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

Isabel Palmeirim,* Domingos Henrique,†§
David Ish-Horowicz,† and Olivier Pourquié‖∥

Introduction

Isabel Palmeirim,* Domingos Henrique,†§
David Ish-Horowicz,† and Olivier Pourquié‖∥

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.

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 germ band insects such as the fly, segments are determined essentially simultaneously in a syncytial unicellular embryo, prior to gastrulation. In more primitive short germ band 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
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
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:S0), 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:S1).

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*-hairy1expressing 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 antero-posterior level in both the left and right PSM with DI. 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 DI was then photoconverted to an insoluble DAB precipitate, and both halves were hybridized with the *c*-hairy1 probe. In all observed cases (n = 8), DI 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
The above results show that or more in the presence of cycloheximide often differs
Periodic Oscillations of c-hairy1

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. Rosstral to the top. Bar = 350 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

Figure 4. Cyclic Expression of c-hairy1RNA in the Presomitic Mesoderm Correlates with Somite Formation

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

their circuitry involves unstable components that are subject to negative autoregulation (reviewed in Sas- sone-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 cyclohexi- mide 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 [35S]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)
Molecular Clock Linked to Vertebrate Segmentation

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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, 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 increase 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.

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).

Explanets cultured for 30 min within 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.

More recently, it has been proposed that PSM cells are synchronized using the cell cycle as an internal clock 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.
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 0 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 germ band 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 intrasomatic 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 pattern 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...

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’-CGG/CIGAT/NNAA CAAN/CTGC(T/C)/T/TT-3’ and 5’-ACIG/CTCTC/NCAGAT/S/TC/NGC (C/T)TT. These primers correspond, respectively, to the sequences RAR/I/MIN(K/N)CL and KAI/D/E/I(M)LEKT, 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 primer 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, J A57, 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 instruction. We thank Dr. Julian Lewis for helpful criticisms and for providing embryo facilities to Monique Coltey, C. The Christiane Béraud, and Pascale Malapert for their excellent technical assistance. Funds were provided by the Centre National de la Recherche Scientifique (CNRS), the Association Francophone contre les myopathies (AFM), the Association Française contre les cancers (AFCA), the Fondation pour la Recherche contre le Cancer (FRCA), the Recherche Médicale (FRM), and the Imperial Cancer Research Fund. I. P. was funded by the Portuguese Gulbenkian PhD program in Biology and Medicine and the French Embassy in Portugal. D. I. H. is an International Research Scholar of the Howard Hughes Medical Institute.

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References


GenBank Accession Numbers

Accession numbers for the sequences used for the phylogenetic tree are: c-hairy1, AF032966; Mhes1, D16464; RHes1, L04527; Hues1, L19314; ZFher6, X97333; X-Hairy1, L36194; Hes2, D14029; Dhm hairy, X15904; Trib hairy, S29712; Deadpan, S48025; Espl-m5, X16552; Espl-m8, X16550; Espl-m7, X16553; Hes3, D13418; ZHher3, X97331; Hes3, Q03062; Zher2, X97330; Zher4, X97332; Zher5, X95301; Zher1, X97329.