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Avian *hairy* Gene Expression Identifies a Molecular Clock Linked to Vertebrate Segmentation and Somitogenesis

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Summary

We have identified and characterized *c-hairy1*, an avian homolog of the Drosophila segmentation gene, *hairy*. *c-hairy1* is strongly expressed in the presomitic mesoderm, where its mRNA exhibits cyclic waves of expression whose temporal periodicity corresponds to the formation time of one somite (90 min). The apparent movement of these waves is due to coordinated pulses of *c-hairy1* expression, not to cell displacement along the anteroposterior axis, nor to propagation of an activating signal. Rather, the rhythmic *c-hairy* mRNA expression is an autonomous property of the paraxial mesoderm. These results provide molecular evidence for a developmental clock linked to segmentation and somitogenesis of the paraxial mesoderm, and support the possibility that segmentation mechanisms used by invertebrates and vertebrates have been conserved.

Introduction

Identification and characterization of the Drosophila melanogaster segmentation genes has led to a recent revival of interest in mechanisms underlying vertebrate segmentation (Lewis, 1978; Nüsslein-Volhard and Wieschaus, 1980; Tautz and Sommer, 1995; Kimmel, 1996; De Robertis, 1997). However, the process of segmentation in Drosophila differs significantly from that of more primitive insects or vertebrates. In long germband insects such as the fly, segments are determined essentially simultaneously in a syncytial unicellular embryo, prior to gastrulation. In more primitive short germband insects like orthopterans, in other arthropods such as crustaceans, and in vertebrates, segment determination occurs in a cellularized embryo, and posterior segments are laid down sequentially from a terminal growth zone during the course of development.

In vertebrate embryos, the most obvious metameric structures are the somites. They constitute the basis of the segmental pattern of the body and give rise to the axial skeleton, the dermis of the back, and all striated muscles of the adult body (Christ and Ordahl, 1995). Individual pairs of somites, located symmetrically on either side of the neural tube, emerge from the rostral end of the presomitic mesoderm (PSM), while new mesenchymal cells enter the caudal paraxial mesoderm, as a consequence of gastrulation. In the chick embryo, a somite pair is laid down every 90 min in a rostro-caudal progression, and a total of 50 somite pairs are formed during embryogenesis. The presomitic mesoderm appears as a long strip of mesenchymal tissue, and surgical experiments have shown that approximately 10–12 prospective somites are contained within the 2-day-old chick PSM (Packard, 1976; I. P. et al., unpublished data). Its length becomes progressively reduced during later development. Also, it has been suggested that the PSM includes up to 12 "somitomeres," segmented arrangements of cells that can be visualized using the electron microscope and that may correspond to prospective somites (Meier, 1984).

Although various models have been proposed to account for segmentation in vertebrates, little is currently known about underlying molecular mechanisms (see Keynes and Stern, 1988; Tam and Trainor, 1994, and references therein; Discussion below). Numerous vertebrate homologs of the Drosophila segmentation genes have been identified but are not expressed during somitogenesis. However, homologs of the neurogenic genes, *Notch*, *Delta* (*Delta like-1*:*Delta1*) and *RBPjk* genes, which are not involved in segmentation in the fly, have been implicated in vertebrate somitogenesis. Targeted inactivation of these genes in mice leads to a disruption of somitogenesis (Conlon et al., 1995; Oka et al., 1995; Hrabe de Angelis et al., 1997). Nevertheless, although somitogenesis is disrupted in *Delta1*$^{-/-}$ mice, paraxial mesoderm derivatives such as muscles or skeleton retain a segmented pattern (Hrabe de Angelis et al., 1997). These results, therefore, support the view that segmentation occurs independently of somitogenesis, and were also taken as confirmation of the widely held view that segmentation arose independently in vertebrates and invertebrates.

In this paper, we report the identification and analysis of the chick *c-hairy1* gene, an avian homolog of the Drosophila *hairy* segmentation gene. In Drosophila, *hairy* is a member of the pair-rule genes, which are the first to reveal the prospective metameric body plan of the fly (Nüsslein-Volhard and Wieschaus, 1980; Ish-Horowicz et al., 1985). Here, we show that *c-hairy1* mRNA is expressed in a highly dynamic manner in the chick PSM, appearing as a caudo-rostral wave, which is reiterated during the formation of every somite. We demonstrate that this wavefront is not due to cell movements within the PSM, nor to the periodic production of an anterior-to-posterior diffusing signal, but is an autonomous property of the cells in this tissue. We show

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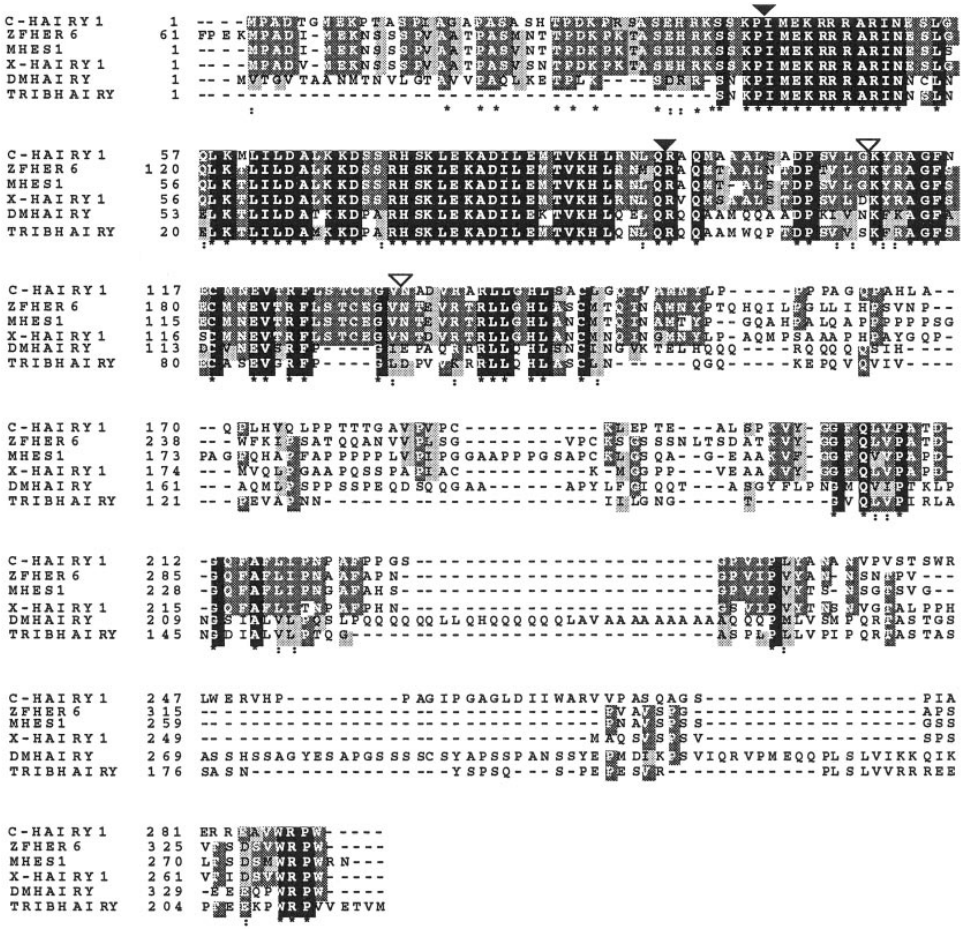

Figure 1. Sequence Analysis of *c-hairy1*

Comparison of the *c-hairy1* protein sequence with that of other vertebrate homologs belonging to the *Hairy/Enhancer-of-split* (HES) family and the insect *hairy* proteins using the ClustalX programme (Higgins et al., 1996). *c-hairy1* shows highest homology with the Xenopus *x-hairy1*, the zebrafish *Her6*, and the mammalian *HES* genes. The bHLH domain (between black arrowheads) and the orange domain (Fisher et al., 1996) (between white arrowheads) are well conserved between all the HES proteins, as well as the tetrapeptide WRPW at the carboxyl terminus, essential to recruit the corepressor Groucho and exert its negative effect on the transcriptional apparatus (Paroush et al., 1994; Fisher et al., 1996).

that blocking protein synthesis in embryo explants leads to an arrest of somitogenesis but that the oscillations of *c-hairy1* expression persist. This provides evidence against the cyclic *c-hairy1* expression being under negative autoregulatory control. Together, these results demonstrate that cells of the PSM undergo a defined and constant number of *c-hairy1* expression cycles between emergence from the primitive streak and incorporation into a somite. The rhythmic oscillations of the *c-hairy1* messenger RNA in prospective somitic cells provide the first molecular evidence in favor of a developmental clock involved in vertebrate segmentation.

Results

Identification of an Avian *hairy* Homolog (*c-hairy1*) Expressed in the Paraxial Mesoderm

To identify chick homologs of the fly pair-rule gene *hairy*, we used a PCR-based approach with degenerate oligonucleotides that correspond to sequences conserved between the two *hairy*-like genes in Drosophila (*hairy* and *deadpan)*. An initial PCR fragment was used to screen a random-primed cDNA library prepared from chick embryonic mRNA, and several positive clones were isolated. Sequence analysis of a fraction of these cDNAs revealed that they arise from a new gene, named *c-hairy1*. Comparison with other vertebrate *Hairy*-like genes reveals that *c-hairy1* is most similar to the Xenopus laevis *hairy1*, the mammalian *HES*, and the zebrafish *Her6* genes (Figure 1).

The putative c-hairy1 protein is 291 amino acids long, including a bHLH domain and the tetrapeptide WRPW at the carboxyl terminus, which are characteristic features of the *hairy*-related class of bHLH transcription factors in flies and vertebrates (Figures 1 and 2). Analysis of the c-hairy1 sequence suggests that it belongs to a subgroup of the WRPW-containing bHLH proteins, which includes mammalian HES1 and HES2, Xenopus X-hairy1, zebrafish Her6, and the fly and tribolium hairy (Figure 1). The *Enhancer-of-split* and the zebrafish *Her1* genes are only distantly related to these *hairy*-like genes (Figure 2). In Drosophila, these proteins act as transcriptional repressors in a variety of developmental contexts (Ohsako et al., 1994; Paroush et al., 1994; Van Doren et

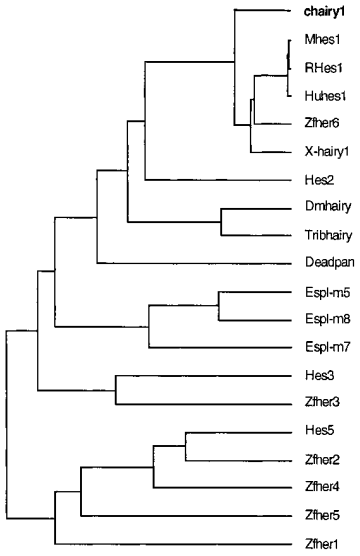

Figure 2. Phylogenetic Tree Analysis of b-HLH Proteins of the Hairy and Enhancer-of-Split Families Indicate that c-Hairy1 Belongs to the Hairy Family of Proteins

The tree was generated using the Distances and Growtree progammes of the GCG package Version 9.0 (Madison, Wisconsin). Another bootstrapped tree calculated on a Power Macintosh using the ClustalX programme (Higgins et al., 1996) generated a substantially similar tree (not shown). All compared sequences are accessible in Genbank. Espl, *Enhancer-of-split*; HES, *Hairy* and *Enhancer-of-split* genes; Her, *Hairy* and *Enhancer-of-split* related genes; X, Xenopus; M, mouse; Zf, zebrafish; Hu, human; Dm, *Drosophila Melanogaster*; Trib, *Tribolium Castaneum*; *Hes1*, *Hes2*, *Hes3*, and *Hes5* are the rat genes.

al., 1994; Jiménez et al., 1996; Fisher et al., 1996). Given the structural conservation, it is likely that c-hairy1 functions similarly during chick development. Expression of the *c-hairy1* gene was analyzed during chick embryonic development and was detected in several tissues (data not shown). In this paper, we focus our attention on mesoderm expression, in particular on the presomitic mesoderm, where *c-hairy1* revealed a very dynamic mRNA expression pattern.

Cyclic *c-hairy1* mRNA Expression in the Paraxial Mesoderm Is Correlated with Somite Formation

Analysis of the *c-hairy1* mRNA expression pattern in the paraxial mesoderm was carried out by whole mount in situ hybridization of embryos containing between 1 and 25 somites. *c-hairy1* expression is detected in the caudal part of somites at all stages examined, where it persists in the caudal sclerotome for at least 15 hr (Figure 3 and data not shown). By contrast, *c-hairy1* expression in the presomitic mesoderm is highly dynamic, as reflected by the variety of expression patterns that are seen in embryos with an identical number of somites. The domain of *c-hairy1* expression has the appearance of a wavefront beginning in the broad, caudal PSM, progressing anteriorly and intensifying into the narrow anterior PSM (Figure 3, top). Finally, in each cycle, expression decays sharply throughout the PSM except for a thin stripe corresponding to the posterior part of the forming somite (somite 0).

This dynamic expression sequence is reiterated during the formation of every somite and can be represented as a cycle of three successive stages (Figure 3, bottom). In stage I, *c-hairy1* transcripts are detected in a broad domain comprising the posterior 70% of the PSM (corresponding to at least eight prospective somites) and in a narrow band in the prospective caudal part of the forming somite (somite 0). In stage II, the posterior band of *c-hairy1* expression has narrowed to about 3 somite-equivalents in length and has moved anteriorly, so it now lies in the rostral half of the PSM. In stage III, the *c-hairy1* expression domain becomes narrower than a somite-equivalent and moves further anteriorly, forming a stripe coincident with the caudal part of prospective somite 0.

Transitions between these stages are observed, indicating that *c-hairy1* is expressed as a continuous and dynamic sequence rather than abrupt switches from one stage to the other. For example, the broad caudal stripe observed in stage I begins to appear during stage III (Figure 3C), indicating that stage III is indeed a precursor to the next stage I and that the anterior *c-hairy1* stripe in stage III is a precursor to the stripe in somite 0 of stage I. In addition, the intensity of *c-hairy1* expression increases between stage I and stage III. Out of 71 embryos analyzed, 24 were found in stage I, 22 in stage II, and 25 in stage III. Based on a cycle time of 90 min, we estimate that each stage lasts about 30 min.

The reiterated patterns of *c-hairy1* expression at the different stages examined suggest that the wavefront of *c-hairy1* in the unsegmented mesoderm occurs in a cyclic fashion correlated with somite formation. To investigate this further, we cultured bilaterally divided avian embryos in vitro, under conditions where the PSM yields at least three new somites according to in vivo kinetics (one somite per 90 min). The caudal parts of 2-day-old embryos including the PSM were removed and separated surgically along the midline into two halves. One embryonic half was fixed immediately, and the other half cultured on a filter for 30–270 min prior to fixation. Both halves were then hybridized with the *c-hairy1* probe, and the expression pattern on the two sides was compared (Figure 4).

After culturing for 30 to 60 min, the patterns of *c-hairy1* expression in the cultured and uncultured presomitic mesoderm always differ (Figure 4A, $n = 18$), demonstrating the extremely dynamic nature of the expression of this mRNA. However, when half embryos are cultured for 90 min, the time required to form one somite, the *c-hairy1* expression patterns in the PSMs of cultured and uncultured halves are identical, reflecting the cyclic property of this expression pattern (Figure 4B; $n = 25$). The same rhythmicity of *c-hairy1* expression profile is also observed when half embryos are cultured for 270 min, corresponding to the time required to form three somites in vivo ($n = 3$; data not shown). Therefore, the wavefront of *c-hairy1* expression in the PSM occurs in a cyclic fashion, with a periodicity that correlates precisely with somite formation.

The Wave of *c-hairy1* mRNA Expression in the Presomitic Mesoderm Is Independent of Cell Movement

Several mechanisms could account for the kinetics of *c-hairy1* expression in the PSM. One simple possibility

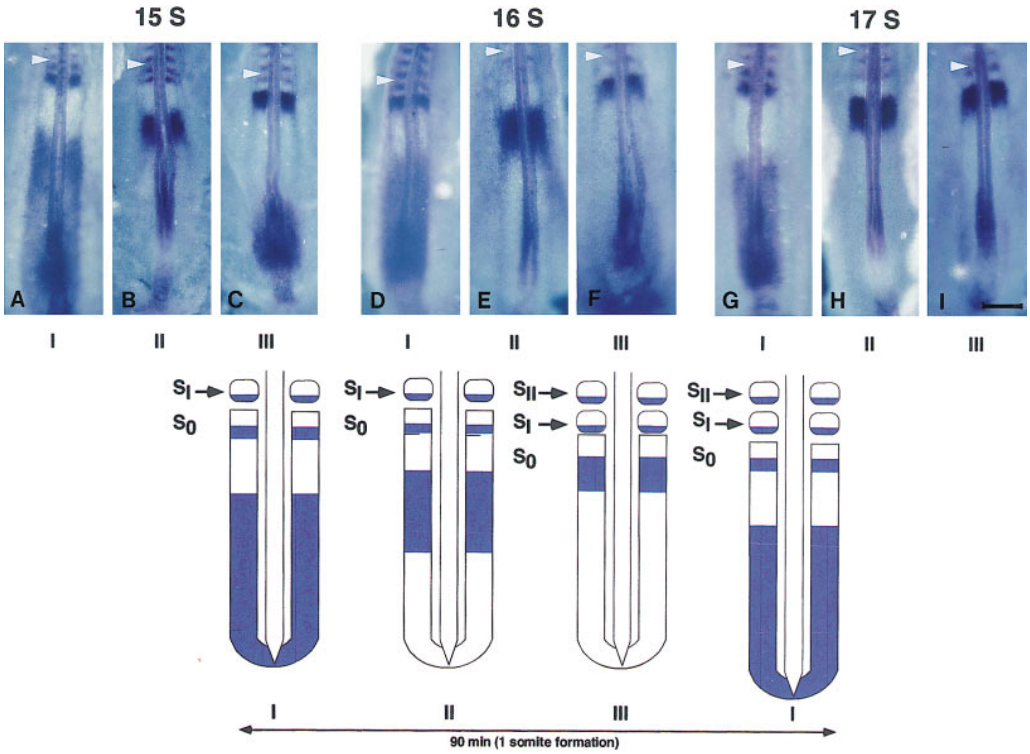

Figure 3. *c-hairy1* mRNA Expression in the Presomitic Mesoderm Defines a Highly Dynamic Caudal-to-Rostral Expression Sequence Reiterated during Formation of Each Somite

(Top) In situ hybridization with *c-hairy1* probe showing the different categories of *c-hairy1* expression patterns in embryos aged of 15 (A, B, and C), 16 (D, E, and F), and 17 (G, H, and I) somites. Rostral to the top. Bar = 200 μm.

(Bottom) Schematic representation of the correlation between *c-hairy1* expression in the PSM with the progression of somite formation. While a new somite is forming from the rostral-most PSM (somite 0:S_0), a narrow stripe of *c-hairy1* is observed in its caudal aspect, and a large caudal expression domain extends rostrally from the tail bud region (stage I; A, D, and G). As somite formation proceeds, as evidenced by the visualization of the appearing caudal fissure, the *c-hairy1* expression expands anteriorly, the caudal-most domain disappears, and *c-hairy1* appears as a broad stripe in the rostral PSM (stage II; B, E, and H).

When somite 0 is almost formed, the stripe has considerably narrowed, and *c-hairy1* is detected in the caudal part of the prospective somite (stage III; C, F, and I) while a new caudal expression domain arises from the tail bud region (in C can be seen the beginning of stage I of the next cycle). This highly dynamic sequence of *c-hairy1* expression in the PSM was observed at all stages of somitogenesis examined (from 1 to 25 somites), suggesting a cyclic expression of the *c-hairy1* mRNA correlated with somite formation. Arrowheads point to the most recently completely formed somite (somite I:S_I).

is that the wavefront reflects extensive caudo-rostral movement of *c-hairy1* expressing cells during somite formation. This appears unlikely because previous work has indicated that cell movement within the PSM is restricted (Tam and Beddington, 1986; Stern et al., 1988). If the *c-hairy1*-expressing cells in stage II were to derive from cells in stage I, they would have to move across about 50% of the PSM, a distance greater than 450 μm, in less than 30 min.

To exclude the possibility that cell migration contributes to the dynamics of *c-hairy1* expression, we have marked small clusters of cells at the same anteroposterior level in both the left and right PSM with DiI. The caudal part of these embryos was then separated into its two halves as described previously, and one half was immediately fixed while the other was cultured for 30 min prior to fixation. The DiI was then photoconverted to an insoluble DAB precipitate, and both halves were hybridized with the *c-hairy1* probe. In all observed cases (n = 8), DiI labeled cells are found at exactly the same level in the two halves whereas the *c-hairy1* expression patterns differ (Figure 5). This experiment clearly indicates that the progression of the *c-hairy1* wavefront occurs independently of cell movement. It also confirms and extends the results of cell grafting experiments in the mouse and of tracer injection into single PSM cells, which demonstrated that their progeny never encompass more than two consecutive segments (Tam and Beddington, 1986; Stern et al., 1988).

Rhythmic Expression of *c-hairy1* Is an Autonomous Property of the Presomitic Mesoderm

What might drive the caudal-to-rostral wavefront of *c-hairy1* expression in the PSM? One possibility is that it results from a periodic signal originating at the posterior end of the PSM, which spreads and activates *c-hairy1* in successively more anterior cells. This relay hypothesis predicts that a discontinuity within the PSM would interrupt spreading of the signal and halt the anterior progression of *c-hairy1* expression. To test this idea, *c-hairy1* expression was assayed in half embryos in which the caudal part of the PSM including the tailbud

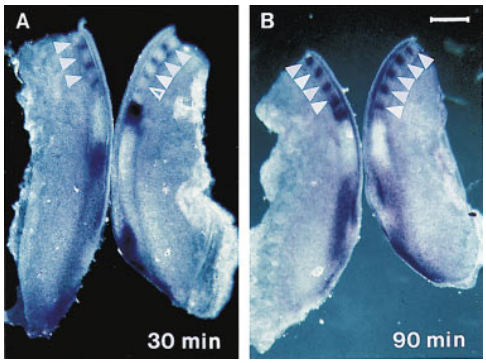

Figure 4. Cyclic Expression of *c-hairy1* RNA in the Presomitic Mesoderm Correlates with Somite Formation

The caudal regions of 15- to 20-somite embryos (including the presomitic mesoderm and the last few somites) were sagittally divided into two halves. One half (left side) was immediately fixed, and the other half (right side) was incubated on top of a millipore filter. Both halves were hybridized with *c-hairy1* probe.

(A) Experimental half-embryo cultured for 30 min. A different expression pattern is observed between the two halves, indicating the extremely dynamic nature of *c-hairy1* expression.

(B) Experimental half-embryo cultured for 90 min (the time required for the formation of one somite). The same expression pattern is found in both halves, indicating that *c-hairy1* expression pattern cycles over a period exactly corresponding to somite formation.

Open arrowhead, somite 0; arrowheads, segmented somites. Rostral to the top. Bar = 350 μm.

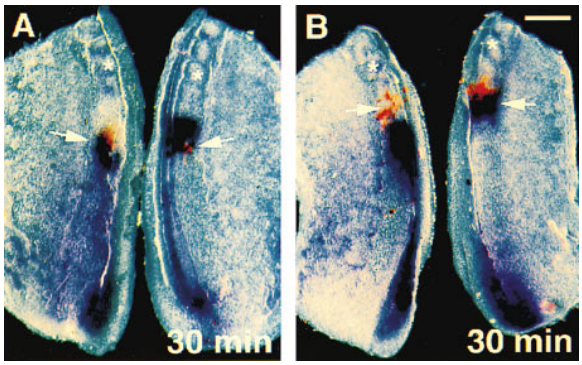

Figure 5. Cell Movements Do Not Account for *c-hairy1* Expression Kinetics

The caudal regions of 15- to 20-somite embryos were sagittally divided into two halves after DiI labeling of a small group of cells at the same anteroposterior level in the left and right PSM. One half (left side) was immediately fixed, and the other half (right side) was incubated on top of a millipore filter for 30 min. Both halves were hybridized with *c-hairy1* probe after photoconversion of the DiI. In (A), the *c-hairy1* expression pattern changes from stage I to stage II+ while in (B), it progresses from stage II to stage III. Cells labeled with DiI appear brown owing to the formation of DAB precipitate and are indicated by white arrows. In both examples, the cells are located at exactly the same anteroposterior level after a 30 min culture period while the *c-hairy1* expression pattern has progressed, indicating that expression dynamics are not due to cell movements in the PSM. Asterisk marks the last formed somite. Bar = 150 μm.

was surgically ablated (n = 8). The same expression pattern is observed in ablated and unoperated halves, even after extended culture (Figures 6A–6C). Therefore, cycling of *c-hairy1* expression in the rostral PSM is independent of the presence of a caudal PSM, and the progression of the *c-hairy1*-expressing wavefront during somite formation is not related to the spreading of a signal originating in the posterior part of the embryo and travelling anteriorly along the cells in the PSM.

These experiments suggest that the dynamic *c-hairy1* expression sequence reflects an autonomous property of the PSM. We therefore studied the *c-hairy1* expression pattern in explant cultures of presomitic mesoderm isolated from all the surrounding tissues that might be providing extrinsic signals. The presomitic mesoderm of one-half of 15- to 25-somite embryos was separated from ectoderm, endoderm, neural tube, notochord, lateral plate, and tail bud while the other half remained intact. The two halves were cultured separately for periods between 30 and 180 min (n = 31). *c-hairy1* expression patterns are similar in both types of explant (Figures 6D–6F), suggesting that the kinetics of *c-hairy1* expression are independent of surrounding tissues, and derive autonomously from the PSM. Moreover, these cultures cycle normally although Hensens node is absent, showing that cycling in the caudal PSM does not depend on a signal from the node (Figure 6).

Periodic Oscillations of *c-hairy1* Are Independent of Protein Synthesis

The above results show that *c-hairy1* mRNA is expressed cyclically in cells of the PSM and are consistent with clock models for somitogenesis (see Discussion). Studies of other clock control mechanisms indicate that their circuitry involves unstable components that are subject to negative autoregulation (reviewed in Sassone-Corsi, 1994; Dunlap, 1996). The dynamic pattern of *c-hairy1* expression and the likelihood that c-hairy1 is a transcriptional repressor led us to ask whether *c-hairy1* is itself a central component of the clock mechanism or if its cyclical transcription reflects an output from the clock. To address these questions, we examined the effects of blocking protein synthesis on *c-hairy1* expression.

Half-embryo explants were incubated in cycloheximide for up to 90 min while the contralateral half was fixed immediately. When explants are cultured for less than 75 min, the fixed and incubated halves show different patterns, indicating that inhibiting protein synthesis does not block *c-hairy1* oscillations (n = 4/4; Figure 7A). We confirmed this result by studying half-embryos cultured for equal times in the presence or absence of cycloheximide. For the first 60 min of culture, treated and untreated halves show the same patterns of *c-hairy1* expression (n = 11/11; Figures 7C and 7D), suggesting that the periodicity of *c-hairy1* pulsing is initially independent of de novo protein synthesis.

Nevertheless, protein synthesis may be required for continued periodicity of *c-hairy1* expression. Explants cultured in cycloheximide for 90 min (one somite equivalent) usually show a different pattern of expression from halves fixed immediately (n = 6/9; Figure 7B). Also, *c-hairy1* expression in half-embryos cultured for 90 min or more in the presence of cycloheximide often differs from that in the matched half-embryos incubated without the drug (n = 8/16; Figures 7E and 7F). Thus, a 90 min periodicity is not maintained in such longer term cultures.

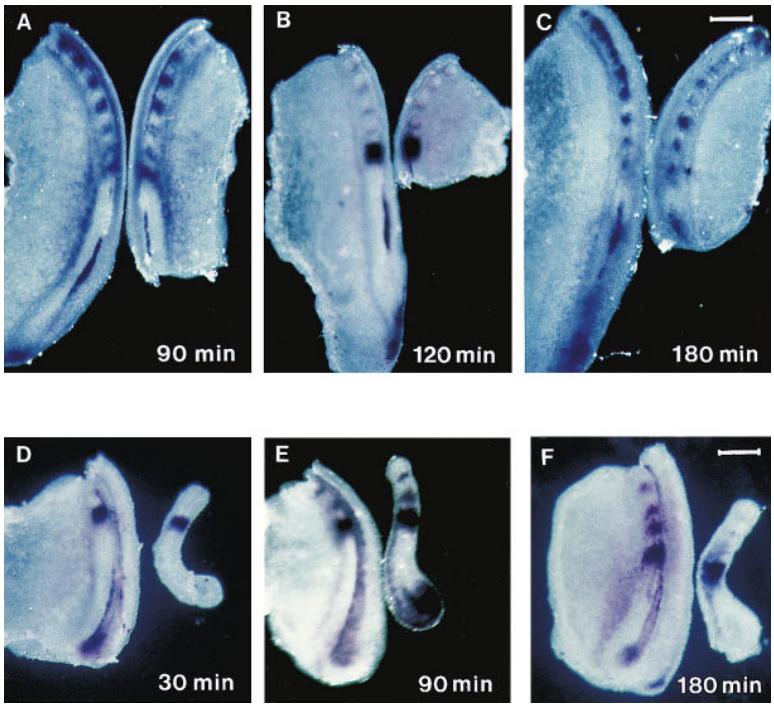

Figure 6. The Cyclic Expression of the *c-hairy1* Gene Is an Autonomous Property of the Presomitic Mesoderm Independent of the Anterior-Posterior Integrity of This Tissue

Caudal parts of 15- to 20-somite embryos including the PSM were sagittally divided into two halves and were cultured in parallel.
(A–C) The caudal part of the right embryonic half was surgically removed, and the remaining part was cultured in parallel with its contralateral half during 90 min (A), 120 min (B), and 180 min (C). Expression pattern of *c-hairy1* is similar in operated and control halves independent of the culture period.
(D–F) In the experimental embryonic half, the presomitic mesoderm was isolated from the surrounding tissues and cultured with the contralateral half during 30 min (D), 90 min (E), and 180 min (F). The expression pattern of *c-hairy1* gene is preserved in the isolated presomitic mesoderm, showing that the expression of this gene is an autonomous property of the presomitic mesoderm. Rostral to the top. Bar = 150 μm.

To verify that protein synthesis was efficiently blocked during such short time periods in explant culture, we measured [^{35}S]methionine incorporation in half-embryo explants incubated with or without cycloheximide (n = 36). At concentrations of 5 or 10 μM cycloheximide, progression of the *c-hairy1* wavefront was not affected after 30 min in culture while 71% and 84% of the protein synthesis was blocked, respectively (data not shown). Increasing the concentration to 20 μM did not increase the efficiency of the inhibition. Since cycloheximide does not block all protein translation (i.e., mitochondrial protein synthesis), we consider that treatment efficiently blocked protein synthesis in our explants. Two other lines of evidence indicate that the persistence of *c-hairy1* wave of expression after cycloheximide treatment is not due to a failure of the drug to block protein synthesis. First, somitogenesis is blocked in the treated embryos (Figure 7F). Second, treated explants show strongly increased levels of *c-hairy1* transcripts indicating mRNA stabilization (Figures 7B, 7E, and 7F; note that staining times are reduced by at least 5-fold for treated explants). Together, these results indicate that during one cycle of expression, the dynamic regulation of *c-hairy1* mRNA is unlikely to involve feedback regulation by the c-hairy1 protein. Indeed, the failure of the cycloheximide treatment to stop the clock suggests that *c-hairy1* is more likely to be an output of the clock than a component of the clock.

Discussion

We report here the identification of *c-hairy1*, an avian homolog of the fly segmentation gene *hairy*. This gene is expressed in a cyclic fashion in the presomitic mesoderm with a periodicity corresponding to the formation time of one somite. The periodic expression of *c-hairy1* mRNA appears as a wavefront travelling along the anteroposterior axis, and this scheduled expression constitutes an autonomous property of the paraxial mesoderm. We discuss these results in terms of a developmental clock linked to segmentation of the paraxial mesoderm.

Rhythmic *c-hairy1* mRNA Expression Provides Molecular Support for a Developmental Clock Driving Segmentation

Prospective somitic cells begin to express pulses of *c-hairy1* mRNA as soon as they leave Hensen's node and rostral primitive streak territory to enter the paraxial mesoderm (Figure 8). Thus, PSM cells exhibit periodicity immediately after gastrulation, well before they are incorporated into a somite. This result is in good agreement with earlier studies which showed that prospective somites are determined almost concomitantly with paraxial mesoderm formation (reviewed in Keynes and Stern, 1988). However, *c-hairy1* mRNA is not expressed according to the postulated prepattern of the PSM defined in the somitomere hypothesis (Meier, 1984).

Rather, we propose that the periodic nature of *c-hairy1* mRNA expression in the PSM, which correlates precisely with the time it takes to form a somite, is driven by an underlying molecular clock linked to somitogenesis. Various experiments in amphibian embryos have led to the idea of such a clock or an oscillator that would govern the behavior of the cells that are destined to segment together and form a somite (Cooke and Zeeman, 1976; see Davidson, 1988 for a review). In the "clock-and-wavefront" model, cells oscillate synchronously according to the clock while they are in the PSM and then halt their oscillation as they become mature for somite formation. The boundary between oscillating (immature, presomitic) and arrested (mature, somitic)

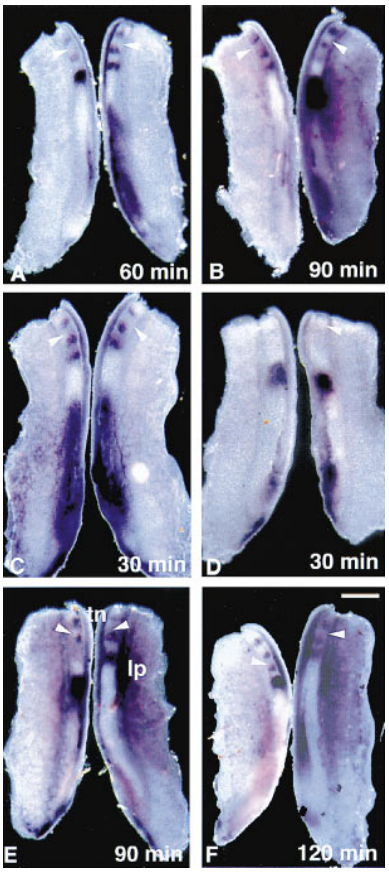

Figure 7. Blocking Protein Synthesis Using Cycloheximide Treatment Does Not Block the *c-hairy1* Wave Progression

(A–D) The caudal region of stage-12 embryos was separated into two halves along the midline. The left half was immediately fixed while the right half was cultured in medium containing cycloheximide (5 μM) for 60 min (A) or 90 min (B). Both halves were then hybridized with the *c-hairy1* probe and their expression pattern was compared. (A) Progression of the wavefront is not stopped since the control explant (left) is in stage III while the explant cultured in presence of cycloheximide for 60 min is in stage I (right). (B) In the majority of explants (6 of 9), a different expression pattern is found in the fixed (left, stage III) and cultured (right, stage II) halves after a 90 min culture period. Similar explants as above were cultured for the same time period in absence (left) or in presence of cycloheximide (right). Explants cultured for 30 min with or without cycloheximide show the same expression pattern, stage I (C) or stage III (D), confirming the wavefront progression observed in the previous experiment (A and B).

(E–F) When explants are cultured for longer periods of time, such as 90 min (E) or 120 min (F), treated and control sides are found in a different stage in 50% of the cases. In (E), the control side (left) is in stage II, and the treated side (right) is in stage III. Segmentation is blocked by the cycloheximide treatment in these longer cultured explants (F). The control (left) and treated (right) explants are in stage III but are out of register by one somite, owing to the block of segmentation.

Arrowhead points to somite I. Note that in all explants cultured for longer periods of time, *c-hairy1* transcripts become accumulated in the neural tube and lateral plate (B, C, E, and F). Staining of control explants lasted 5 times longer than that of treated ones, indicating transcript stabilization. Rostral to the top. Bar = 300 μm.

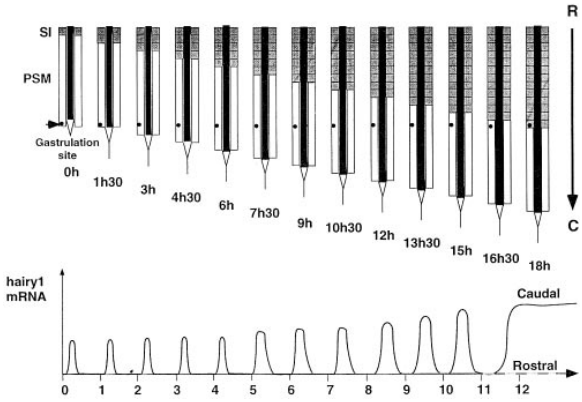

Figure 8. Rhythmic *c-hairy1* mRNA Expression Identifies a Developmental Clock Driving Segmentation and Somite Formation

The PSM is a rod of mesenchymal cells thought to contain about 12 prospective somites. As a new somite is formed every 90 min at its rostral extremity, PSM length is maintained by a continuous addition of new cells arising caudally from the gastrulation site. PSM cells begin to rhythmically express *c-hairy1* mRNA as soon as they exit the gastrulation site (Hensen's node and rostral primitive streak) and stop cyclic expression once they are incorporated into a somite. Therefore, between the moment a cell enters the PSM (0h, arrowhead) and the time it is incorporated into a somite (18h), a total of 12 somites will have formed, and consequently, 12 pulses of *c-hairy1* expression will have occurred in the cell. During this time period, *c-hairy1* mRNA expression levels increases progressively, as cells pass first through stage I, then stage II, and finally stage III before being incorporated into a somite. *c-hairy1* expression is retained only by cells that lie in the caudal somitic portion. We propose that the *c-hairy1* pulses identify a molecular clock linked to vertebrate segmentation and somitogenesis. The purpose of such a clock could be to synchronize cells fated to belong to the same somite as postulated in somitogenesis models, such as the clock-and-wavefront, and also to act as a time counting system for PSM cells to coordinate the moment of somite formation. R, rostral; C, caudal.

cells sweeps slowly back along the embryo in an anteroposterior direction. In this model, the wavefront corresponding to the anteroposterior gradient of maturation of the vertebrate embryo can in principle be either propagatory (extrinsic) or "kinematic" (independent of the propagation of a signal and not stopped by a cut across its path; Cooke and Zeeman, 1976). *c-hairy1* expression is kinematic because it continues to follow an endogenous program, even in parts of the PSM that are isolated from the rest.

Strikingly, the observed pattern of *c-hairy1* expression is mimicked by a simple mathematical simulation based on a kinematic clock-and-wavefront model of this type, in which the wavefront serves to smoothly slow down and finally freeze the clock. An appendix describing the model and a movie generated by this simulation (composed by Dr. Julian Lewis, ICRF, London) are available on the Internet at http://www.cell.com/cgi/content/full/91/5/639. The latter conveys, more clearly than is possible with static images, the remarkable spatio-temporal pattern of the oscillations of *c-hairy1* expression that we observe and serves as proof that the observed pattern can be generated by a clock-and-wavefront mechanism. Although the nature of both clock and wavefront remains undefined in the model, the spatiotemporal pattern of *c-hairy1* expression provides molecular evidence for their existence.

More recently, it has been proposed that PSM cells are synchronized using the cell cycle as an internal clock

(Primmett et al., 1989). This proposal is based on experiments in avian embryos in which a heat shock induces repeated segmental defects separated by regular intervals of 6–7 somites, an interval corresponding to the length of one cell cycle in the PSM (9 hr according to Primmett et al., 1989). These experiments led to a model for somitogenesis in which PSM cells are intrinsically synchronized by a clock based on the cell cycle, so that when groups of cells enter a similar phase of the cycle, they increase their adhesive properties and segregate together to form a somite. Indeed, some synchrony in the cell cycle is observed in the PSM.

Our observations are in agreement with these models postulating the existence of an intrinsic clock responsible for the coordinated behavior of the PSM cells. However, the period of the cell cycle in the PSM is 9 hr while that of the *c-hairy1* cycle is only 90 min. A direct link between the cell cycle and the clock driving *c-hairy1* expression in the PSM is therefore not obvious. Together, these considerations tend to argue against a direct role for the cell cycle in defining segmental periodicity.

Our results also argue against models in which a reaction-diffusion mechanism patterns the rostral PSM into two states that lead to the segregation of alternating anterior and posterior somitic compartments (Meinhardt, 1986). However, an attractive possibility is that *c-hairy1* plays a role in the specification of the posterior somitic identity (see below).

Progression of the *c-hairy1* wavefront and operation of the *c-hairy1* clock are insensitive to blocking protein synthesis by cycloheximide. Not only does this tend to exclude *c-hairy1* playing a role in the clock mechanism itself, but also it places significant limits on the oscillator mechanism. Theoretical and experimental studies of circadian clocks indicate that cycling machineries use unstable components and negative feedback. Several circadian clocks are clearly dependent on transcription factors that regulate their own transcription via delayed negative feedback (Sassone-Corsi, 1994; Dunlap, 1996). Our cycloheximide experiments argue against such a model and any other in which clock activities are regulated by cyclic regulation of protein levels, either by regulated translation or degradation. More likely, the clock mechanism revealed by *c-hairy1* expression acts posttranslationally, using protein modifications such as phosphorylation.

Potential Functions for *c-hairy1* During Segmentation and Somitogenesis

As somitogenesis occurs autonomously within the anterior PSM, the moment of segmentation must be determined intrinsically (Deuchar and Burgess, 1967; Packard, 1976; Menkes and Sandor, 1977). *c-hairy1* could play a role as part of a counting mechanism in which cells would use time to measure their positions within the presomitic plate to determine when they should start somitogenesis, for example, by regulating expression of a more stable component whose accumulation triggers somitogenesis when a threshold concentration is exceeded. Alternatively, *c-hairy1* might play a direct role in counting, whereby successive pulses last longer and lead to higher levels of *c-hairy1* accumulation (see results and Figure 8).

In addition, expression in the posterior of somite 0 and then in the caudal part of the newly formed somite suggests that the posterior boundary of *c-hairy1* expression may mark the site at which a new somite boundary should form. *c-hairy1* could also contribute to patterning within somites. Segments in both long and short germband insects are subdivided into anterior and posterior compartments, domains of lineage restriction that are required to establish and maintain metamerism and also to allow further patterning within segments. Although there is no evidence of such lineage restriction in the early somite, the intrasomitic anteroposterior difference in *c-hairy1* expression is maintained during somite maturation (Figure 3) and may help polarize somitic cells into anterior and posterior populations whose interactions lead to further pattern refinements such as peripheral nervous system segmentation (Keynes and Stern, 1988).

Evolutionary Implications for Mechanisms of Segmentation in Invertebrates and Vertebrates

The striking and intriguing pattern of *c-hairy1* expression during somitogenesis suggests that it is likely to play an important role in mesoderm segmentation in vertebrates. Thus, *hairy*-like genes may function during metamerization in both invertebrates and vertebrates, whose segmentation mechanisms may have more in common than previously thought.

Most vertebrate homologs of the fly segmentation genes do not exhibit expression patterns or mutant phenotypes indicative of a role in somitogenesis (Patel et al., 1989; Kimmel, 1996; De Robertis, 1997). It is currently thought that, whereas some of the major patterning systems involved in dorsoventral and anteroposterior patterning have been conserved during evolution between arthropods and vertebrates, segmentation arose independently in these two phyla (see Weisblat et al., 1994 for a discussion). However, there is increasing evidence that *Urbilateria*, the common ancestor of invertebrates and vertebrates, was segmented (Kimmel, 1996; Muller et al., 1996; De Robertis, 1997). Moreover, the recent identification of vertebrate homologs of Drosophila segmentation genes that are expressed during somitogenesis, including the zebrafish *Her1* gene (Muller et al., 1996), the avian *c-hairy1* gene (this report), and the amphioxus *engrailed* gene (Holland et al., 1997), raises the possibility that part of the machinery involved in the segmentation process may be conserved between insects and vertebrates.

Her1 is expressed in the paraxial mesoderm in alternating segment primordia as expected for a pair-rule gene, rather than in every segment as seen with *c-hairy1*. However, Her1 is only very distantly related to c-hairy1 and is also very different from Drosophila Hairy, so it may belong to a different family of WRPW-containing bHLH proteins (Figure 2). Moreover, *Her1* expression constitutes the only evidence for a pair-rule type of mechanism in vertebrates and, in fact, outside of the more evolved insects species such as dipterans or coleopterans. Other vertebrate pair-rule homologs such as the *even-skipped*-like *evx* genes do not exhibit pair-rule expression (Bastian and Gruss, 1990), nor do currently

identified homologs of pair-rule genes in primitive short germ band insects like orthopterans (Patel et al., 1992). The universality of the pair-rule phenomenon therefore remains controversial (Sander, 1988).

By contrast, the segmental pattern of *c-hairy1* expression and, more particularly, its cyclical anticipation of segmentation in the PSM and its expression in the posterior of the newly forming somite, are clear hints that it plays a role in segmentation and/or somitogenesis. This reactivates the debate as to whether vertebrate somitogenesis is closely related to more "primitive" insect modes of segmentation in which segments are added successively from a terminal growth zone. More evolved insect groups such as dipterans may have subsequently acquired pair-rule patterns of expression in order to allow extremely rapid segmentation in a syncytial embryo. Of course, this raises the question of whether *hairy* and *c-hairy1* play conserved roles in segmentation and, even more intriguingly, whether aspects of their transcriptional regulation might have been conserved. The latter appears paradoxical because *hairy* is directly regulated in the syncytial embryo, whereas spatial regulation of *c-hairy1* in the chick embryo must depend on intercellular signals. Future experiments will indicate how the dynamic pattern of *c-hairy1* transcription is achieved.

Experimental Procedures

Cloning of *c-hairy1*

First-strand random-primed cDNA was synthesized from mRNA prepared from 1.5-day-old chick embryos. The cDNA was used in a PCR reaction (94°C for 30 sec, 50°C for 2 min, 72°C for 1 min, 40 cycles) with the following degenerate primers: 5′-CGIGCICGIATNAA CAANTG(C/T)(C/T)T-3′ and 5′-ACIGTCTTCTCNAGNAT(S)TCNGC (C/T)TT. These primers correspond, respectively, to the sequences RAR(I/M)N(K/N)CL and KA(D/E)(I/M)LEKTV, located on helices 1 and 2 of the fly hairy/deadpan proteins. A fragment of 117 bp derived from a *hairy*-like cDNA was obtained and used to screen a random-primed cDNA library in lambda gt10, prepared from stage 10–14 chick embryos. Three overlapping cDNA clones, which cover the entire coding region of the *c-hairy1* gene, were obtained and fully sequenced using the Sequenase kit (Amersham).

Eggs and Embryos

Fertilized chick (*Gallus gallus*, JA57, Institut de Sélection Animale, Lyon, France) eggs, obtained from commercial sources, were incubated for up to 48 hr in a humidified atmosphere at 38°C. The embryos were staged by the number of somite pairs formed and according to the developmental table of Hamburger and Hamilton (1992).

In Vitro Culture of Chick Explants and DiI Labeling

Chick embryos ranging from 15 (HH12−) to 20 (HH13+) somites were used throughout this study. Different types of explants were precisely delimited, excised, and cultured for 30 min–3 hr in 35 mm culture dishes on Polycarbonate filters (0.8 μm; Millipore) floating on top of culture medium composed of Medium 199 (Sigma) supplemented with 5% heat inactivated fetal calf serum, 10% chicken serum, 1% L-glutamine and 1% penicillin 5000 IU/ml, streptomycin 5000 IU/ml. Under these conditions, somitogenesis proceeds normally, and up to 3 somites can be formed in 270 min.

In the first series of experiments, embryos were divided into two halves by cutting across the three germ layers at the midline level. One half was immediately fixed. The other one was cultured, as described above. A similar series of experiments was performed in which small groups of cells at different A/P levels of the the PSM were DiI labeled. In each embryo, the injection site was precisely the same on both left and right PSM before separation of the two halves. One half was immediately fixed, and the other was cultured for 30 min prior to fixation. DiI was photoconverted in both halves prior to in situ hybridization with the *c-hairy1* probe. DiI labeling and photoconversion were performed as described in Ispizua-Belmonte et al (1993).

In the second series of experiments, embryos were also divided sagittally, and in one of the halves, the caudal part including the tailbud was removed. Both halves, the entire and the truncated one, were incubated for the same period of time.

In the third series of experiments, embryos were separated into two halves, and the PSM was dissected from the surrounding tissues from one of the two halves after a brief treatement with 4× pancreatin (Gibco). The isolated PSM and the contralateral intact half of the embryo were cultured for the same time period as described above.

Cycloheximide Treatment of Explant Cultures

The caudal part of stage 12–13 embryos was separated into two halves. The experimental half was incubated in the presence of cycloheximide (Sigma, 5, 10, or 20 μM), and the contralateral half was either cultured for the same time period in normal medium or fixed immediately. In all series, explants were processed for whole mount in situ hybridization as described below with the *c-hairy1* probe.

To monitor efficiency of protein synthesis inhibition, explants were incubated for 30 min in 100 μl DMEM without cysteine and methionine complemented with 1% glutamine and 14 μCi [^{35}S]Met and Cys (Amersham) with or without cycloheximide (5, 10, or 20 μM). Explants were then lysed in hot sample buffer, and proteins were precipitated in 10% trichloroacetic acid (TCA) overnight. The protein pellet was recovered by centrifugation and washed consecutively with 5% TCA, ethanol, ethanol/ether (1:1), and ether and resuspended in 10% SDS prior to counting. Explants were also incubated in DMEM with 1% glutamine and hybridized with the *c-hairy1* probe to ensure that absence of serum did not affect the cycling expression pattern.

Whole Mount In Situ Hybridization

c-hairy1 probe was produced from an 850 bp fragment of the coding sequence cloned in Bluescript KS, linearized using Hind III, and transcribed with T7 polymerase. Embryos and explants were fixed overnight at 4°C in 4% formaldehyde-2mM EGTA, rinsed in PBS, dehydrated through a methanol series, and stored in 100% methanol at −20°C. Whole-mount in situ hybridization was performed according to the procedure described by Henrique et al. (1995). Embryos were photographed as whole mounts in PBT (PBS, 0.1% Tween20) using a Wild stereomicroscope.

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GenBank Accession Numbers

Accession numbers for the sequences used for the phylogenetic tree are: *c-hairy1*, AF032966; *Mhes1*, D16464; *RHes1*, L04527; *Huhes1*, L19314; *ZFher6*, X97333; *X-hairy1*, U36194; *Hes2*, D14029; *Dmhairy*, X15904; *Tribhairy*, S29712; *Deadpan*, S48025; *Espl-m5*, X16552; *Espl-m8*, X16550; *Espl-m7*, X16553; *Hes3*, D13418; *ZfHer3*, X97331; *Hes5*, Q03062; *Zfher2*, X97330; *Zfher4*, X97332; *Zfher5*, X95301; *Zfher1*, X97329.