Supporting Information

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SI Materials and Methods

Measurement of Time Delays. The velocity of movement of the luciferase expression domain relative to the posterior tip of the PSM was measured from the spatiotemporal profiles of Movie S1 (Fig. S1) so that the space distance can be converted into the time distance. The propagation speed was found to be ∼1 unit/20 min (= 0.05 unit/min) during phase A and 1 unit/40min (= 0.025 unit/min) during phases B and C. One unit is defined as one somite length. For definition of phases A–C, see Fig. 2 legend. To determine the space distance between the reporter expression and *Hes7* transcription, the PSM samples were subjected to in situ hybridization with the first intron as a probe after imaging of reporter expression. To determine the space distance between the reporter expression and Hes7 protein expression, the PSM samples were subjected to immunostaining with Hes7 antibody after imaging of reporter expression. Because the expression domains were propagated into the anterior region, the distance from the last somite border to the anterior end of the reporter, the intron, and Hes7 protein expression was measured. During in situ hybridization and immunostaining, the size of the PSM was changed and therefore normalized to the original size. The distance between the anterior ends of the reporter expression and the intron/Hes7 protein expression was measured and then converted into the time difference on the basis of the propagation speed.

Mathematical Simulation: Significance of Delays. Hes7 oscillations are simulated with the following equations (1),

$$\frac{dp(t)}{dt} = am(t - T_p) - bp(t)$$
$$\frac{dm(t)}{dt} = f(p(t - T_m)) - cm(t),$$

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

$$b = \frac{\ln 2}{\tau_p},\ c = \frac{\ln 2}{\tau_m}.$$

Because transcription is inhibited by Hes7 protein, which acts as a dimer, we assume

$$f(p) = \frac{k}{1 + (p/p_{crit})^2},$$

where k is the number of molecules of *Hes7* mRNA synthesized per unit time in the absence of inhibition and p_{crit} is the amount of protein that gives half-maximal inhibition. We set a = 4.5 protein molecules per mRNA molecule per min, p_{crit} = 40 molecules per cell, k = 33 mRNA molecules per cell per min, and τ_m = 3 min. We assume that the Hes7 protein half-life τ_p = 20 min, T_p = 8 min, and T_m = 29 min. Under these conditions, oscillatory expression continues (Fig. S3*A*). In contrast, when T_m = 10 min (19 min shorter), oscillations are abolished (Fig. S3*C*).

Mathematical Simulation: Distributed Delays. The amplitude of the Hes7 reporter oscillation was different depending on whether the introns were present (Fig. 1 *C* and *D*). We hypothesized that the cause of these differences is related to the nature of the introns (discrete vs. distributed). The average signal of both reporters seemed to increase, at least during the first three peaks (Fig. 1 *C* and *D*). On the other hand, the amplitude seemed to be different between pH7-UbLuc-In(+) and pH7-UbLuc-In(−). In the pH7-UbLuc-In(−) reporter, the average signal was ∼2 at 3 h and ∼3 at 5 h, so that there was an approximate increase of 0.5-fold units every hour. In the pH7-UbLuc-In(+) reporter, the average signal was ∼1.5 at 2 h and ∼2.5 at 4.5 h, so that there was an approximate increase of 0.4-fold units every hour. To model this observation, we hypothesized that this relatively similar signal increase is due to increasing amounts of luciferase substrate inside the cells [function $s(t)$ in Eq. **S1**]. Regarding the amplitude differences, the amplitude of the pH7-UbLuc-In(−) reporter was ∼4-fold whereas the amplitude of the pH7-UbLuc-In(+) reporter was ∼2-fold. To model this observation, we hypothesized that the intronic delay is distributed around an average value.

We used the previously described model (1) with an additional variable l for the luminescence:

$$\begin{aligned} \frac{dp}{dt} &= am(t - T_p) - \frac{ln2}{T_p}p(t) \\ \frac{dm}{dt} &= \frac{k}{1 + (p(t - T_m)/p_{crit})^2} - \frac{ln2}{T_m}m(t) \\ \frac{dl}{dt} &= \frac{ks(t)}{1 + \left(\int_0^\infty p(t - T_l)g_\upsilon^r(T_l)dT_l/p_{crit}\right)^2} - \frac{ln2}{T_m}l(t). \end{aligned} \quad \textbf{[S1]}$$

The function $s(t)$ stands for the intracellular luciferase substrate. To account for the distributed delay, we integrated a delayed protein concentration $p(t - T_l)$ weighted with a probability distribution $g_\upsilon^r(T_l)$ from T_l = 0 to $T_l = \infty$ (2, 3). The probability distribution $g_\upsilon^r(T_l) = (\nu^{r+1}/r!)T_l^r e^{\nu T_l}$ showed a maximum at $T_l = r/\nu$ and was zero for T_l = 0 and for $T_l \to \infty$. The value of r influenced the shape of the probability distribution $g_\upsilon^r(T_l)$ from a broad distribution for low r to a very sharp distribution approximating a discrete delay for high r. To simulate the integral differential equation, we need to convert it into a set of ordinary differential equations using the linear chain trick (2), which results in

$$\begin{aligned} \frac{dl}{dt} &= \frac{ks(t)}{1 + (x_r/p_{crit})^2} - \frac{ln2}{T_m}l(t) \\ \frac{dx_0}{dt} &= \upsilon(p(t) - x_0(t)) \\ \frac{dx_j}{dt} &= \upsilon(x_{j-1}(t) - x_j(t)),\ j = 1,\ \ldots,\ r. \end{aligned} \quad \textbf{[S2]}$$

For r = 1 and $\nu = r/T_l$, the set of equations becomes

$$\begin{aligned} \frac{dp}{dt} &= am(t - T_p) - \frac{ln2}{T_p}p(t) \\ \frac{dm}{dt} &= \frac{k}{1 + (p(t - T_m)/p_{crit})^2} - \frac{ln2}{T_m}m(t) \\ \frac{dl}{dt} &= \frac{ks(t)}{1 + (x_1/p_{crit})^2} - \frac{ln2}{T_m}l(t) \\ \frac{dx_0}{dt} &= \frac{r}{T_l}(p(t) - x_0(t)) \\ \frac{dx_1}{dt} &= \frac{r}{T_l}(x_0(t) - x_1(t)). \end{aligned} \quad \textbf{[S3]}$$

To simulate the previous equation system, we chose the same parameter values as previously described (1); that is, a = 4.5, p_{crit} = 40,

$k = 33$, $\tau_m = 3$, $\tau_p = 20$, $T_p = 8$, and $n = 2$. For the distributed delay, the parameter is $r = 1$. We carried out two simulations for the pH7-UbLuc-In(−) and pH7-UbLuc-In (+) case where $T_l = 10$ min (Fig. 1*C*) and $T_l = 29$ min (Fig. 1*D*), respectively. To remove transient effects, the model was simulated for 9,400 min before plotting. During the transient simulation, the intracellular substrate was held at $s(t) = 0$ and after that the substrate concentration followed the function $s(t) = 0.283t$. These results suggest that the increase of the average signal is related to the concentration increase of the intracellular luciferase substrate, whereas the differences of amplitude between pH7-UbLuc-In(−) and pH7-UbLuc-In(+) constructs are related to the distributed nature of the reporter delay (Fig. S3*D*).

Primers for Genotyping. Genotypes were determined by PCR using the following primers:

For pH7-UbLuc-In(+) and pH7-UbLuc-In(−) mice,
LucF, 5′-TACTGGTCTGCCTAAAGGTG-3′;
LucR, 5′-CCACCAGAAGCAATTTCGTG-3′.
For wild-type, *Hes7*-null, and *Hes7* intronless mice (Fig. S5),
Primer 1, 5′-GTCACCCGGGAGCGAGCTGAGAAT-3′;
Primer 2, 5′-AAGTTGGGCTCTGACCCTGCCCTC-3′;
Primer 3, 5′-AGCGCGGAGAAAAGCTGGGAGCGTG-3′;
Primer 4, 5′-AGAAAGGGCAGGGAGAAGTGGGCGAG-CCAC-3′;
Primer 5, 5′-GTTCTGAGAGCGAGAGGGGGTCTGGGA-TGG-3′;
Primer 6, 5′-TTGGCTGCAGCCCGGGGGATCCACTAGT-TC-3′.

For rescue experiments with pH7-HA-Hes7-In(+) and pH7-HA-Hes7-In(−), the following primers as well as primers 2, 3, and 5 were used:

Primer 7, 5′-ACCCTGCAGCGGCGGGATATAAGG-3′;
Primer 8, 5′-GTCCACCGAAGGGTCCGGAGGAGCAAT-GGT-3′.

Primers 2, 3, and 7 were used for pH7-HA-Hes7-In(+) (319 bp), pH7-HA-Hes7-In(−) (486 bp), and the wild-type and *Hes7*-null alleles (266 bp). Primers 5 and 8 were used for the wild-type endogenous *Hes7* allele.

Western Blotting. The posterior parts of embryos were mixed with 30 μL of lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 0.5% Nonidet P-40, 1× Proteinase inhibitor mixture, 1 mM PMSF, 250 units/mL Benzonase) and incubated on ice for 30 min. After addition of 3 μL of 10% SDS, the samples were boiled, and the protein concentrations were measured. The protein solution after boiling was run on 12.5% SDS/PAGE. After transferring protein from the gel to PVDF membrane (Millipore; IPVH15150), the membrane was immersed in buffer containing 5% skim milk, anti-Hes7 antibody (1/500) (4), and peroxidase-conjugated anti-guinea pig IgG (1/5,000; Chemicon) in sequence. Immunoreactive bands were visualized with ECL-plus (GE Healthcare) and LAS 3000 mini (Fujifilm). Intensity of each band was calculated with Image Gauge (Fujifilm). After stripping antibodies, the membrane was immersed in buffer containing 5% skim milk, anti–β-tubulin IgG (Santa Cruz), and peroxidase-conjugated anti-rabbit IgG (GE Healthcare) in sequence. Immunoreactive bands were visualized with ECL (GE Healthcare). The intensity of Hes7 protein was normalized by that of β-tubulin.

1. Hirata H, et al. (2004) Instability of Hes7 protein is crucial for the somite segmentation clock. *Nat Genet* 36:750–754.
2. Fall CP, Marland ES, Wagner JM, Tyson JJ (2000) *Computational Cell Biology* (Springer, Berlin).
3. Rateitschak K, Wolkenhauer O (2007) Intracellular delay limits cyclic changes in gene expression. *Math Biosci* 205:163–179.
4. Bessho Y, Hirata H, Masamizu Y, Kageyama R (2003) Periodic repression by the bHLH factor Hes7 is an essential mechanism for the somite segmentation clock. *Genes Dev* 17:1451–1456.

Fig. S1. Spatiotemporal profiles of *Hes7* oscillations in the PSM. The bioluminescence intensity of pH7-UbLuc-In(−) along the anterior–posterior axis was plotted according to the time. The profiles were made from Movie S1. *Hes7* oscillation was propagated from the posterior end to the anterior PSM. The space between red lines corresponds to one somite length. The arrowhead indicates the position of a newly formed boundary around time = 5. On the basis of the spatiotemporal profiles from Movie S1, the velocity of movement of the luciferase expression domain (propagation speed) was measured. The average propagation speed was ∼1 unit/20 min (= 0.05 unit/min) during phase A and 1 unit/40 min (= 0.025 unit/min) during phases B and C. One unit is defined as one somite length. For definition of phases A–C, see Fig. 2 legend.

Fig. S2. Introns delay the timing of gene expression. *Hes1* promoter-driven Ub-Luc reporters pH1-UbLuc-In(–), which has no intron, and pH1-UbLuc-In(+), which has all *Hes7* gene introns, were transfected into C3H10T1/2 cells, and the reporter protein expression after serum stimulation was quantified. The average peak of Luc occurred at time = 56 min in pH1-UbLuc-In(–) and at time = 77 min in pH1-UbLuc-In(+). Thus, introns led to an ~20-min delay in expression. The average with a SE of three independent experiments was determined at each time point.

Fig. S3. pH7-UbLuc-In(+) reporter expression in the presence of PTC124. To prevent nonsense-mediated mRNA decay, the pH7-UbLuc-In(+) reporter explant was cultured in the presence of 0.5 μg/mL PTC124 (Enzo Life Sciences). We also performed the culture in the presence of 5.0 μg/mL PTC124 and obtained similar patterns. The amplitude was not improved by PTC124.

Fig. S4. Mathematical simulation of *Hes7* mRNA and Hes7 protein oscillations. (*A*) When a transcriptional delay (T_m) is 29 min (the wild-type condition), oscillations of *Hes7* mRNA and Hes7 protein expression continue in a sustained manner (*SI Materials and Methods*). (*B*) A 12-min shorter delay (T_m = 17 min) makes the amplitude much smaller and damps oscillatory expression. (*C*) A 19-min shorter delay (T_m = 10 min, the ΔIn/ΔIn condition) abolishes oscillations of *Hes7* mRNA and Hes7 protein expression. (*D*) Simulation of the pH7-UbLuc-In(–) reporter (T_I = 10 min) (*Left*) and the pH7-UbLuc-In(+) reporter (T_I = 29 min) (*Right*). Solid curves show the simulations and dots stand for experimental data. The simulation values were normalized by dividing by the minimal value of the simulation.

Fig. S5. Deletion of all introns from the *Hes7* locus. (*A*) Strategy for generation of the intronless *Hes7* allele. Solid and open squares indicate coding and noncoding regions of *Hes7*, respectively. A neomycin-resistant gene cassette (Neo^r) flanked by LoxP sites was inserted into the SacI site in the 3′-downstream region in the targeting vector. The diphtheria toxin A (DT-A) gene was used as a negative selection marker. The targeting vector was introduced into mouse ES cells, and after obtaining homologous recombinant ES cells, Neo^r was removed by Cre. Bands with sizes detected by 5′ and 3′ probes are indicated at the bottom. (*B*) Southern blotting analysis of genomic DNA extracted from embryos or ES cells. DNA was digested with SacI for the 5′ probe and KpnI for the 3′ probe.

Fig. S6. Genotyping of *Hes7*-mutant mice and verification of the intronless mutation. (*A* and *B*) PCR analysis for genotyping. Genomic DNA from the ES cells (*A*) and mouse tails (*B*) was used as a template. (*C*) The positions of primers are indicated by arrows. (*D*) Verification of the absence of the introns. Genomic DNA of the intronless mice was isolated and sequenced by PCR. In these mice, all introns were removed from the *Hes7* locus.

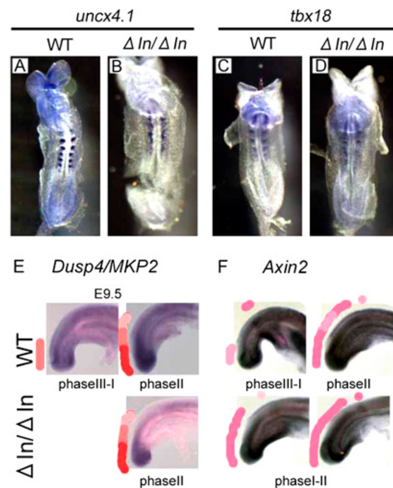


Fig. S7. Patterning defects in *Hes7* intronless mice. (*A–D*) *Uncx4.1* (*A* and *B*) and *Tbx18* (*C* and *D*) expression in E8.5 wild-type (*A* and *C*) and *Hes7* intronless (*B* and *D*) embryos was examined by in situ hybridization. *Uncx4.1* and *Tbx18* expression was not properly segmented in *Hes7* intronless mice. Anterior is to the top in *A–D*. (*E*) *Dusp4/MKP2* and *Axin2* expression. *Dusp4/MKP2* expression oscillates in wild-type embryos at E9.5. *n* = 4/9 (phase III-I) and *n* = 5/9 (phase II). (*Lower Left*) By contrast, *Hes7* intronless mice lose the oscillation of *Dusp4/MKP2* expression. *n* = 4. (*F*) *Axin2* expression was dynamic in wild-type embryos at E9.5. *n* = 7/9 (phase III-I) and *n* = 2/9 (phase II). (*Lower Right*) *Axin2* expression was still variable but less dynamic in *Hes7* intronless embryos. *n* = 2/7 (phase I) and *n* = 5/7 (phase II).

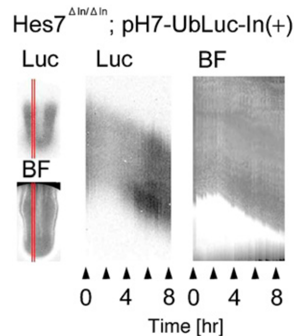


Fig. S8. Reporter expression in *Hes7* intronless mice. pH7-UbLuc-In(+) reporter was introduced into *Hes7* intronless mice. Real-time imaging of *Hes7* promoter activity in the PSM explant culture was performed (Luc), and spatiotemporal profiles of the reporter expression were made. Oscillatory reporter expression was not detectable, although some difference in amplitude was observed. Clear segmentation was not observed in a bright field (BF).

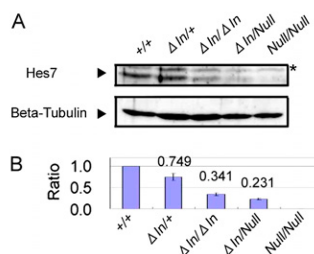
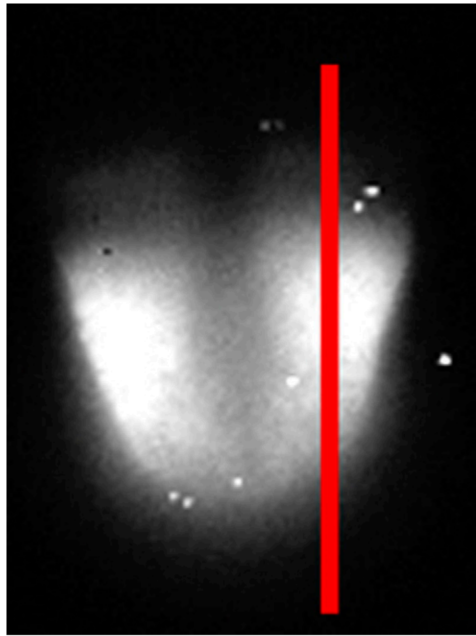
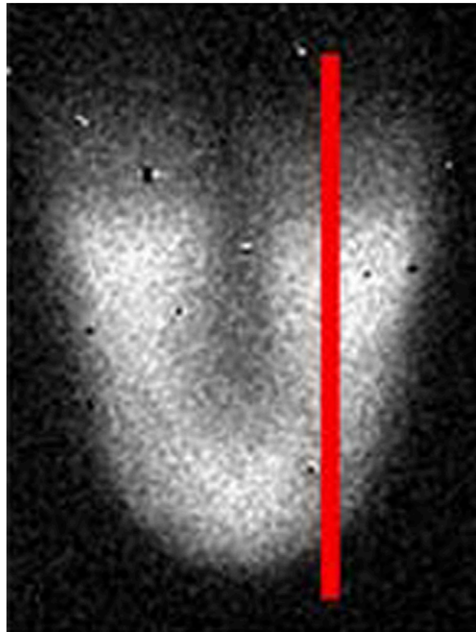


Fig. S9. Western blot analysis of Hes7 expression in the PSM. (*A*) Proteins were extracted from the posterior parts of embryos, and the average expression levels of Hes7 protein were analyzed by Western blot. The asterisk indicates a nonspecific band. (*B*) Relative average levels of Hes7 protein expression. The average of three independent samples with a SE was determined for each genotype. Each value was normalized with that of β-tubulin. About 34% of the wild-type levels of Hes7 protein were expressed in *Hes7* intronless mice (ΔIn/ΔIn).



Movie S1. Time-lapse images of the PSM of a pH7-UbLuc-In(−) embryo at E10.5. Images were taken by 20-min exposure and binning of pixels 4 × 4 over a period of 10 h. Oscillations were propagated from the posterior to the anterior PSM.

Movie S1



Movie S2. Time-lapse images of the PSM of a pH7-UbLuc-In(+) embryo at E10.5. Images were taken by 20-min exposure and binning of pixels 4 × 4 over a period of 10 h. Oscillations were propagated from the posterior to the anterior PSM.

Movie S2