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Autoinhibition with Transcriptional Delay: A Simple Mechanism for the Zebrafish Somitogenesis Oscillator

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Summary

Background: The pattern of somites is traced out by a mechanism involving oscillating gene expression at the tail end of the embryo. In zebrafish, two linked oscillating genes, *her1* and *her7*, coding for inhibitory gene regulatory proteins, are especially implicated in genesis of the oscillations, while Notch signaling appears necessary for synchronization of adjacent cells.
Results: I show by mathematical simulation that direct autorepression of *her1* and *her7* by their own protein products provides a mechanism for the intracellular oscillator. This mechanism operates robustly even when one allows for the fact that gene regulation is an essentially noisy (stochastic) process. The predicted period is close to the observed period (30 min) and is dictated primarily by the transcriptional delay, the time taken to make an mRNA molecule. Through its coupling to *her1*/*her7* expression, Notch signaling can keep the rapid oscillations in adjacent cells synchronized. When the coupling parameters are varied, however, the model system can switch to oscillations of a much longer period, resembling that of the mouse or chick somitogenesis oscillator and governed by the delays in the Notch pathway. Such Notch-mediated synchronous oscillations are predicted even in the absence of direct *her1*/*her7* autoregulation, through operation of the standard Notch signaling pathway that is usually assumed simply to give lateral inhibition.
Conclusions: Direct autorepression of a gene by its own product can generate oscillations, with a period determined by the transcriptional and translational delays. Simple as they are, such systems show surprising behaviors. To understand them, unaided intuition is not enough: we need mathematics.

Introduction

Somites, the future segments of the vertebrate body axis, are laid down in head-to-tail succession. The somite rudiments are marked out in alternating bands of expression of genes that distinguish anterior from posterior portions of somites. This pattern, like the trace on a magnetic recording tape, represents the record of a temporal oscillation, manifest in cyclic changes in levels of gene expression in the cells of the presomitic mesoderm, or PSM, from which the somites derive (Figure 1) [1, 2]. As growth continues at the tail end of the embryo, cells exit from the anterior end of the PSM and cease oscillating, probably because of a decline in the concentration of Fgf8 when the distance from the tail bud becomes large [3]. According to the phase of the oscillation cycle in which they become arrested, the cells leaving the PSM switch on sustained expression of different sets of genes, marking them as front end or back end of a nascent somite.

The oscillation in the PSM is called the somite *clock*, and the moving interface between the PSM and the determined somite tissue is called the somitogenesis *wavefront*; somite patterning is thus said to depend on a *clock and wavefront* mechanism [4]. A single complete somite rudiment consists of the set of cells that emerge from the PSM in the course of one complete clock cycle, that is, in one complete cycle of the posterior PSM oscillation. What then is the mechanism that drives this oscillation and defines its timing; in other words, how does the clock work?

The Oscillator Depends on Components of the Notch Signaling Pathway

In the zebrafish, the only genes so far reported to cycle in the posterior PSM code for components of the Notch signaling pathway or for downstream genes whose expression is regulated by Notch signaling. Thus, *deltaC*, coding for a Notch ligand, oscillates, and so do *her1* and *her7*, coding for inhibitory bHLH gene regulatory proteins of the Hairy/E(spl) family that are controlled by Notch [5–8]. The mRNA levels of these genes go up and down synchronously in the posterior PSM with a period of 30 min (at 28°C), corresponding to the time taken to generate one additional somite.

The set of genes known to be required for normal oscillations in the posterior PSM is somewhat larger, but, again, all or almost all of them lie in the Notch pathway [5–11]. All this suggests that the oscillator, in zebrafish at least, is somehow based on Notch pathway components.

In the Zebrafish, PSM Cells May Continue to Oscillate Even When out of Synchrony with Their Neighbors

A gene whose expression oscillates may be merely a downstream read-out of the core oscillator, like the hands on a mechanical clock. Moreover, a gene required for oscillation may merely code for necessary supporting apparatus, like the clock frame, cogs, and bearings. The problem is to identify the genes whose products actually generate the oscillations.

In mutants in which Notch signaling is disrupted, the pattern of *deltaC* expression, although abnormal, does not correspond to what one might expect for a nonfunctional oscillator. The cells in the PSM, instead of all adopting a maximal, minimal, or at least uniform level of expression of *deltaC*, show a pepper-and-salt mixture

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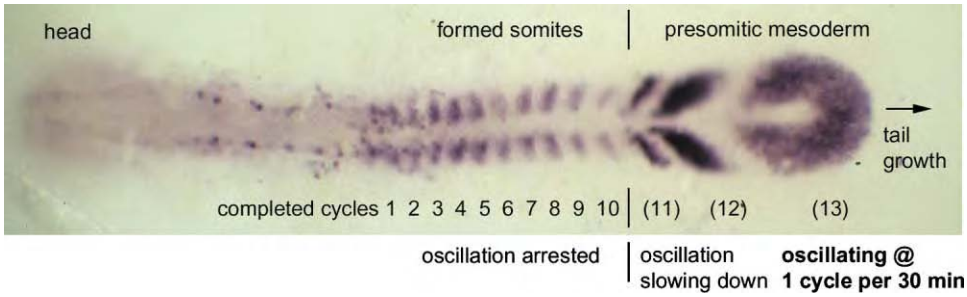

Figure 1. A Zebrafish Embryo at the 10-Somite Stage, Stained by In Situ Hybridization for *deltaC* mRNA, Coding for the Notch Ligand DeltaC

The expression pattern of this gene is like that of *her1* and *her7*: oscillation occurs in the PSM and is arrested as cells become recruited into the region of formed somites. The arrest is not abrupt, but it is foreshadowed by a gradual slowing of the oscillation in the transition region (the anterior PSM). This gives rise to a graded phase lag, such that cells in different parts of the PSM at any given instant are in different phases of their oscillation cycle. The maximal rate of oscillation occurs at the tail end of the PSM, and this defines the periodicity of the whole process: in the course of one oscillation cycle of the posterior PSM, the entire spatiotemporal pattern of gene expression in the PSM goes through a complete cycle of changes and returns to its initial configuration, but with an increase of one in the number of formed somites ([1], Appendix). The n^{th} somite consists of cells arrested after the n^{th} cycle since somitogenesis began. (Photograph courtesy of Yun-Jin Jiang.)

of different levels of expression: the appearance is what one would expect if the cells were individually continuing to oscillate almost normally but had lost synchrony [5]. This suggests that some intracellular mechanism generates oscillations in each cell, and that the function of cell-cell communication via the Notch pathway is to synchronize neighbors so that they oscillate in phase with one another.

If there is such an intracellular oscillator mechanism, how might it work? Oscillations in biological systems are typically generated by negative feedback loops [12]. As explained in Figure 2, the length of the delay in the feedback loop dictates the period of the oscillation. One might therefore hope to find at the heart of the oscillator some molecule that, with a delay, directly or indirectly inhibits its own production or activation. The feedback loop should be intracellular, so that oscillation can occur even without any coordinated periodic prompting from neighboring cells, as it seems to do in Notch pathway mutants in which the neighbors are in a random assortment of different phases. On the other hand, the oscillators in neighboring cells must normally be capable to some extent of influencing one another via the Notch pathway, so that when the pathway functions, synchrony is preserved. Thus, the central oscillating component should be sensitive to signals from neighbors, delivered via the Notch pathway, so as to bring its own oscillation into step with theirs; and it should itself drive an oscillation of the signals for activation of Notch—for example, oscillation of expression of the Notch ligand Delta—so that the cell can reciprocally tell its neighbors what phase of the oscillation cycle it is itself in.

Cell-Autonomous Oscillations in the Zebrafish May Be Generated by *her1* and/or *her7*

Recent papers have identified *her1* and *her7* as a pair of genes in the zebrafish that jointly satisfy all these conditions to be central components of the oscillator. Both genes code for bHLH gene regulatory proteins in the E(spl)-related subfamily of the hairy/E(spl) inhibitory class [13]; they lie only 11 kb apart in the genome, with opposite polarity and possibly sharing common regulatory elements [14, 15]. Their levels of expression, as indicated by their mRNA concentrations, normally oscillate in synchrony in the PSM, with a periodicity and phase matching those of *deltaC*, as though the mRNA levels of all three genes are coordinately regulated [8]. When both *her1* and *her7* are lost through a chromosomal deletion [9], or when both gene products are knocked down by morpholino injections, oscillation breaks down: *deltaC* expression in the PSM fails to oscillate, the spatially periodic pattern of expression of marker genes in the maturing somite tissue is lost, and the physical pattern of somite segmentation becomes grossly irregular. Morpholino knockdown of *her1* by itself or *her7* by itself produces similar but weaker effects [6, 8, 9]. Both genes are positively regulated by Notch signaling [8, 10], and, most importantly, they appear to negatively regulate their own expression [6, 8]. The evidence for this negative autoregulation in the zebrafish comes mainly from studies of *her1* and *her7* mRNA expression in the *her1* or *her7* morpholino knockdown experiments. Although there is some debate about these experiments and there may be differences between *her1* and *her7* in the way they function in the anterior and posterior parts of the PSM [8, 15], the evidence for the corresponding gene in the mouse is clear cut. Like *her1* and *her7*, the mouse *Hes7* gene, the ortholog of *her1*, shows oscillating expression in the PSM and is necessary for regular somite segmentation; and studies with a *lacZ* knockin mutation, as well as protein stabilization experiments, show clearly that Hes7 protein inhibits *Hes7* expression [16, 17]. As Holley et al. [6], Hirata et al. [18], and Bessho et al. [17] have emphasized, negative feedback regulation of this sort provides the basis for an oscillator.

Direct Autoinhibition of Gene Expression Can Generate Oscillations

The simplest possibility one might envisage would be a feedback loop in which Her1 or Her7 protein directly binds to the regulatory DNA of its own gene to inhibit transcription. Indeed, direct binding of Hes7 protein to the *Hes7* promoter has been demonstrated in the mouse

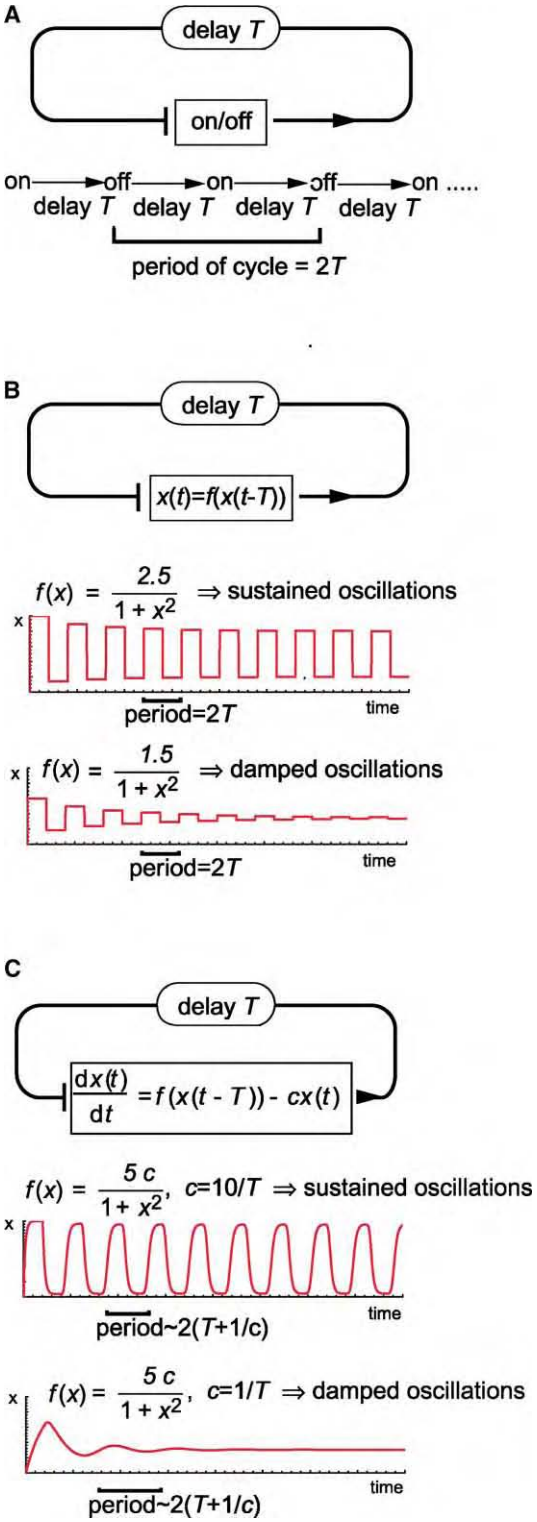

Figure 2. Idealized Oscillators Illustrating Basic Principles: The Period Is Determined by the Delay in the Feedback Loop

(A) On/off switch. Transition to the "on" state creates a delayed negative feedback signal, causing transition to the "off" state after time T, and vice versa; the time for one complete cycle (on → off → on) is $2T$.

(B) System described by a continuous variable, $x(t)$, autoregulated by negative feedback with delay T such that $x(t) = f(x[t - T])$, where f is a monotonically decreasing function. If, for example, $f(x) = K/(1 + x^2)$, oscillations will be sustained and will have period $2T$ whenever $K > 2$. (This is the condition under which the pair of equations $x_1 = f[x_2]$ and $x_2 = f[x_1]$ has solutions $x_1 \neq x_2$; the system then alternates between state $x = x_1$ and state $x = x_2$).

(C) A chemical system in which the rate of synthesis of a substance x is regulated by delayed negative feedback and degradation occurs at rate $cx(t)$: $dx(t)/dt = f(x[t - T]) - cx(t)$. If degradation is rapid in relation to the delay time T, so that $c >> 1/T$, the concentration closely follows the instantaneous value of $f(x[t - T])/c$ and the behavior approximates that of system (B). Thus, if $f(x) = K/(1 + x^2)$, the system oscillates with a period of $\sim 2T$ provided $K/c \gtrsim 2$. If degradation is too slow (specifically, if $c < r/T$, where $r = 2\pi/3\sqrt{3} \sim 1.2$), sustained oscillation becomes impossible even for very large values of K/c (see [12], pg. 192). For further discussion, see also [48] and references therein.

[17]. But could such a simple system really generate oscillations? To answer, one has to do some mathematics. For the given autoregulatory gene, let m be the number of mRNA molecules in the cell at any instant, and let p be the number of molecules of the corresponding protein. The rate of change of m and p might then be supposed to obey the equations:

$$\frac{dp}{dt} = am - bp$$
$$\frac{dm}{dt} = f(p) - cm \qquad (1)$$

where the constants b and c are the decay rates (inverse lifetimes) of the protein and message molecules, a is the rate of production of new protein molecules per mRNA molecule, and $f(p)$ is the rate of production of new mRNA molecules, which is assumed to be a decreasing function of the amount of protein p. For example, we might take $f(p) = k/(1 + p^2/p_0^2)$, with k and p_0 as constants, to represent the action of an inhibitory protein that acts as a dimer (as bHLH proteins do).

It can be proved from Bendixson's Negative Criterion, however, that it is impossible for this pair of differential equations to generate sustained oscillations ([19], p. 109). This conclusion holds good for any form of the function $f(p)$, provided only that a and c are both positive numbers. Other investigators of gene expression oscillations have therefore postulated more complex mechanisms, involving larger numbers of components (e.g., [18]), and have even created artificial bacterial systems in which oscillations are generated by, for example, a ring of three genes, the protein product of each one regulating the transcription of the next [20].

There is, however, a much simpler way to arrive at sustained oscillations, by taking more careful account of the delays involved in the synthesis of mRNA and protein (Figure 3A). The amount of regulatory protein p in the cell dictates the rate of initiation of transcription, but a significant time, T_m, elapses between the initiation of transcription and the arrival of the mature mRNA molecule in the cytoplasm. Thus, the rate of increase in the number of mature mRNA molecules at any instant reflects the value of p at a time that is earlier by an amount T_m. Likewise, there is a delay, T_p, between the initiation of translation and the emergence of a complete

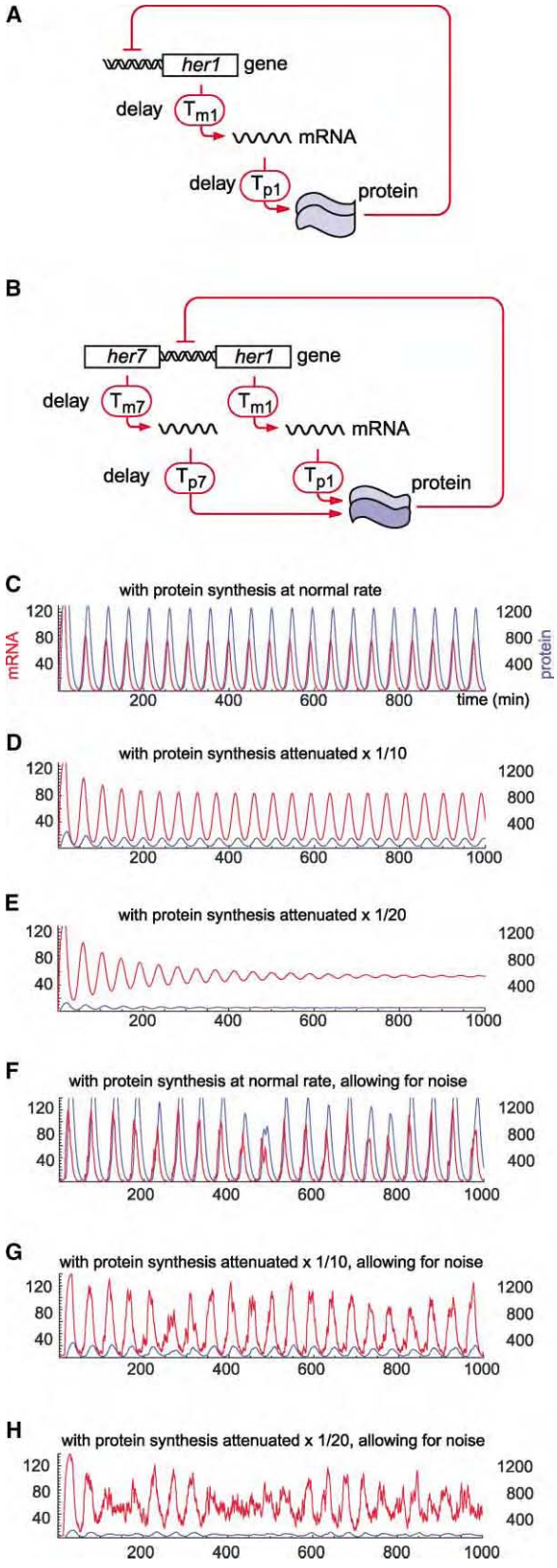

Figure 3. A Cell-Autonomous Gene Expression Oscillator: Effects of Partial Blockade of Translation and Effects of Noise

(A) Molecular control circuitry for a single gene, *her1*, whose protein product acts as a homodimer to inhibit *her1* expression.

(B) Molecular control circuitry for a pair of coregulated genes, *her1* and *her7*, whose protein products combine as a heterodimer to inhibit *her1* and *her7* expression.

(C–E) Computed behavior of system (A), showing the number of *her1* mRNA molecules per cell in red and of Her1 protein molecules in blue. Drastic changes in the rate constant for protein (or mRNA) synthesis (such as might be produced by partially blocking translation with cycloheximide) cause little or no change in the period of oscillation, though beyond a certain critical severity they may convert the sustained oscillation into a damped oscillation. The results are computed for parameter values appropriate to a *her1* homodimer oscillator (as specified in the text) and show outcomes for three different values of a, the number of protein molecules synthesized per mRNA molecule per minute. Results are similar for a *her7* homodimer oscillator and for the *her1*/*her7* heterodimer oscillator shown in (B). The protein and the mRNA are assumed absent initially, with the gene becoming abruptly available for transcription at time 0. Thereafter, the amounts of protein and mRNA are assumed to change according to the equations 2 in the text. In general, for sustained oscillations, three conditions must be satisfied: (i) $ak/bc > 2\,p_0$; (ii) $b \geq 1.7/T$; and (iii) $c \geq 1.7/T$, where $T = T_m + T_p$. The period of oscillation is then given to a good approximation (within ~15%) by the formula $2(T + 1/b + 1/c)$.

(F–H) Computed behavior of system (A) when the stochastic nature of the control of gene expression is taken into account. Results are shown for the case in which the repressor protein dissociates from its binding site on the DNA with a rate constant $k_{off} = 1\ \text{min}^{-1}$, corresponding to a mean lifetime of 1 min for the repressor bound state. Other parameters are set to be the same as in (C)–(E). Note that oscillation now continues with high amplitude, though noisily, even under conditions in which the deterministic model predicts that oscillation will be damped (compare [H] with [E]). With lower values of k_{off}, corresponding to longer lifetime for the repressor bound state, this phenomenon is even more pronounced.

For details of the computation and proofs of the analytical results, see the Supplemental Data.

functional protein molecule. The equations 1 are easily modified to take account of these delays:

$$\left.\begin{aligned}\frac{dp(t)}{dt} &= am(t - T_p) - bp(t)\\ \frac{dm(t)}{dt} &= f(p(t - T_m)) - cm(t)\end{aligned}\right\}, \qquad (2A)$$

where the time dependencies of the variables are now shown explicitly.

Delay differential equations such as these cannot be solved analytically, but they are easily solved numerically for any given choice of parameters. Oscillations occur easily and robustly provided certain simple conditions are satisfied. In the discussion below, it will be assumed that

$$f(p) = \frac{k}{1 + p^2/{p_0}^2}, \qquad (2B)$$

which represents the action of the inhibitory protein as a dimer; however, the behavior is qualitatively similar for other cases, such as $f(p) = k/(1 + p^n/{p_0}^n)$ for $n > 1$.

The Period of Oscillation Is Determined by the Sum of the Transcriptional and Translational Delays

The behavior is easiest to analyze in the limit in which the lifetimes of the mRNA and protein are very short compared with the total delay time $T = T_m + T_p$. Oscilla-

tions, in this extreme, take the form of square waves: the system flip-flops between two quasisteady states, and the period of oscillation is simply $2T$, in accordance with the basic principles illustrated for a still simpler system in Figure 2. There is just one additional condition that must be satisfied for oscillation to occur: the maximal rates of mRNA and protein synthesis must be high enough to be capable of raising the protein concentration beyond the critical value, p_0, at which the inhibition of mRNA synthesis starts to operate in earnest. Specifically, we require $ak/bc > 2p_0$.

As the lifetimes of the protein and mRNA become longer, so that they are no longer negligible in comparison with the total delay T, the square-wave form of the oscillations becomes smoothened into something more like a sine wave (see Figures 2C and 3A). Sustained oscillations will still occur provided that the lifetimes are not too long and the product of the rate constants for mRNA and protein synthesis is high enough. The period of the oscillations ranges between a minimum of $2T$ (when the lifetimes are very short) and a maximum of approximately $4T$ (as the lifetimes approach their upper limit). In fact, one can show analytically that for $1/b << T$ and $1/c << T$, the period is approximately equal to $2(T + 1/b + 1/c)$ (see the Supplemental Data available online). Thus, the delay T is the key determinant of the period. The values of the other parameters can be varied by orders of magnitude, with very little effect on the period, provided only that the conditions for sustained oscillation are satisfied (Figures 3C–3E).

The Predicted Period for a *her1*/*her7* Oscillator Is Close to the Observed Period of the Somite Oscillator

To see whether *her1* and/or *her7* could generate oscillations by the simple mechanism mentioned above, we have to estimate the parameters a, b, c, k, p_0, T_m, and T_p. The observed period of the somite oscillator is short, and it is therefore reasonable to assume that the rates a, b, c, and k are close to the upper limits reported for mRNA and proteins in eukaryotic cells. Plausible values are: $a = 4.5$ protein molecules per mRNA molecule per minute [21]; $b = c = 0.23$ molecules per minute, corresponding to protein and mRNA half-lives of 3 min [22, 23]; $k = 33$ mRNA molecules per diploid cell per minute, corresponding to 1000 transcripts per hour per gene copy in the absence of inhibition [21]; $p_0 = 40$ molecules, corresponding to a critical concentration of $\sim 10^{-9}$ M within a 5 μm-diameter cell nucleus.

To estimate the delays T_m and T_p, note that for animal cells, RNA polymerase II moves along DNA at a rate of roughly 20 nucleotides per second [21, 24, 25]. Though data are scanty, it has been estimated from a study of two different genes in mammalian cells that each successive intron takes between 0.4 and 7.5 min (half-life) to splice out [26]. A further delay, estimated at about 4 min (half-life), elapses after completion of splicing before the mature mRNA emerges into the cytosol [26]. The mRNA is translated by ribosomes moving at roughly 6 nucleotides per second [21]. Given the genomic sequence of *her1* and *her7* (GenBank accession number AF292032), and assuming similar rates for fish and mammal, we arrive at the following estimates: for *her1*, which has a primary transcript of length 6005 nt, with 3 introns, and codes for a protein of 328 amino acids, we have $10.2 < T_{mher1} < 31.5$ min and $T_{pher1} \approx 2.8$ min; for *her7*, which has a primary transcript of length 1280 nt, with 2 introns, and codes for a protein of 204 amino acids, we have $5.9 < T_{mher7} < 20.1$ min and $T_{pher7} \approx 1.7$ min. Thus, the estimated total delay T for *her1* is between 13.0 and 34.3 min, and, for *her7*, it is between 7.6 and 21.8 min.

By inserting parameter values anywhere within these ranges in the equations 2 we obtain oscillations. Moreover, it is easy to extend equations 2 to describe the case in which *her1* and *her7* are coordinately regulated by a heterodimer of Her1 protein with Her7 protein; again, the system oscillates. If we assume that it takes 1 min to splice out each intron for *her1* and *her7*, so that $T_{mher1} \approx 12$ min and $T_{mher7} \approx 7.1$ min, we get a computed period of 47 min for the pure Her1 dimer oscillator, 30 min for the pure Her7 dimer oscillator, and 37 min for the Her1/Her7 heterodimer oscillator. Given the uncertainties in the parameter values, the periods predicted for all these variant mechanisms represent good agreement with the observed period of 30 min for the zebrafish somite oscillator at 28°C. The *her1*/*her7* heterodimer oscillator model has the virtue that it accounts for the observed joint involvement of *her1* and *her7* in a simple and plausible way; its behavior is generally similar to that of a simple homodimer oscillator based on *her1* or *her7* alone.

The Oscillator Is Surprisingly Insensitive to Blockade of Protein Synthesis

The properties of an oscillator of the type described can be quite surprising. What, for example, will be the consequence of a drastic general reduction of protein synthesis? One might expect that this would stop the oscillator, which depends on protein synthesis, or at least that it would cause a big change in the period and amplitude. In fact, if we reduce the protein synthesis rate constant a to one tenth of its standard value (from 4.5 to 0.45 protein molecules per minute per ribosome) and use the model to recompute the predicted mRNA levels (taking the *her1* homodimer oscillator as an example), we find none of these effects. Sustained oscillation is still seen, the period is practically unchanged, and even the amplitude of the mRNA oscillation is almost the same as before (Figure 3D). A further reduction, to a twentieth of the standard value, does indeed cause a qualitative change in the behavior: oscillations now are damped, so that the system tends toward a nonoscillating steady state (Figure 3E). But the period of the damped oscillations is practically the same as before, and the damping (for $a = 0.225$) is only about 25% per cycle, so that, for example, it takes 4 cycles for the amplitude to fall to one third of its initial value if the system is initially far from its steady state.

Palmeirim et al. [1] have tested experimentally the effects of inhibiting protein synthesis with cycloheximide during chick somite formation: an 84% reduction in protein synthesis, maintained for the duration of a full cycle of the normal oscillator, allows oscillation of *c-hairy1* mRNA levels to continue. This finding was originally taken to imply that the oscillation could not depend

on oscillating synthesis of a regulatory protein. From the mathematical model, we can see that (regardless of the details of the chick somitogenesis oscillator) this is not a safe inference. Indeed, subsequent work in chick and mouse has strongly suggested that the oscillation is after all based on oscillating synthesis of mRNA and protein [27–30].

Figure 3E also illustrates another counterintuitive feature. If Her1/Her7 protein represses transcription of the *her1/her7* gene(s), one might expect that when translation of *her1* or *her7* mRNA is blocked with an antisense morpholino, as in the experiments mentioned earlier, repression would be lost and levels of the mRNA would rise dramatically. In fact, as the figure shows, the negative feedback operates in such a way that even with a 95% block of translation, the mRNA in our model system tends only to a middling value, intermediate between the extremes of the normal oscillation. To get the mRNA concentration to rise above the levels normally encountered, the morpholino block must be even more severe. It can be difficult, therefore, to draw conclusions from morpholino experiments as to the presence or absence of negative feedback.

Damped Oscillations or Progressive Loss of Synchrony May Explain the Normality of the First Few Somites in Somitogenesis Mutants

As we have just seen, when the parameters of the model system are altered to such a degree that sustained oscillation becomes impossible, damped oscillations can still occur. These may have a large amplitude if the system is started far from the steady state, and their decay may be slow. Moreover, the period of these damped oscillations is almost the same as the period of the sustained oscillations that occur for other parameter values, provided only that the delay T in the feedback loop is unchanged.

This suggests a simple interpretation of a curious observation: in most of the mutants listed earlier that show a disrupted pattern of somite segmentation, as well as in analogous mouse mutants, the first few somites appear practically unaffected. The mathematical model shows that there is no need to invoke any region-specific molecular control machinery to explain this phenomenon: the normal formation of the first few somites can simply reflect the occurrence of a damped oscillation at the onset of somitogenesis, followed by a failure of patterning as the amplitude of oscillation declines to zero.

This explanation is attractive in principle, but experimental observations indicate that it is not quite right in fact, for the zebrafish at least. In the mutants and morpholino-injected embryos in which somitogenesis is progressively disrupted, the oscillations of the individual cells in the PSM do not all damp down to a common steady level. Instead, as noted earlier, the cells drift into a pepper-and-salt pattern of gene expression [5, 8], in a manner suggesting that they are still oscillating individually but have lost coordination and become randomized with respect to their phase in the oscillation cycle. On this basis, we proposed in a previous paper that the first few somites form normally, and the subsequent somites abnormally, not because of damped oscillations in the individual cells, but because individual cell oscillators, having been set going in synchrony, take time to drift out of synchrony [5].

The two explanations, damping of individual cell oscillations and damping of the population average oscillation through loss of synchrony, seem quite different, but they are not mutually exclusive. In fact, a closer analysis of the principles of the feedback inhibition oscillator shows that the same type of genetic defect can give rise to either or both of these phenomena, according to the parameter values of the system. To see how this can be, and to explain why so many types of genetic disturbance that disrupt somite segmentation in the zebrafish give the pepper-and-salt result, we must take account of one further essential feature of real cells: noise in the control of gene expression.

Noisy Control of Gene Expression Favors Continued Oscillation

In any transcriptional control system, random fluctuations will arise from random variation, or noise, in the regulation of gene expression [31]. The binding and dissociation of a gene regulatory protein to and from its site on DNA are stochastic processes, each described by a certain probability of occurrence per unit time, so that we should expect *her1* and *her7* to flicker between "off" (repressor bound) and "on" (repressor dissociated) states. In writing down our deterministic equations that describe the gene regulation in terms of a smooth dependence of transcription rate on protein concentration, we are in effect assuming that we can replace the underlying on/off flickering behavior by an instantaneous time average, in which the rate of transcription is proportional to the fraction of time the gene is currently spending with repressor protein bound to it. The slower the average flicker rate, the more the real system will depart from the idealized deterministic behavior.

Figures 3F–3H show how the predictions of the deterministic model for a single cell are altered when we take into account the noisy nature of gene regulation. As explained in detail in the Supplemental Data, to model the stochastic effects, we have to specify just one further independent parameter in addition to those characterizing the deterministic model: the rate constant k_{off} for dissociation of the repressor protein from its binding site in the regulatory DNA. The results in Figures 3F–3H represent the case where $k_{off} = 1\ min^{-1}$, corresponding to a mean lifetime of 1 min for the repressor bound state. Three main points are apparent:

First, when protein synthesis is at the full normal rate (Figure 3F), the noisy system oscillates in the same way as the deterministic system; the only obvious difference consists in some random variability in the amplitude and shape of individual oscillation peaks. The smaller the value we assign to k_{off}, the larger these noise effects become (data not shown). But even for a k_{off} as small as 0.1 min^{-1}, corresponding to a mean lifetime of 10 min for the repressor bound state, well-defined (though noisy) oscillations still occur and have practically the same mean period as in the deterministic model, reflecting the length of the delays in the feedback loop.

Second, when protein synthesis is attenuated (Figure

3G), the noisy system still oscillates with the same mean period as the deterministic system, but with more random variation in the amplitude and shape of the individual peaks and in the spacing between them.

Third, when protein synthesis is attenuated severely (Figure 3H), to the point where the deterministic system shows only damped oscillation, the noisy system does not tend to a steady state, but shows persistent strong fluctuations in the level of mRNA. A large random fluctuation can initiate a damped train of oscillations; although these are noisy, they still show the standard periodicity. Thus, oscillation continues but is episodic, with periods of regularity and high amplitude separated by intervals of more random fluctuation. In fact, when k_{off} is made smaller, intensifying stochastic effects, the behavior approximates still more closely to sustained oscillation. Far from disrupting oscillation, noise helps it to occur.

Conversely, the larger we make k_{off}, the smaller the stochastic effects become, until for $k_{off} \gtrsim 10\ min^{-1}$, the behavior approximates that of the deterministic system. Thus, according to the dissociation rate of the repressor protein from its DNA binding site, the same mutation may lead to oscillations that are simply damped, or to oscillations that continue with substantial amplitude but reduced regularity.

Zebrafish with mutations in the Notch pathway [5] or injected with anti-*her1* or anti-*her7* morpholinos ([8], and B. Aerne, personal communication) presumably correspond to the latter case, with oscillation continuing noisily. In these embryos, it is not surprising that synchrony between neighbors is lost: there is not only a defect in the production of proteins needed for cell-cell communication, but also an increased tendency for the cells to be erratic in their individual rhythms.

Delta-Notch Communication Can Synchronize *her1*/*her7* Oscillations in Adjacent Cells

If the clock-and-wavefront mechanism is to guide regular somite segmentation, neighboring PSM cells must oscillate in synchrony. It is easy to extend the basic single-cell oscillator model to show how, through Notch signaling mechanisms that are known to operate in the PSM, synchronization can be achieved (Figure 4).

For simplicity, let us disregard stochastic effects and consider two adjacent PSM cells with slightly different free-running oscillation periods, so that in the absence of cell-cell communication their cycles will drift in and out of synchrony (Figure 4B). To model the effect of Notch-mediated communication, note first of all that in the normal zebrafish, levels of mRNA coding for the Notch ligand DeltaC oscillate very nearly in phase with the oscillations of *her1* and *her7* mRNA ([8]; L. Smithers and J.L., unpublished data). It is reasonable to hypothesize, therefore, that *deltaC* expression is regulated in parallel with expression of *her1* and *her7* by Her1/Her7 protein acting directly on the *deltaC* promoter, and that the mRNA and protein lifetimes are short for *deltaC*, as they are for *her1* and *her7*, so that DeltaC protein levels will oscillate. (The effective DeltaC protein lifetime is expected to be short, since Delta proteins delivered to the cell surface are rapidly endocytosed and degraded [32]). DeltaC oscillation will cause oscillation in the level

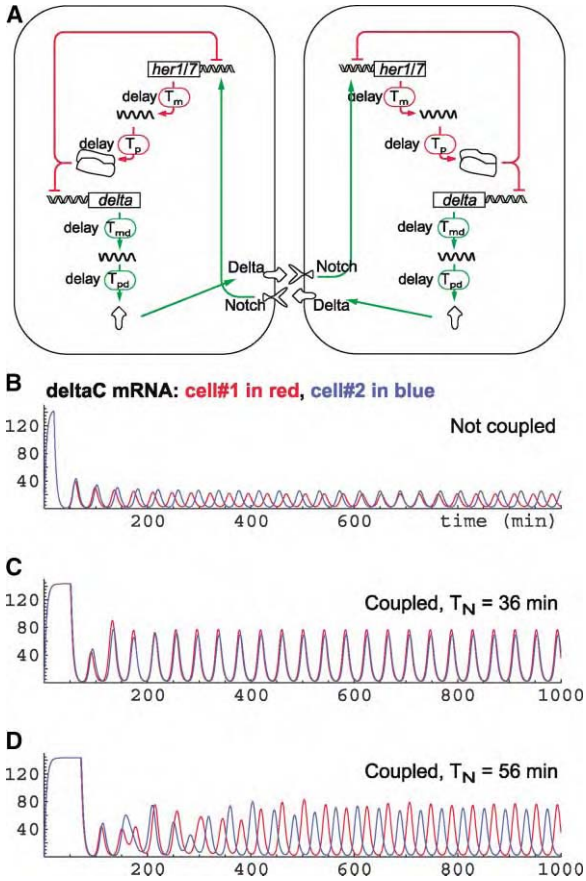

Figure 4. Notch Signaling Can Synchronize Oscillations in Two Adjacent Cells

(A) The molecular circuitry: the two cells are assumed to contain *her1*/*her7* heterodimer oscillators, but with a 10% difference in their values of $T_m + T_p$, corresponding to different free-running oscillation periods. The oscillators are coupled via Notch signaling.

(B) The computed behavior with no Notch-mediated cell-cell communication. The cells drift in and out of synchrony.

(C) Notch signaling active, with a signaling delay of $T_N = 36$ min. The rate of *her1*/*her7* transcription is assumed to be determined by the product of an increasing function of the amount of Delta-Notch activity and a decreasing function of the amount of Her1/Her7 protein dimers. The cells oscillate synchronously, with a period that is an average of their free-running periods.

(D) Notch signaling active, but with a delay of $T_N = 56$ min. The cells now oscillate asynchronously.

See the Supplemental Data for full details.

of Notch activation in each cell according to the level of DeltaC in its neighbor. Activation of Notch stimulates expression of *her1* and *her7* [8, 10]. We may thus suppose that the transcription of *her1* and *her7* is governed by the product of two oscillatory influences: positive regulation by activated Notch and inhibition by Her1/Her7. Figure 4A shows the proposed control scheme; while it is speculative in some respects, and has difficulty explaining some of the results of mRNA injection experiments [10], it seems the simplest way to represent the main experimental facts. Full details of the corresponding system of equations for the two interacting cells are given as Supplemental Data, together with the Mathematica program used to compute the behavior.

Just as single-cell oscillation depends on the delay in the *her1/her7* intracellular feedback loop, so synchronization of two cells depends on the delays in the cell-cell signaling pathway. The total delay in the Notch pathway, T_N, is a sum of four parts: the delay $T_{mdeltaC}$ from initiation of transcription of *deltaC* (the event controlled by Her1/Her7) to emergence of a mature *deltaC* mRNA molecule into the cytoplasm; the delay $T_{pdeltaC}$ from initiation to completion of synthesis of a DeltaC protein molecule; the delay $T_{pexport}$ for delivery of the protein via the secretory pathway to the cell surface; and the delay $T_{NotchActivation}$ from the time when functional DeltaC protein reaches the cell surface to the time when the resultant activated Notch arrives in the nucleus of the neighboring cell to regulate transcription of *her1* and *her7*. $T_{mdeltaC}$ and $T_{pdeltaC}$ can be estimated in the same way as the corresponding delays for *her1* and *her7*, by using the published sequence data (GenBank gi|18858544 and gi|27374724): *deltaC* has a primary transcript of ~4851 nt, with 8 introns, coding for a protein of 664 aa, giving estimates 16 min $< T_{mdeltaC} <$ 68 min and $T_{pdeltaC} \approx$ 5.5 min. Transit times, $T_{pexport}$, in the protein export pathway in vertebrate cells are highly variable, but they may be as short as 15 min [33–35]. Data on $T_{NotchActivation}$ are scanty, but it is likely to be short by comparison with $T_{pexport}$ [36]. Adding these contributions, we conclude that the total delay, T_N, in the Notch signaling pathway could be anywhere from about 36 min up to a few hours. Given the rapidity of other events in the PSM cells, it is reasonable to assume the shorter value.

One might think that such a long delay in the cell-to-cell signaling pathway would make it incapable of synchronizing the rapid oscillations of the individual cells. But again, intuition is a poor guide to the behavior of the system. Numerical modeling with the specified parameters shows that the two adjacent cells are in fact forced into synchrony (Figure 4C). The mechanism is robust: the same effect is obtained if we make T_N longer or shorter by up to 10 min, although not if we make it 20 min longer (Figure 4D) or 15 min shorter. (The "push" that the one cell delivers to its neighbor must be received in the correct part of the neighbor's own oscillation cycle if it is to promote synchronized oscillation.) Moreover, the precise value of T_N has very little effect on the period of oscillation, which is chiefly defined by the *her1/her7* kinetics.

A Small Change in Gene Regulation Can Switch Control of the Oscillator Period from the Intracellular to the Intercellular Feedback Loop

In the circuitry just described for two interacting cells (see Figure 4A), the intercellular signaling pathway via Notch creates an additional feedback loop (activated Notch in cell #1 → *her1/her7* in cell #1 —| *deltaC* in cell #1 → activated Notch in cell #2 → *her1/her7* in cell #2 —| *deltaC* in cell #2 → activated Notch in cell #1, where → denotes stimulation and —| denotes inhibition). This control circuit is capable of generating oscillations in its own right (Figure 5). This is easily verified by eliminating direct *her1/her7* autoinhibition in the model of two interacting cells, leaving *her1* and *her7* regulated directly by Delta-Notch activity alone, and recomputing the behav-

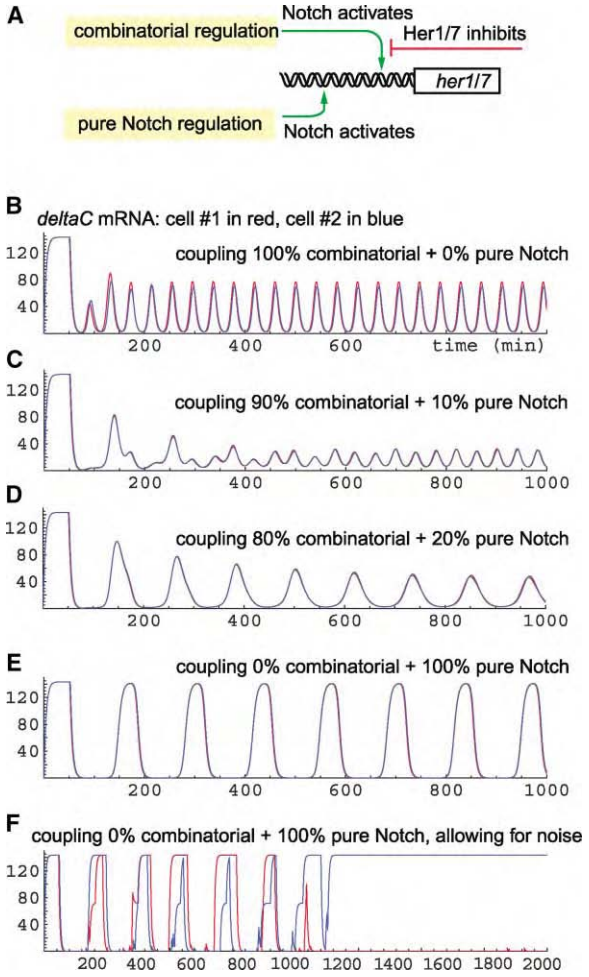

Figure 5. A Small Change in Parameters Can Cause an Abrupt Switch in the Period and Mechanism of Coupled Oscillation

The graphs show the behavior of the same two-cell system as in Figure 4, assuming T_N = 36 min, but varying the quantitative details of the regulation of *her1/her7* expression by Notch.

(A) A diagram of the regulation of *her1/her7* transcription: the rate of transcription in each cell is assumed to be the sum of two contributions, dependent on separate enhancers in the regulatory DNA. One enhancer ("combinatorial") mediates a combined action of activated Notch and Her1/Her7 protein; its contribution is described by a function that represents stimulation by Notch, multiplied by a function that represents inhibition of that effect by Her1/Her7. The other enhancer ("pure Notch") mediates a simple stimulatory effect of activated Notch, independent of Her1/Her7.

(B–E) Computed behavior, for different values of the percentage contribution to transcription due to the "pure Notch" enhancer. The cells oscillate synchronously, but as the "pure Notch" contribution rises above about 10%, they switch abruptly to a much longer cycle time, dictated by the delay $T_p + T_m + T_N$ in the *her1/her7* → Notch → *her1/her7* feedback loop, instead of the delay $T_p + T_m$ in the direct intracellular *her1/her7* → *her1/her7* loop.

(F) Computed behavior for the same case as shown in (E), but allowing for noise in the control of gene expression and assuming that the two cells are intrinsically identical at the outset. k_{off} is assumed to be equal to 0.1 min^{-1} for the regulation both of *her1/her7* by activated Notch and of *delta* by Her1/Her7. After a few synchronous oscillations, the system undergoes a switch and the two cells become committed to opposite steady states—the standard outcome of lateral inhibition.

See the Supplemental Data for full details.

ior. The cells again oscillate in synchrony, but with a much longer period than before (133 min instead of 40 min), dictated by the delay in the feedback loop that goes via Notch, instead of the delay in the direct intracellular *her1/her7* autoregulatory loop. If the contribution to the *her1/her7* transcription rate that is regulated directly by Delta-Notch activity independently of Her1/Her7 is gradually increased, a transition occurs: when this contribution becomes more than about 10% of the Her1/Her7-regulated contribution, the system switches from rapid oscillation with period $\sim 2(T + 1/b + 1/c)$ to slow oscillation with period $\sim 2(T + T_N + 1/b + 1/c + 1/b_{deltaC} + 1/c_{deltaC})$. Between these two regimes, there is a narrow transitional domain in which the first few oscillations of the system are irregular, with mixed periodicity, and the later oscillations are weak (Figure 5). This is reminiscent of the disorders of somite segmentation reported for *her1* + *her7*-deficient zebrafish embryos [9].

The above conclusions are computed for the deterministic case, with no noise in the control of gene expression. How must they be modified if we make the model more realistic and take account of noise? A full analysis will be presented elsewhere, but it seems that noise has little effect so long as the flicker rate is reasonably high (k_{off} > about 1 min^{-1}). An important effect of noise is seen, however, for smaller values of k_{off} in the case where *her1* and *her7* are regulated directly by Delta-Notch activity alone, with no direct *her1/her7* autorepression. Figure 5F shows results for $k_{off} = 0.1$ min^{-1}. Whereas the deterministic model gives sustained slow Delta-Notch-mediated oscillations (Figure 5E), the noisy model displays an instability. After a variable number of noisy oscillation cycles with a similar slow period, it switches to a steady state representing the standard outcome of Notch-mediated lateral inhibition, with the two cells adopting opposite characters (one expressing *delta* but not *her1/her7*, the other *her1/her7* but not *delta*).

The Somite Oscillator in Chick and Mouse May Be More Complex

The somitogenesis oscillator in mouse and chick, as in the zebrafish, depends on components of the Notch signaling pathway [2]. Like *her1/her7* in the fish, the mouse *her1* ortholog *Hes7* oscillates, negatively regulates its own expression, and is critical for the production of oscillations [16, 17]. But there are also important differences between the species:

1) Mutations in Notch pathway components such as Delta1 and $RBPJ_K$ appear to abolish oscillations in the mouse rather than merely desynchronize them as in the fish [37, 38]. This may reflect a lower level of noise in gene regulation, as explained above.

2) Delta genes are expressed at a steady mRNA level in the posterior PSM of mouse and chick [39, 40], instead of oscillating as in zebrafish.

3) The *Lunatic fringe* (*Lfng*) gene, coding for a glycosyltransferase that modulates Delta-Notch signaling, shows oscillating expression in the posterior PSM of mouse and chick and is critical for the genesis of oscillations [27–29, 41–43]; whereas in the corresponding region in zebrafish, the homologous gene does not appear to be expressed at all [44, 45].

4) In the mouse, the Wnt signaling pathway also has been reported to play a critical part in the genesis of the oscillations [30], perhaps through a negative feedback loop controlling the expression of *Axin2*, which oscillates, or perhaps simply in a permissive role, since it is required to maintain *Fgf8* expression in the PSM. There is (as yet) no evidence for such an involvement of Wnt signaling in the zebrafish.

5) The period of oscillation in mouse and chick is 90–120 min, 3–4 times longer than in the zebrafish, allowing time for more complex chains of transcriptional regulation.

How, then, does the oscillation mechanism proposed for the zebrafish relate to the mechanism operating in these other species? One possibility is that a switch of the type illustrated in Figure 5 has occurred during the evolution of birds and mammals – a switch from rapid oscillation with a period governed by *her1/her7* direct autoregulation to slower oscillation with a period governed by intercellular signaling via the Notch pathway and Lfng. As we have seen (Figure 5), a modest change in parameters, even without any change in the circuit diagram of the control system, can lead to qualitatively different behavior, with a different part of the control circuitry acting as pacemaker.

Another strong possibility is that the pacemaker mechanism in the mouse is essentially the same as in the zebrafish. Bessho et al. [16, 17] have analyzed the role of *Hes7* in detail, and they find persuasive evidence that it functions in the mouse as a negative feedback oscillator of just the sort described above for *her1/her7* in the fish model, but with longer delays and slower kinetics. They show, moreover, that Hes7 and Notch proteins regulate expression of the *Hes7* gene combinatorially in just the way depicted in Figure 5A for *her1/her7*, implying that Notch signaling might have a similar synchronizing role in both systems.

Other workers have proposed still other mechanisms for the mouse or chick somite oscillator, advocating a central pacemaking role for Lfng [27, 28] or for Axin2 [30]. This is not the place to judge between these various theories. All of them have experimental backing, and it is possible that more than one of them is correct, since several mutually supportive mechanisms may operate in parallel. The general principles discussed in this paper are relevant to all of them, since all of them postulate that oscillations arise from feedback in the control of transcription.

The Theory for Zebrafish Is Simple and Testable

In the zebrafish, the somitogenesis clock has a remarkably short period for a transcriptional oscillator, and its rapidity places constraints on the mechanism. If the oscillator is based on a transcriptional feedback loop, as the evidence indicates, that loop has to be simple, as there is no time for long, complex cascades. Moreover, to explain how the loop generates oscillations, one has to postulate molecular lifetimes that are short, synthesis rate constants that are high, and gene regulatory proteins that act at low concentrations; in each

case, the values need to be close to the limits of what has been reported in other systems. By measuring these various quantities, as Bessho et al. [16, 18] have begun to do in the mouse, and as we are currently attempting to do in the zebrafish, we can put the model to the test.

The model is simple, and there is little in it that is arbitrary. Nevertheless, while there is evidence for all the postulated ingredients, and these ingredients seem sufficient to explain the observations, they may not be the whole story. We cannot, for example, exclude a role for Wnt signaling or for posttranscriptional controls in the genesis of the oscillations, and some data hint at an involvement of components of the cell-cycle control machinery [46, 47]. By making transgenic fish with modified *her1*/*her7* genes, it should be possible to test specific quantitative predictions of the model and to discover whether the *her1*/*her7* feedback loop is truly the clock for somitogenesis.

The simplicity of the proposed oscillator mechanism implies that it may be commonplace, operating in other systems also: any gene whose product inhibits its own transcription in the way described for *her1*/*her7* should oscillate, provided the transcriptional (or translational) delay is long enough and the mRNA and protein lifetimes are short enough. The oscillations of *Hes1* expression seen by Hirata et al. in cultured mammalian cells [18] are a possible example. A detailed discussion of this idea, including mathematical analysis of some aspects not covered in the present paper, is given by Monk [48] in this issue of the journal and points out at least three instances where there is strongly suggestive experimental evidence.

Standard Notch Signaling Can Generate Coordinated Oscillations instead of Lateral Inhibition

As a byproduct of the analysis of the somitogenesis oscillator, one stumbles over a surprising fact about Notch signaling that forces a reconsideration of how the Notch pathway may function in other systems. The control circuitry corresponding to the result shown in Figure 5E is simply the standard Notch signaling pathway. This is famous in other contexts for its role in lateral inhibition, forcing neighboring cells to become different [49, 50]. But in Figure 5E, we see a completely different outcome, synchronous oscillation, albeit transiently if noise effects are strong (Figure 5F). This is predicted for a wide range of parameter values, as soon as one allows for delays in the signaling pathway (N. Monk, personal communication). Such delays are always present. So does Notch signaling in fact generate transcriptional oscillations in any of the other innumerable tissues where it is known to act? To answer this question, it will be necessary to reexamine the evidence for each system in the light of the revised theoretical analysis that takes account of delays.

Conclusion

In this paper, we have seen that robust oscillating gene expression is easy to produce. But it is not easy to observe, and with present techniques, it is often likely to go unnoticed, especially where there is nothing to synchronize adjacent cells. Intracellular transcriptional oscillations may thus be more common than we imagine [51]. The principles illustrated here for our model of the zebrafish somitogenesis oscillator may help to clarify the behavior of other genes in other tissues and organisms. They may also serve to emphasize that in studying these biological feedback phenomena, intuition without the support of a little mathematics can be a treacherous guide.

Supplemental Data

The Supplemental Data include mathematical analysis of the oscillator mechanisms discussed, as well as the Mathematica programs used to compute their behavior, both for deterministic and for stochastic models of gene regulation; readers with Mathematica can use the programs to explore the behavior for different parameter values. Supplemental Data are available at http://www.current-biology.com/cgi/content/full/13/16/1398/DC1/.

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The single-cell single-gene oscillator: mathematical analysis and a calculation of the period for the deterministic (no-noise) system

Define:

$p[t]$= number of protein molecules per cell at time t

$m[t]$ = number of mRNA molecules per cell at time t

a = protein synthesis initiation rate per mRNA molecule (i.e. number of protein molecules whose synthesis is initiated per unit time per mRNA molecule)

$f[p]$= mRNA synthesis initiation rate per cell (i.e. number of mRNA molecules whose synthesis is initiated per unit time), as a function of p

b = protein degradation rate constant (inverse lifetime)

c = mRNA degradation rate constant (inverse lifetime)

T_p = delay from initiation of synthesis of a protein molecule to its completion

T_m = delay from initiation of synthesis of an mRNA molecule to delivery of the mature mRNA to the cytosol.

Then the rates of change of p and m are:

$$p'[t] = a\, m[t-T_p] - b\, p[t]$$

$$m'[t] = f[p[t-T_m]] - c\, m[t]$$

Assume that transcription is inhibited by the protein p acting as a dimer, so that

$$f[p] = \frac{k}{1 + p^2 / {p_0}^2}$$

where k and p_0 are constants.

To simplify, define a phase-shifted protein concentration

$$p_{\text{advanced}}[t] = p[t + Tp]$$

so that

$$p_{\text{advanced}}[t] = am[t] - bp_{\text{advanced}}[t]$$

$$m[t] = f[p_{\text{advanced}}[t - T_m - T_p]] - cm[t]$$

If we define

$T = T_m + T_p$,

we can now convert to non-dimensional variables as follows:

$\tau = t / T$

$\beta = bT$

$\gamma = cT$

$\alpha = ak / bcp_0$

$\pi[\tau] = p_{\text{advanced}}[\tau T] / p_0$

$\mu[\tau] = cm[\tau T] / k$

Then

$$\dot{\pi}[\tau] = \alpha\mu[\tau] - \beta\pi[\tau]$$

$$\dot{\mu}[\tau] = \frac{1}{1 + \pi[\tau - 1]^2} - \gamma\mu[\tau].$$

(Note that the steady-state values of m and p in the absence of inhibition by p are:

$m_{\max} = k/c$

$p_{\max} = ak/bc$,

so that

$\mu = m / m_{\max}$

$\pi = p_{\max} / p_0$.)

The task is to explore the behaviour as a function of the three parameters, α, β and γ.

In the limit where α and β are big, the system flip-flops between two quasi-steady states (μ_1, π_1) and (μ_2, π_2), where

$\alpha_1=\alpha\beta_1$, $\alpha_2=\alpha\beta_2$,

$\beta_1=1/(1+\alpha_2^2)$, $\beta_2=1/(1+\alpha_1^2)$.

Hence it is easily shown that, for $\alpha>2$, either

$\alpha_1=\alpha_2$

or

$$\alpha_{1,2}=\frac{1\pm\sqrt{\alpha^2-4}}{2},$$

while for $\alpha<2$ only the $\alpha_1=\alpha_2$ solution is possible.
Thus in the extreme where β and γ are big, we have oscillation iff $\alpha>2$, and the period of that oscillation ($\alpha_1>\alpha_2>\alpha_1$) is simply 2 time units, i.e. $2T$.

In the general case, for any α, β, γ, we can eliminate q between the differential equations for p and q to get a single second-order equation for p:

$$\ddot{p}[t]+\left(\frac{1}{\beta}+\frac{1}{\gamma}\right)\dot{p}[t]+\frac{1}{\beta\gamma}p[t]=\frac{\alpha}{1+p[t-1]^2} \qquad (1)$$

This has oscillatory solutions provided α, β and γ are sufficiently large. To discover the period of oscillation, we examine the interval from one extremum of $p[t]$ to the next.
Since the right-hand side of (1) is a monotonically decreasing function of p, the left-hand side is a minimum when $p[t-1]$ is a maximum, and vice-versa.
Let us define

$$\Pi[t]=p[t]+\left(\frac{1}{\beta}+\frac{1}{\gamma}\right)\dot{p}[t]+\frac{1}{\beta\gamma}\ddot{p}[t].$$

Then $\Pi[t_0]$ is a minimum if $\Pi[t_0-1]$ is a maximum, and vice-versa.
We know (from the preceding discussion, and from numerical analysis) that when β and γ are large, the interval between extrema is approximately 1, so that if $\Pi[t_0-1]$ is an extremum, then $\Pi[t_0]$ will be close to an extremum. In other words, $p[t]$ will have an extremum at $t=t_0+u$, where u is small and

satisfies $f'[p_0+u]=0$. In the neighbourhood of p_0+u, therefore, we can use a Taylor expansion to approximate f thus:

$$f[p]=f[p_0+u]+(p-p_0-u)^2 f''[p_0+u]/2.$$

Substituting in the expression for $f[p]$, we get

$$f[p] = f[p_0+u] + \tfrac{1}{2}(p-p_0-u)^2 f''[p_0+u] + \left(\frac{1}{a}+\frac{1}{b}\right)(p-p_0-u) f'[p_0+u] + \frac{1}{ab} f[p_0+u]$$

But $f[p]$ has a minimum at $p=p_0$. Therefore

$$f'[p_0] = \left(-u+\frac{1}{a}+\frac{1}{b}\right) f'[p_0+u] = 0 .$$

Assuming that $f''[p_0+u]\neq 0$, it follows that

$$u=\frac{1}{a}+\frac{1}{b} .$$

Thus if $f[p]$ has a minimum at $p=p_0$-1, it has a maximum at $p=1/a+1/b$, and vice-versa. The period of oscillation, therefore, is $2\left(1+\frac{1}{a}+\frac{1}{b}\right)$ units of the dimensionless variable p, i.e. $2\left(T+\frac{1}{b}+\frac{1}{c}\right)$ in ordinary time units.

Although this formula is an approximation derived on the assumption that a and b are large (i.e. that the protein and mRNA lifetimes are short compared with the delay time T), numerical solution of the equations shows that it is accurate to within about 15% over practically the whole of the range of parameter values for which sustained oscillations occur.

The key point that emerges is that the period is practically independent of the rate constants a and k for synthesis of protein and mRNA, and independent also of the critical protein concentration p_0, so long as these parameters are such as to permit sustained oscillations.

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Segmentation Clock: Insights from Computational Models

Dispatch

Olivier Pourquié[1] and Albert Goldbeter[2]

Two new theoretical models show how negative feedback loops incorporating a time delay can account for a variety of transcriptional oscillations, such as the mechanism of the segmentation clock in zebrafish and a number of recently identified transcriptional oscillators.

The study of biological oscillators has for long been a major focus of interest for theoretical biologists [1]. Complex models of the cell cycle [2] or circadian clocks [1] have been elaborated in the past few years. In developmental biology, few examples of oscillators have been identified [3]. The best characterized example so far is the segmentation clock, a transcriptional oscillator involved in the control of the segmentation of the body axis [4]. While a wealth of data has accumulated on this oscillator over the past few years, no modelling attempts based on these data have been reported — until now.

A paper by Lewis [5] in this issue of *Current Biology* proposes a theoretical model which integrates the different components of the zebrafish oscillator. The proposed model is based on a negative feedback loop with a transcriptional delay. It accounts for the transcriptional oscillations produced by the segmentation clock. This study is complemented by a report by Monk [6], which extends this modelling approach to other oscillations based on transcriptional loops recently uncovered. The articles by Lewis and Monk illustrate the usefulness of theoretical models for comprehending the dynamics of regulated cellular processes. Both studies show that mathematical models provide an important tool for analyzing dynamic phenomena that cannot be predicted on the basis of sheer intuition.

The body of a vertebrate animal is formed by a series of repeated blocks called segments, which include structures such as vertebrae, muscles and peripheral nerves. This segmental pattern of the body axis is established early in embryogenesis through the rhythmic production of the somites, paired blocks of paraxial mesoderm which bud off sequentially from the anterior extremity of the presomitic mesoderm. The segmentation clock drives the periodic transcription in the presomitic mesoderm of so-called 'cyclic genes', most of which are related to the Notch signalling pathway (see [4] for a review).

The cyclic genes include downstream targets of Notch signalling, such as genes of the *hairy* and *Enhancer of Split* family — including *c-hairy1*, *c-hairy2*, *hes1*, *hes7*, *her1*, *her7* and *hey* in chick, mouse and zebrafish — and also genes encoding regulators of Notch signalling, such as the ligand DeltaC in zebrafish and the glycosyl-transferase Lunatic fringe in mouse and chick. One proposed role of the clock is to drive the periodic activation of Notch signalling in the rostral presomitic mesoderm, thus setting the pace of boundary formation. The Notch pathway plays a central role in the core mechanism of the oscillator, and it has been suggested that it coordinates oscillations between neighboring presomitic mesoderm cells [7–13].

The theoretical model proposed by Lewis [5] entirely relies on Notch signalling (Figure 1). It accounts for the generation of the cyclic gene oscillations, and for their coordination in the presomitic mesoderm. This model is based on two interacting loops: a negative feedback loop established by the basic-helix–loop–helix transcription factors HER1 and HER7 on their own promoters, and an intercellular loop involving regulated Notch activation [10,11,13]. Lewis [5] models first the HER1/HER7 direct negative auto-regulatory loop. He shows that transcriptional oscillations with a period similar to that seen *in vivo* (around 30 minutes) can be obtained with parameters in a physiological range, if the delays in production of Her mRNA and protein are taken into account. The oscillations are robust even when one allows for the noisy, flickering character of gene regulation, as the regulatory protein binds to and dissociates from its site in the DNA.

By varying the parameter values, Lewis [5] shows that the model is compatible with a number of experimental observations, some of which are counter-intuitive. For instance, the model indicates that oscillations can be maintained, with period practically unchanged, even if the protein synthesis rate is drastically reduced. This prediction of the model is compatible with the published effects of cycloheximide treatments, in which *c-hairy1* oscillations were not completely halted [14]. Another prediction of the model is the progressive dampening or loss of regularity of the oscillations if Notch signalling is impaired — dampening if noise effects are small, loss of regularity if noise effects are larger. This may account for the observation that, in zebrafish or mouse *notch* mutants, the severity of the segmentation defects observed increases along the antero-posterior axis, with the cells — in the zebrafish mutants at least — becoming progressively uncoordinated so as to create a random, pepper-and-salt pattern of expression of the oscillatory genes [9].

The second feedback loop is based on Notch activation by Delta in an adjacent cell, regulating the expression of the *her* genes in this cell. This loop drives the periodic expression of *deltaC*, resulting in rhythmic activation of Notch and of its downstream target *her* genes. Physiological parameters can be found for which synchronized oscillations of the HER-based loop can be triggered by this circuitry in adjacent cells. The Notch-based loop is able to sustain

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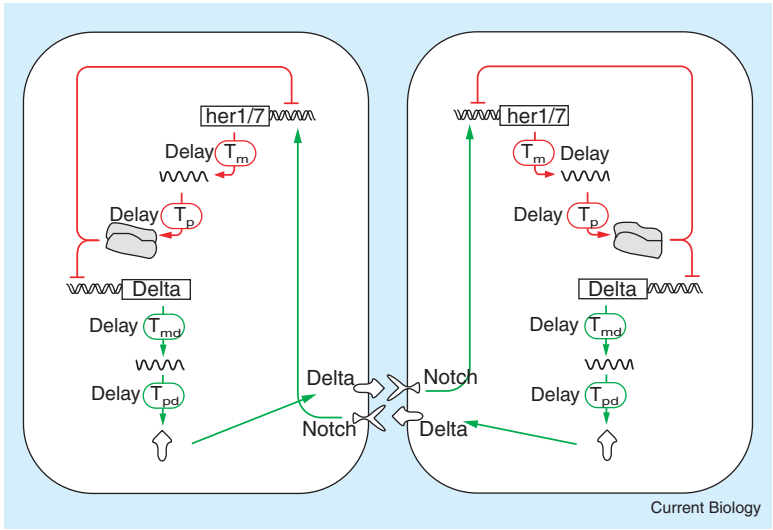

Figure 1. A schematic representation of the molecular network constituting the segmentation clock in two adjacent cells in zebrafish.

The circuitry constituting the HER1/HER7-based oscillator — which also requires components of the Notch pathway — is shown in red while the pure Notch-based oscillator is shown in green. (Adapted from [5].)

oscillations on its own and to synchronize these oscillations between adjacent cells. But these oscillations have a much longer period than those driven by the HER-based loop, closer to the period seen in the chick or mouse somite clock (around 2 hours). Remarkably, the Notch-based synchronous oscillations can occur even if there is no direct HER autoregulation: that is, they can be generated by the standard Delta–Notch signalling circuitry which is usually assumed to mediate lateral inhibition [15].

The model simulations thus show that, in the zebrafish somitogenesis clock, oscillatory gene expression may result either from direct negative autoregulation of *her1/her7* expression within each cell of the presomitic mesoderm, or from intercellular communication via the Delta–Notch pathway. A slight change in parameter values modulating the respective weights of the two types of regulation can produce an abrupt change in the period of oscillatory gene expression, as the mechanism switches from a 'pure' internal one to one based primarily on the intercellular mechanism. This leads Lewis [5] to speculate that, during evolution, the use of one or the other loop might have varied between species, thus accounting for the diversity of speed of somite formation seen among vertebrates.

A parallel can be made between the work reported by Lewis [5] and models for circadian oscillations, which have recently uncovered the possibility that there are multiple sources of oscillatory behavior in the genetic regulatory network of the circadian clock in *Drosophila* and mammals [16]. Wnt signalling has recently been shown to act upstream of Notch in the segmentation clock mechanism in the mouse embryo [17]. Such a role for the Wnt pathway has not been established yet in the zebrafish clock, but it is certainly possible that it has one. If this is the case, the model will have to be refined and its complexity will drastically increase.

A second report by Monk [6] nicely complements the theoretical approach of Lewis [5] on the zebrafish somitogenesis oscillator. Monk describes models in which a transcriptional delay is introduced into negative feedback loops to explain the recently described oscillations of the mRNAs coding for the transcription factors HES1, p53 and NFκB. These oscillations have been reported to occur with a period of 2–3 hours in cultured cells [18–20]. Monk's simulations based on such a modeling approach nicely fit the experimental observations in the original papers.

In simple models based on a negative feedback involving two variables, sustained oscillations can be obtained only if there is a time delay in the negative feedback loop. This time delay is a critical feature of the models presented by Lewis [5] and Monk [6]. A system with three or more variables, however, can show sustained oscillations even in the absence of such delays. Accordingly, in a recent model describing *hes1* oscillations in cell cultures, Hirata *et al.* [18] invoked the existence of an unknown intermediate to obtain oscillations. The third variable need not be a new molecular species: it suffices to distinguish the cytoplasmic and nuclear forms of the regulatory protein, as shown by a study [1] of models for circadian oscillations based on negative autoregulation of gene expression.

A highly useful aspect of the studies by Lewis [5] and Monk [6] is their attempt to provide experimentally based estimations for the parameter values, including the time delays in transcription and translation. Both authors estimate that the transcription delay is of the order of 10–20 minutes, and show that when the half-life of the protein and mRNA is sufficiently short, the period of the oscillations is largely set by this delay. With regard to oscillatory gene expression controlled by negative feedback, there is a continuum of possible situations: the oscillations are either primarily timed by the transcriptional delay or, at the other extreme, they can arise in the absence of this type of delay, when the chain of events forming the feedback loop is sufficiently long — this constraint is already satisfied in the presence of three variables — and when the degree of cooperativity of repression is sufficiently large. Transcriptional or translational delays probably become negligible when the period of the oscillations becomes very long, as in the case of circadian rhythms. The

incorporation of delays may be important, but the modeller pays a price as they make the numerical integration of the equations more cumbersome.

An interesting point emphasized by these two studies [5,6] is the likelihood of the wide occurrence of oscillatory gene expression resulting from transcriptional delays in regulated genetic networks. The question is: why have such oscillations not been reported more often? A possible explanation is that, given the possibility of cell desynchronisation, it might be necessary to resort to measurements in individual cells to uncover further evidence for the occurrence of oscillatory gene expression. The results of these delay-driven oscillator models show that it might be critical to incorporate delays in the description of genetic regulatory networks. This is important for predicting the impact of time delays on both the dynamic behavior of these networks and the parameter values predicted by the models.

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