. In each batch of embryos, a subset was clearly abnormal when analysed by ISH, corresponding to the expected Mendelian proportion of homozygotes with 100% penetrance. We took these to be the homozygotes.

A full description of the her1hu2124 and her7hu2526 phenotypes is given elsewhere (Choorapukayil et al., 2012); here we describe the main features relevant to our present theme. The segmentation phenotypes of the homozygotes are compared with those of the wild type in Fig. 3A-C. In her1 mutant embryos, we saw no segmentation defects apart from occasionally irregular boundaries of the anteriormost somites (Fig. 3B). In her7 mutant embryos, anterior somite boundaries up to at least the tenth were unaffected; defects were confined to the posterior trunk and tail region, where somite boundaries were still visible and for the most part properly spaced, but often irregular, broken or incomplete (Fig. 3C). Individual embryos of the same mutant genotype varied in the extent of segmentation defects (supplementary material Fig. S1). These abnormalities are similar to those reported in morphants (Gajewski et al., 2003; Henry et al., 2002; Oates and Ho, 2002), although somewhat less severe.

Despite the mildness of the segmentation phenotypes, the expression patterns of segmentation clock components in each of the mutants were distinctly abnormal in much the same way as reported previously for morphants. We assessed these patterns by ISH in whole mounts, using both standard NBT/BCIP staining (Fig. 3D-L’), for comparison with previous studies, and tyramide chemistry with fluorescent detection (Fig. 3M-U’), which allowed optical sectioning. Three main points emerged. First, both her1$^{-/-}$ and her7$^{-/-}$ mutants show clear signs of continuing coordinated oscillation in the expression of all three oscillatory genes (her1, her7 and deltaC) as manifest in variation between siblings (reflecting fixation in different phases of the oscillator cycle) and in the presence of stripes (for her7 and deltaC at least) in the anterior PSM. Second, the number of PSM stripes is reduced, especially in the her1$^{-/-}$ mutants (compare Fig. 3G-I’ with 3D-F’ and 3P-R’ with 3M-O’). This implies that the process of oscillator slowing prior to arrest as cells approach the anterior boundary of the PSM is spread out over a smaller number of oscillator cycles [supplementary information Box 2 in Gomez et al. (Gomez et al., 2008)]. In other words, the cells halt their cycling more abruptly than in wild type. Third, in the her7$^{-/-}$ mutants the ISH pattern, especially that of her1 (Fig. 3J’,S,S’), is less regular and more noisy than normal and shows less variation between siblings, hinting at some loss of cell-cell synchronisation.

Hes6 is required as a binding partner for Her7

Combined loss of function of her1 and her7 results in a failure of regular somite boundary formation all along the embryonic body axis and in failure of the oscillations of deltaC (Gajewski et al., 2003; Henry et al., 2002; Oates and Ho, 2002). Interestingly, however, double knockdown of her1 and the non-cyclic gene hes6 (her13.2) results in the same severe segmentation phenotype (Sieger et al., 2006). Her1 and Hes6 proteins have been reported to bind to one another (Kawamura et al., 2005), suggesting that Hes6 might be required for the function of Her1 and/or Her7. To clarify
**Fig. 3.** *her1* and *her7* embryos show mild defects in somite boundary formation and disturbed expression of oscillatory genes in the PSM. (A-C) Zebrafish embryos fixed at ~24 hpf and stained with cb1045 ISH probe to show somite boundaries (lateral views). (A) Wild type (siblings of *her7*−/−mutant); (B) *her1*−/−; (C) *her7*−/−. Arrows indicate irregularities of segmentation. (D-U) Expression patterns of *her1*, *her7* and *deltaC* in wild type, *her1*−/− and *her7*−/− as shown by conventional ISH (D-L’ and FISH (M-U’; optical sections). Embryos are at the 10-somite stage (14 hpf) and flat mounted. Two specimens from each cohort are shown to illustrate the oscillatory behaviour. Note that the ISH phenotype, like the segmentation phenotype (supplementary material Fig. S1), varied in severity from one batch of embryos to another (compare K,K’ with T,T’). A total of 243 embryos were stained and assessed, including at least 12 for each gene/genotype combination.

In this situation, we transiently transfected HEK293T cells with expression constructs coding for tagged versions of Her1, Her7 and Hes6, and analysed their patterns of association by co-immunoprecipitation. In this overexpression situation, we saw all possible homo- and heterodimeric combinations of Her1, Her7 and Hes6, but with very different preferences: whereas Her1 preferentially homodimerised, Her7 mainly heterodimerised with Hes6 (Fig. 4A).

Thus, in contrast to the previous report (Kawamura et al., 2005), our *in vitro* data suggest that Her1 functions mainly as a homodimer, whereas Her7 functions mainly as a heterodimer with Hes6. Our findings agree with recent data from Trofka et al. (Trofka et al., 2012) (published after the initial submission of our manuscript): they analysed the dimerisation of a larger set of zebrafish Her family proteins, and similarly concluded that the functional dimers are Her1-Her1 homodimers and Her7-Hes6 heterodimers. This explains why deficiency of *her1* and *hes6*, but not deficiency of *her7* and *hes6*, gives the same segmentation phenotype as deficiency of *her1* and *her7* (Sieger et al., 2006) (Fig. 4B). These findings are consistent with the idea that *her1* and *her7* are the central oscillatory components of the segmentation clock but function quasi-redundantly.

**her1 and her7 are each capable, in the absence of the other, of sustaining oscillations with almost exactly the normal period**

According to our previous theory (Lewis, 2003), the period of oscillation of the segmentation clock should depend on the delay in the *her1/her7* negative-feedback loop, and a major component of the delay is expected to be the time taken to make a mature transcript. However, the *her1* and *her7* genes differ substantially in length (6392 bp and 1304 bp, respectively, from the start of the 5’ UTR to the end of the 3’ UTR) (Fig. 2A); thus, one might expect that they should have different transcriptional delays. A commonly quoted textbook value (e.g. Alberts et al., 2008) for the RNA Pol II elongation rate is 1.2 kb/minute, implying that transcription of *her1* should take 4.25 minutes longer than that of *her7*. This discrepancy, by itself, would entail that the period of a pure *her1* oscillator should be ~8.5 minutes longer than that of a pure *her7* oscillator, with the period of the full *her1/her7* oscillator lying somewhere in between (Fig. 2B). Assuming that the total duration of somitogenesis is independent of *her1* and *her7*, this line of argument would imply that the total number of somites generated should be substantially larger (~15%, corresponding to four or five additional somites) for a *her1* mutant than for a *her7* mutant.

This is not what we found. We counted the number of segments in *her1*−/− and *her7*−/− mutants, both in 48-hpf embryos stained by ISH for the somite boundary marker *cb1045* (Riedel-Kruse et al., 2007) (Fig. 3A-C; supplementary material Fig. S1) and in young fish stained with calcein (Fig. 5). The mean total numbers of somites or vertebrae formed by the mutants were almost identical to the wild-type values, differing by ≤3% (Table 1).

These observations as to the clock period in *her1*−/− and *her7*−/− mutants, as well as our finding that Her7 heterodimerises preferentially with Hes6, agree with the recent findings of Schröter et al. (Schröter et al., 2012), whose paper appeared after the initial submission of our manuscript.

**The b21 line contains a her7 transgene with normal exons and regulatory elements but an artificially enlarged intron**

To test more directly whether the oscillation period actually depends on the length of the *her1* or *her7* gene, and with the aim of obtaining proof that these genes are pacemakers of the clock, we created a transgenic zebrafish line in which the *her7* gene has been

**DEVELOPMENT**
artificially lengthened. For this, we took a BAC containing the full-length her1 and her7 genes together with their regulatory DNA, and inserted 21 kb of intronic DNA from the human dystrophin (DMD) gene into the second intron of her7, creating a modified her7 gene that we called her7b21. To avoid complications from the continued presence of functional her1, and to provide, at the same time, a useful fluorescent reporter, we also replaced the her1 coding sequence with d1EGFP (destabilised enhanced GFP; Fig. 6A). We injected the resulting construct into embryos to derive the transgenic line TgBAC_BX537304(her1:d1EGFP)cj1 or b21 for short [which we previously used for the her1:d1EGFP reporter function (Ozbudak and Lewis, 2008)]. We anticipated that by crossing these with b567 mutants, which lack endogenous her1 and her7 (Henry et al., 2002), we could obtain fish in which oscillations were generated by the her7b21 transgene acting alone, with a period that was altered according to the extra time required for transcription of the giant intron.

Although we succeeded in creating b21;b567−/− embryos, the her7b21 transgene failed to rescue the b567 deficiency. The giant intron failed to be spliced out correctly, almost no normal her7 mRNA was produced, and we were unable to detect any sign of oscillations (data not shown).

Spatial offsets of the in situ hybridisation patterns with different probes allow the measurement of relative delays in transcription

The b21 transgenic line did, however, enable us to measure how fast RNA Pol II moves as it transcribes a gene in the intact zebrafish embryo – that is, to determine the RNA Pol II elongation rate.

The stripy pattern of expression of the oscillatory genes in the PSM is a manifestation of a slowing of the oscillations as cells are displaced from the tail bud towards the anterior boundary of the PSM (Lewis, 2003; Palmeirim et al., 1997). The distance from peak to peak of the spatial stripe pattern (the spatial wavelength) corresponds to a phase difference of one complete cycle. If two events within a typical oscillating cell are separated in time by a certain fraction of an oscillation cycle, then they will be seen, in a fixed specimen, in cells separated in space by that same fraction of a spatial wavelength. In this way, knowing the relationship between the period of oscillation and the position of the cells in the PSM, we can use measurements on fixed tissue to deduce the time interval between events of the oscillation cycle (Giudicelli et al., 2007).

This, of course, requires a means of making these events visible. For our present purposes we used fluorescent ISH (FISH) with pairwise combinations of four different probes: probe AI1 (899 nt), which recognises an initial part of the artificially enlarged intron in her7b21; probe AI2 (988 nt), which recognises a middle segment of that intron, centred on a point 10,452 nt downstream from the centre of the site recognised by probe AI1 (Fig. 6A); and probes H1 and H7, which are complementary to full-length (spliced) mRNA from the endogenous her1 and her7 genes, respectively.

Our main focus was on embryos that contained the b21 construct but were otherwise wild type. As expected, these embryos possessed a normal functional segmentation clock, manifest in normal somitogenesis and normal stripy PSM patterns of endogenous Her gene expression as revealed with probe H1 and H7, which are complementary to full-length (spliced) mRNA from the endogenous her1 and her7 genes, respectively.

For our present purposes we used fluorescent ISH (FISH) with pairwise combinations of four different probes: probe A11 (899 nt), which recognises an initial part of the artificially enlarged intron in her7b21; probe A12 (988 nt), which recognises a middle segment of that intron, centred on a point 10,452 nt downstream from the centre of the site recognised by probe A11 (Fig. 6A); and probes H1 and H7, which are complementary to full-length (spliced) mRNA from the endogenous her1 and her7 genes, respectively.
stained with calcein to visualise calcified tissues. These fish are 21-28 days old and measure 10-12 mm from head to tail. Most her7⁻/⁻ fish have skeletal abnormalities affecting their posterior skeleton, such as missing or bifurcated ribs, irregularly sized vertebrae, and malformation and/or misalignment of the neural and haemal spines. her1⁻/⁻ fish rarely have posterior skeletal defects, but often exhibit defects in their anterior vertebrae (e.g. an extra Weberian vertebra, not visible in this specimen). Mean vertebra counts are the same within 3% for the three genotypes (see Table 1).

RNA Pol II in zebrafish progresses at 4.8 kb/minute (at 28.5°C)

To estimate the RNA Pol II elongation rate, we took embryos fixed at the 10- to 12-somite stage, stained them by double FISH with probes AI1 and AI2, and compared the distributions of the two sets of nuclear dots seen in optical sections (Fig. 6C-E). The anterior boundary of each stripe of staining with a given probe reflects the time of onset of production of the pre-mRNA sequence recognised by that probe. The spatial offset between the anterior boundaries of staining with probes AI1 and AI2 therefore reflects the time taken by RNA Pol II to travel the 10,452 bp from the AI1 site to the AI2 site. We measured this offset from confocal images (Fig. 6) and computed the corresponding time intervals, expressing the result as a fraction of the fundamental clock period T₀ (the time taken to make a somite) (see Materials and methods). In case the labelling and fluorescence detection chemistry might distort the result, we repeated the experiment with the labelling reversed. With probe AI1 detected as red fluorescence and probe AI2 as green, we obtained t₁₀kb/T₀=0.100±0.008 (mean ± s.e.m.; n=21). With the labelling reversed, the result was t₁₀kb/T₀=0.088±0.034 (n=8). Combining these two datasets, we get t₁₀kb/T₀=0.097±0.011 (n=29). For zebrafish at 28.5°C, T₀=22.6 minutes (Schröter et al., 2008), implying that the RNA Pol II elongation rate at this temperature is 4.8±0.5 kb/minute.

DISCUSSION

Mathematical modelling predicts oscillations of reduced amplitude and impaired synchrony when her1 or her7 is mutated

Our study of the her1 and her7 mutants confirms that the two genes function quasi-redundantly in the segmentation clock; whereas loss of both genes disrupts segmentation drastically, when either is functionally null then segments form with only minor irregularities. This was unsurprising and consistent with prevailing theory. We were, however, surprised to see that in the single mutants the expression patterns of her1, her7 and deltaC were distinctly abnormal, although they still showed signs of coordinated oscillation. Similar findings have been reported in morphants (Gajewski et al., 2003; Henry et al., 2002; Oates and Ho, 2002). Does the theory explain this aspect of the mutant phenotypes?

To answer this question, we extended our original model (Lewis, 2003; Ozbudak and Lewis, 2008) to describe an array of many oscillatory cells that are individually noisy and coupled and synchronised through Notch signalling (see Materials and methods and supplementary material Model 1). Fig 7 shows the predictions, comparing the wild type with a mutant in which either her1 or her7 is defective. With the chosen parameters, we see that loss-of-function mutation of her1 or her7 indeed still permits collective oscillations, but these have impaired synchronisation and reduced peak-to-trough ratio, matching our experimental observations (see Fig. 3).

Table 1. Numbers of vertebrae/segment boundaries in wild-type, her1⁻/⁻ and her7⁻/⁻ zebrafish

<table>
<thead>
<tr>
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<th>Wild type</th>
<th>her1⁻/⁻</th>
<th>her7⁻/⁻</th>
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<tbody>
<tr>
<td>Calcein-stained juveniles</td>
<td>31.4±0.5 (n=228)</td>
<td>31.5±0.7 (n=76)</td>
<td>32.2±1.2 (n=84)</td>
</tr>
<tr>
<td>cb1045 ISH of embryos</td>
<td>31.5±0.8 (n=226)</td>
<td>31.0±1.1 (n=38)</td>
<td>30.4±1.2 (n=86)</td>
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Shown are the number of segments (mean ± s.d.) in the three genotypes. Specimens were stained either with calcein as juveniles or by ISH with a cb1045 probe as 48-hpf embryos. Mutants and wild-type sibling controls were individually genotyped by PCR. The slight (<3%) differences between mutant and wild-type values might reflect inaccuracies in counting segments when these are malformed (see Figs 3, 5); note the increased s.d. for the her7⁻/⁻ mutants.
resulting in the formation of more or fewer segments. But our expectations were confounded: somite and vertebra counts showed there was little difference in the number of segments formed by embryos of the three genotypes, providing a strong indication that their clocks run at practically the same speed. So which part of our theory was wrong?

One questionable assumption was that a major contribution to transcriptional delay came from the time taken by RNA Pol II to transcribe her1 or her7 from beginning to end. We measured the RNA Pol II elongation rate in the PSM of intact zebrafish embryos and found that it is much faster than previously supposed. As discussed in detail below, it follows that the time taken for transcript elongation (for her1 and her7) is so short as to make no significant contribution to the total transcriptional delay. This resolves the clash between theory and observation, and means that her1 and her7 remain strong candidates to be joint pacemakers of the segmentation clock.

Support for the role of her1 and her7 as pacemakers comes from the recent demonstration that the clock period is altered in mutants lacking functional Hes6 (Schröter and Oates, 2010), which we have shown here, in agreement with others (Trofka et al., 2012; Schröter et al., 2012), to be a dimerisation partner of Her7.

It remains conceivable, however, that in conjunction with the her1/7 transcriptional oscillator and somehow coupled to it, the zebrafish PSM cells might contain an additional molecular oscillator of some different type. The Wnt and Fgf pathway oscillations that occur in parallel with Hes7 oscillations in the mouse PSM seem to provide an example of this phenomenon (Aulehla et al., 2003; Giudicelli and Lewis, 2004; Dequéant et al., 2006). Studies of circadian clocks have revealed other precedents. For example, the cyanobacterium Synechococcus possesses both a transcriptional oscillator and an enzymatic phosphorylation/dephosphorylation oscillator that is independent of transcription; these are normally coupled, but each can generate a circadian rhythm in the absence of the other (Kitayama et al., 2008; Zwicker et al., 2010). Moreover, many organisms, from bacteria to mammals, contain a non-transcriptional oxidation/reduction circadian oscillator that operates in parallel with a transcriptional circadian oscillator (Edgar et al., 2012). Biological clocks, it seems, can have several pacemakers that are loosely coordinated and function together to provide a more reliable measure of time. Thus, while the evidence is compelling that the her1/7 transcriptional feedback loop is a pacemaker of the zebrafish segmentation clock, it might not be the only one.

The RNA Pol II elongation rate is much faster than previously supposed

We have been able to measure the RNA Pol II elongation rate in the zebrafish embryo by taking advantage of the synchronised waves of transcription that occur during somitogenesis. This is, to our knowledge, the first measurement of this quantity in an intact vertebrate. Our value of 4.8±0.5 kb/minute for zebrafish at 28.5°C is roughly four times faster than estimates commonly quoted in textbooks and reviews (Alberts et al., 2008; Bentley, 2005;
Neugebauer, 2002) for eukaryotes in general. It does, however, agree with recent findings for mammalian cells in vitro: 4.3 kb/minute (Darzacq et al., 2007); 3.1 kb/minute (Wada et al., 2009); 3.8 kb/minute (Singh and Padgett, 2009); and 3.3 kb/minute (Ben-Ari et al., 2010).

The time taken for transcription is insignificant compared with splicing and export delays
Several steps contribute to the total transcriptional delay $T_{\text{m}}$: (1) promoter activation; (2) transcription; (3) splicing; (4) 3'-end processing and polyadenylation; (5) release of the transcript from the site of transcription; and (6) exit through a nuclear pore. $T_{\text{m}}$ is not simply the sum of these individual delays, however, because they are not simply sequential. In particular, splicing and transcription occur concurrently, and there is no necessity for splicing of a given intron to be completed before splicing of the next intron downstream can begin (Brody et al., 2011; Kessler et al., 1993; Neugebauer, 2002; Singh and Padgett, 2009; Tennison et al., 1995; Wada et al., 2009).

At the rate that we have measured, the polymerase should complete the synthesis of pre-mRNA for her1 (6392 nt) in only 1.6 minutes, for her7 (1304 nt) in just 0.3 minutes, and for deltaC (4854 nt) in only 1.2 minutes. Splicing is likely to be slower. For cultured mammalian cells, reports of the time taken to splice out an intron once it has been transcribed range from 0.4 to 12 minutes, depending on the intron but regardless of intron length (Audibert et al., 2002; Kessler et al., 1993; Singh and Padgett, 2009; Zeisel et al., 2011). A reasonable compromise estimate for the time taken to splice would thus be 5 minutes.

Completion of splicing is coupled with, and may coincide with, completion of 3'-end processing and polyadenylation and release of the transcript from attachment to the gene (steps 4 and 5 above) (Bird et al., 2005; Brody et al., 2011; Custódio et al., 1999). Following this, the time $T_{\text{export}}$ (step 6) has been estimated (for β-globin mRNA in 3T3 cells) at 4 minutes (Audibert et al., 2002).

Hence, assuming a splicing delay of 5 minutes and export time of 4 minutes, we arrive at the following theoretical estimates for the total transcriptional delays that occur subsequent to promoter activation:

$T_{\text{her1}}=1.3 \text{ minutes} + T_{\text{her1}} + T_{\text{export}} = 10 \text{ minutes},$  
$T_{\text{her7}}=0.1 \text{ minutes} + T_{\text{her7}} + T_{\text{export}} = 9 \text{ minutes},$  
$T_{\text{deltaC}}=1.0 \text{ minutes} + T_{\text{deltaC}} + T_{\text{export}} = 10 \text{ minutes},$

where $T_{\text{her1}}$, $T_{\text{her7}}$, and $T_{\text{deltaC}}$ are the times taken to splice out the introns from each gene. The first term in each case represents the time taken for the polymerase to complete transcription up to the end of the final intron, at which point splicing of this intron can begin. The splicing reaction (if it takes 5 minutes) will not be completed until after the polymerase has finished transcribing, as the terminal exons of all three genes are relatively short. We assume that introns are spliced concurrently and that the splicing of no single intron is so slow compared with that of the last intron as to be rate limiting.

Given the uncertainties in the extrapolation of splicing times and nuclear export times from cultured mammalian cells to intact zebrafish, these timings should be regarded as rough estimates only. As such, they are consistent with the observed value of the clock period and with our formula for the period as a function of the delays in the negative-feedback loop (Giudicelli et al., 2007; Lewis, 2003). We conclude that there is no reason to expect the period of a pure her1 oscillator and that of a pure her7 oscillator to be significantly different.

The splicing delay may be a major determinant of oscillator period and thus of somite spacing and number
Our measurements suggest that the time required to splice out introns constitutes a large component of the total transcriptional delay. Supporting evidence comes from recent experiments (Takashima et al., 2011) focusing on Hes7, which is the mouse counterpart of zebrafish her1 and her7. The introns in Hes7 were found to be responsible for a 19-minute delay in expression of the gene, relative to the expression of an artificial intronless version, and the presence of these introns was shown to be essential for oscillating Hes7 expression, as expected from the theory if a large part of the transcriptional delay depends on them (Hirata et al., 2004; Lewis, 2003).

The zebrafish segmentation clock shows exceptionally rapid switching of gene expression. We have shown that, even on this
time scale, RNA Pol II moves so rapidly that the time taken to transcribe the oscillator genes is insignificant compared with the other delays and molecular lifetimes that set the tempo of gene switching. The same is likely to be true, even more emphatically, for the generality of gene switching events. Most types of mRNA and protein molecules have a half-life of many hours [median value 9 hours for mammalian mRNAs, 46 hours for mammalian proteins (Schwanhäusser et al., 2011)], whereas the time taken to transcribe a gene of average length (28 kb (Lander et al., 2001; Venter et al., 2001)) should be less than 10 minutes. Thus, gene length is likely to make a negligible contribution to switching delays for the majority of genes, and its contribution is insignificant even in determining the period of the zebrafish segmentation clock.

Acknowledgements

We thank Sharon Amacher for a major part of the problem; Michael Staubert for DMD intronic DNA; David Iosh-Horowicz and Ned Hoyle for discussions; our aquarium staff for excellent fish care; Emel Esen for initial optimisation of FISH for the intronic probes; and Stephan Knierer for help in characterisation of the b21 transgene insertion.

Funding

The work was funded by Cancer Research UK and EMBO Long-Term Fellowships for A.H. and E.M.O.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at http://dev.biologists.orglookup/suppl?doi=10.1242/dev.077230/D1C1

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