Molecular Targets of Vertebrate Segmentation: Two Mechanisms Control Segmental Expression of Xenopus hairy2 during Somite Formation

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Summary

Vertebrate hairy genes are expressed in patterns thought to be readouts of a “segmentation clock” in the presomitic mesoderm. Here we use transgenic Xenopus embryos to show that two types of regulatory elements are required to reconstitute the segmental pattern of Xenopus hairy2. The first is a promoter element containing two binding sites for Xenopus Su(H), a transcriptional activator of Notch target genes. The second is a short sequence in the hairy2 3′ untranslated region (UTR), which most likely functions post transcriptionally to modulate hairy2 RNA levels. 3′ UTRs of other hairy-related, segmentally expressed genes can substitute for that of hairy2. Our results demonstrate a novel mechanism regulating the segmental patterns of Notch target genes and suggest that vertebrate segmentation requires the intersection of two regulatory pathways.

Introduction

Segmentation along the anteroposterior body axis is a basic feature in the development of several major phyla, such as insects, arthropods, annelids, and chordates. The early Drosophila embryo is synchronously partitioned into smaller domains by a hierarchical gene network (Lawrence, 1992). However, in most insects, many arthropods, cephalochordates, and all vertebrates, segmentation occurs gradually from anterior to posterior. The temporal regularity of segmentation has long suggested control by a clock-like mechanism (Cooke, 1981, 1986; Stern and Vasil’ev, 2000).

In vertebrates, contiguous paraxial mesoderm forms on both sides of the unsegmented neural tube and notochord. Sequentially, segments of this mesoderm differentiate into somites, which are further subdivided into skeleton, muscle, and dermis derivatives (Brand-Saberi and Christ, 2000; Stockdale et al., 2000). The robust properties of segmentation have long defined simple explanation. For instance, the number of somites in amphibians is independent of cell number, demonstrated by changes in ploidy (Fankhauser, 1945), or removal of up to 40% of the prospective mesoderm at gastrulation (Cooke, 1975, 1981).

Molecular markers that reveal a prepattern in the unsegmented, or presomitic, mesoderm (PSM) most prominently involve the Notch signaling pathway (Jiang et al., 1999; McGrew and Pourquie, 1998). A dramatic observation related to segmentation concerns c-hairy1, a chicken homolog of the Drosophila hairy gene (a bHLH-WRPW transcriptional repressor [Fisher and Caudy, 1998]). c-hairy1 RNA appears and then disappears in the PSM multiple times before cells are incorporated into a new somite (Palmeirim et al., 1997). During the time to form two somites (one expression cycle) three observed patterns of c-hairy1 RNA suggest a posterior to anterior PSM wavefront (Palmeirim et al., 1997; Pourquie, 1999). For one cycle, the c-hairy1 pattern in plants is unaltered by cycloheximide treatment, supporting the idea that c-hairy1 expression is a readout, but not itself a component, of a clock that governs segmentation. Similar cycling has been shown for a mouse hairy homolog, HES1 (Jouve et al., 2000).

Cycling has also been shown for lunatic fringe, a modulator of Notch signaling (Aulehla and Johnson, 1999; Forsberg et al., 1998; McGrew et al., 1998). lunatic fringe cycling has been proposed to connect Notch signaling to the “segmentation clock” (Jiang et al., 1998; Pourquie, 1999). Additional evidence supporting this connection has been gathered from studies in the frog and fish (Sparrow et al., 1998; Jen et al., 1999; Takke and Campos-Ortega, 1999; Holley et al., 2000; Jiang et al., 2000).

Here we address molecular mechanisms of segmentation by analyzing how segmental gene patterns are controlled. We have focused on Xenopus hairy2, the frog ortholog of c-hairy1. In contrast to c-hairy1, hairy2 is expressed in PSM cells only once, in an anterior region stripe, prior to the formation of each somite (Jen et al., 1997). This stripe moves posteriorly in a wavefront at a constant distance from the most recently formed somite. Using transgenic Xenopus embryos (Kroll and Amaya, 1996), we have defined sequences sufficient to recapitulate the hairy2 pattern in the PSM.

While the hairy2 expression pattern is complex, the hairy2 promoter is remarkably simple. It contains a single upstream element with two binding sites, in inverse orientation, for Xenopus Suppressor-of-hairyless, a transcriptional activator of Notch target genes (Wettstein et al., 1997). While this simple promoter is sufficient to recapitulate hairy2 expression in other tissues, it is insufficient to reproduce the segmental pattern. To achieve this highly dynamic PSM pattern, a 25 bp motif in the hairy2 3′ untranslated region (UTR) is required in combination with the promoter. The 3′ UTR functions post transcriptionally to regulate hairy2 RNA levels. The modulation of expression by the 3′ UTR represents a control mechanism most likely utilized by all vertebrates for segmental patterning and for the regulation of specific Notch target genes.
Results

The *hairy2* Pattern in Segmenting Mesoderm

*Xenopus laevis* possesses two *hairy* homologs, *hairy1* (Dawson et al., 1995) and *hairy2* (Turner and Weintraub, 1994); for *hairy2*, there are a and b copies in the allotrapeloid *Xenopus* genome. Figure 1A shows a tailbud stage embryo after whole-mount in situ hybridization. *hairy2a* RNA is expressed in various neuroectoderm derivatives and in the mesoderm, where expression is limited to the PSM and pronephros.

In the anterior PSM, *hairy2a* RNA appears as a chevron-shaped stripe (Figure 1A); there is no staining of the posterior PSM. The RNA is also detected at much lower levels in somites (more obvious in this cleared embryo). The PSM stripe is easily seen in uncleared embryos, too (Figure 1B). The pattern of *hairy2b* is identical to *hairy2a* in the PSM (data not shown). *hairy1* is different from *hairy2a* in the neuroectoderm but is also expressed in a chevron-shaped stripe, though at a much lower level (Figure 1C). The *hairy2a* stripe is posterior to the last formed somite, displaced one to two somite widths, and maintains this relative position throughout segmentation (Figure 1D).

The stripe shows a limited, reproducible variability, suggesting a dynamic pattern of appearance and disappearance of *hairy2a* RNA. In Figures 1E–1H, we have arranged a series of images to indicate a proposed order, consistent with intermediate patterns. Expression begins in the distal regions of the dorsal and ventral “half-stripes” (relative to the dorsal-ventral midline of the chevron; Figure 1E). These half-stripes meet (Figure 1F), and then the dorsal half-stripe begins to fade, while expression begins in the next posterior dorsal half-stripe (Figure 1G). Then the next posterior ventral half-stripe forms (Figure 1H). This proposed order is reinforced by the pattern in some embryos that show three stripe domains: an anterior ventral half-stripe, a complete middle stripe, and a posterior dorsal half-stripe (Figure 1I).

Strikingly, the *hairy2a* stripe varies consistently between the two sides of an embryo (Figure pairs 1J, 1K and 1L, 1M). The phase-advanced stripe pattern on the right side in the embryos shown, and in all embryos we have analyzed (n > 100), suggests that the right side of the embryo is temporally advanced in segmentation. We observe this asynchrony as early as stage 18–19, soon after somite formation begins (data not shown).

Sequence of the *hairy2a* Promoter

The proximal promoter of a *hairy2a* genomic clone is shown in Figure 2A. Promoter function depends on the putative TATA box and transcription start site indicated (data not shown). 5′ of the TATA box are two binding sites, in inverse orientation, for *Xenopus* Suppressor-of-hairless [XSu(H)] (Wettstein et al., 1997). Su(H) sites have two types: single binding sites, and paired motifs like that in the *hairy2a* promoter (also called SPS sites; Bailey and Posakony, 1995; Nellesen et al., 1999). The *hairy2a* paired Su(H) motif has a variant of the SPS-conserved hexamer (Bailey and Posakony, 1995; Nellesen et al., 1999; see Figure 2C). There are also three
Figure 2. Sequence of the hairy2a Promoter

(A) Sequence of the hairy2a promoter used in H2pm constructs, with features labeled. The raised rightward arrow shows the presumptive transcription start site. The upward arrowhead indicates the 5' end of the hairy2a cDNA. The asterisk indicates the normal position of the hairy2a translation start site, which has been mutated in the reverse primer for PCR amplification (from CAT to CAA). The single leftward bracket shows the junction between the hairy2a 5'UTR and the eGFP cDNA.

(B) Schematic of transgene constructs. The hairy2a promoter and all of its 5' UTR are fused to eGFP, followed usually by a Xenopus globin gene intron and the SV40 late polyadenylation signal. In most cases, there is also a gene-specific 3'UTR between eGFP and the globin intron.

(C) Comparison of paired Su(H) motifs (X: Xenopus, H: Human, M: Mouse, D: Drosophila). The motifs are oriented so that transcription begins 3'. The binding sites for Su(H) protein are in larger type, and the hexamer is underlined. The gaps in the vertebrate motifs are based on comparisons in Nellesen et al. (1999).
Reconstruction of the hairy2a Pattern in Transgenic Xenopus Embryos

We have made numerous transgenic Xenopus embryos with hairy2a promoter-eGFP plasmids (see Figure 2B) (Kroll and Amaya, 1996). The pattern of eGFP RNA is compared to endogenous hairy2a RNA by whole-mount in situ hybridization. The hairy2a sequences of the initial construct (H2pm) included only the promoter shown in Figure 2A. With this construct, eGFP is expressed in neuroectoderm derivatives and the pronephros (Figures 3A and 3D), similar to endogenous hairy2a. By contrast, the transgene is never expressed in the floorplate (compare Figure 3D to 3C).

We do not observe the anterior PSM stripe with the H2pm construct (0/252 embryos), although there is diffuse expression across the PSM and posterior somites (Figure 3A). Transgenes with additional 5’ genomic sequence give similar results (data not shown). We considered the notion that a cis regulatory element for the PSM stripe might be contained within the hairy2a transcript. Therefore, we fused hairy2a sequences (including introns) between its start codon and polyadenylation signal, 3’ of and in-frame with eGFP (H2pmH2G). Transgenic embryos with this construct routinely have a chevron-shaped stripe of eGFP RNA in the anterior PSM (Figure 3B; 49/179 embryos), showing similar dynamics and bilateral differences as endogenous hairy2a, as well as overlapping it (Figures 3E–3H and 7C; see below). By deleting the hairy2a exons and introns individually or in combination, we found that it is the hairy2a 3’ UTR, in conjunction with the promoter, that is both necessary and sufficient for the PSM stripe (H2pm3U; Figure 3I; 29/96 embryos).

Because both hairy2a and hairy2a promoter transgenes are expressed more strongly in the neuroectoderm than in the PSM, we use transgene neuroectoderm levels as a guide for evaluating detection of PSM expression. For PSM stripe-expressing transgenes, 20%–30% of transgenic embryos have a detectable stripe. Those transgenic embryos with no stripe have lower neuroectoderm expression than those with a stripe, and this lower expression is below that of the endogenous hairy2a gene. Most likely, the difference in the frequency of PSM stripe-expressing transgenic embryos and total transgenic embryos reflects a sensitivity limit for in situ hybridization. Thus, the proximal promoter and 3’ UTR contain all of the hairy2a sequences required for the normal spatial pattern in the PSM. It is possible that additional genomic elements not in our constructs may modulate expression (and specify floorplate expression), but they are not required to reconstitute the hairy2a PSM pattern.
Analysis of the hairy2a Promoter

Transient assays of plasmid-injected embryos showed that the hairy2a promoter is stimulated by coexpression of constitutively activated Su(H) protein (Wettstein et al., 1997; data not shown). By deleting the paired Su(H) motif entirely (ΔSu(H)) or introducing point mutations in both Su(H) binding sites (mutSu(H)), almost all expression in transgenic embryos was lost (Figure 4; Figure 6A), as well as stimulation by activated Su(H) protein in transient assays (data not shown). Therefore, the hairy2a promoter is dependent on activation by Su(H) in vivo. We also deleted, or made the same point mutation in, either of the Su(H) binding sites (Δ5, Δ3, mut5, and mut3Su(H)). In each case, the PSM stripe was absent, even in embryos showing normal neuroectoderm expression (Figure 4; Figure 6B). These results suggest that both Su(H) binding sites of the paired motif are required for the PSM stripe, while either is sufficient for neuroectoderm expression.

Point mutations in the paired Su(H) motif hexamer abolished PSM stripe expression and substantially reduced neuroectoderm expression, suggesting that the hexamer has a general role (Figure 4; Figure 6C). Substitution of the hairy2a paired Su(H) motif with paired Su(H) motifs from Drosophila Enhancer-of-split [E(spl)] genes (Nellesen et al. 1999; see Figure 2C) also abolished PSM stripe expression and significantly reduced neuroectoderm expression (Figure 4). In contrast to the hairy2a motif, the E(spl) m3 motif has no hexamer, while the E(spl) m4 motif has a hexamer located in a different position (Figure 2C). Substitution of the intervening sequence (but not the hexamer) with that of the mouse HES1 paired Su(H) motifs had no effect (mhes-int., Figure 4). In fact, substituting the entire hairy2a motif with the similar mouse HES1 motif (Figure 2C) had no effect (mhes, Figure 4; Figure 6D). Taken together, these results argue that the precise architecture of the hairy2a paired Su(H) motif is required for the PSM stripe.

In cell culture, mouse HES1 negatively autoregulates expression through N box motifs in its promoter (Takebayashi et al., 1994). We observe similar repression of the hairy2a promoter by coexpressed hairy2a in plasmid-injected embryos, and this repression requires the N box motifs in the hairy2a promoter (data not shown). However, simultaneous mutation of all the N boxes has no apparent effect on transgene expression (mut-Nbox, Figure 4; Figure 6E). We cannot rule out that negative autoregulation plays some role at later times in development.

The hairy2a 5’ UTR is not required for the PSM stripe, since it can be replaced by vector-derived sequences from the CS2 plasmid (Rupp et al., 1994; Turner and Weintraub, 1994) (Figure 4). To test if the paired Su(H) motif is the only required element in the hairy2a promoter, we inserted one copy of the motif 5’ to a small synthetic promoter, also driving eGFP fused to the
Figure 5. *hairy2a* 3′ UTR Variants

The top schematic (H2pm3U) shows the general layout of *hairy2a* promoter constructs (Pr: promoter, eGFP marker, 3′ UTR, Int: intron, SV-pA: SV40 polyadenylation signal). Total is the number of transgenic embryos analyzed. Specific mutations or substitutions are shown in black. Numbering for the deletion mutants refers to the first base pair of the 3′ UTR after the stop codon.

**hairy2a** 3′ UTR. Transgenic embryos with this simple construct showed a normal PSM stripe [Su(H)Sy, Figure 4; Figure 6F]. The somewhat lower neuroectoderm and pronephros levels probably reflect differences in basal promoter activities.

We conclude that the paired Su(H) motif, including the hexamer, is the only required *hairy2a* promoter element for the PSM stripe, and most of the neuroectoderm expression, as well as pronephros expression. The paired Su(H) motif is necessary for expression in the PSM, but it is insufficient to drive the stripe pattern, which requires additional sequences in the *hairy2a* 3′ UTR.

**Analysis of the hairy2a 3′ UTR**

We repositioned the *hairy2a* 3′ UTR as follows: (a) in antisense orientation relative to eGFP (3Urev), (b) immediately 3′ of the SV40 late polyadenylation signal (SV3U), (c) 5′ of the *hairy2a* promoter (3UPr), and (d) into the 5′ UTR, just 5′ of eGFP (as a functional 90 bp fragment, 5U390, see below). In no case was a PSM stripe evident, though neuroectoderm expression was normal (Figure 5). The SV40 sequence was tested by substituting it with the polyadenylation and transcription termination region of the *Xenopus* larval type I β-globin gene. In this case, the PSM stripe still required the *hairy2a* 3′ UTR (Figure 5, 3UXglo-pA versus Xglo-pA).

To map the 3′ UTR sequences necessary and sufficient for the PSM stripe, a series of deletion mutants was tested. Deletion of the 5′ 90 bp eliminated the stripe but had no effect on neuroectoderm expression (3U90-316, Figure 5). Deletion of all 3′ UTR sequences except the 5′ 90 bp had no effect on the stripe (3U1-90, Figure 5; Figure 6G); therefore, the 5′ 90 bp is both necessary and sufficient for the PSM stripe. Other deletions supported this conclusion, while further 3′ truncation abolished the stripe (3U1-50, Figure 5).

*hairy2a* promoter transgenes with the *hairy2a* 3′ UTR fused downstream of eGFP show reduced expression levels compared to those without the UTR. To test if this effect is specific to the *hairy2a* promoter, we included the UTR in transgenes expressing eGFP from three other promoters. For both the sCMV and *Xenopus EF1α* promoters, expression levels are also significantly reduced when the UTR is present. Because both of these
promoters express uniformly in the epidermis, it is difficult to observe any specific pattern imposed by the UTR (data not shown).

By contrast, the Xenopus cardiac actin promoter restricts eGFP transgene expression to the PSM, somites, and developing heart (Kroll and Amaya, 1996; Figure 6H, bottom). Transgene expression from this promoter is also reduced when the UTR is present; however, in most embryos expression is consistently higher in the PSM and newly formed somites compared to more anterior, older somites (Figure 6H, top). Thus, the hairy2a 3’ UTR can impose a posterior pattern bias on expression from the cardiac actin promoter.

UTRs of Other Genes with Segmental Expression Can Replace the hairy2a UTR
Several other Xenopus genes have chevron-shaped stripe patterns in the PSM. hairy1 expression overlaps hairy2a, while E(spl) related genes esr4, esr5, and clone 8C9 are in stripes just posterior to hairy2a (Gawantka et al., 1998; Jen et al., 1999). Transgenes substituting the hairy2a 3’ UTR with the hairy1, esr5, and 8C9 3’ UTRs also show PSM stripe expression (Figure 5; Figure 6I), although there is no obvious primary sequence relationship among these UTRs. To demonstrate sequence specificity, the Xenopus β-major globin (3UXβglo), MyoD (3UMD), or cardiac actin (3Uca) 3’ UTRs were substituted for the hairy2a UTR. Each of these UTRs failed to give a PSM stripe, while neuroectoderm expression was unaffected (Figure 5).

Does the chimeric transgene with the hairy2a promoter and the esr5 3’ UTR give the hairy2a or esr5 stripe? To show the position of transgene expression, we performed double in situ hybridization for the transgene and endogenous esr5. Figure 7A shows the endogenous hairy2a and esr5 PSM stripes. The hairy2a promoter:esr5 3’ UTR transgene is expressed in a stripe anterior to the endogenous esr5 stripe, therefore overlapping the hairy2a stripe (Figure 7B). The same result was obtained with a hairy2a promoter:hairy2a 3’ UTR
transgene (Figure 7C). Similar double in situ hybridizations for the transgene and endogenous *hairy2a* demonstrated one stripe from overlapping stains (data not shown).

If the function of the *hairy2a* 3′ UTR is conserved through evolution, one might expect that 3′ UTRs of *hairy*-like genes in other vertebrates should substitute for the *hairy2a* UTR. The 3′ UTRs from *c-hairy1* (3Uchry1), mouse *HES1* (3Umhes), and human *HES4* (3Uhhes4) genes were tested. In each case, a PSM stripe was observed (Figure 5; Figure 6I). However, the stripe was consistently broader for the *c-hairy1* and mouse *HES1* UTRs, usually about two somite widths (Figure 6I).

**A 25 bp Motif in the *hairy2a* 3′ UTR Is Sufficient to Drive the Stripe Pattern**

Because a 90 bp domain of the *hairy2a* 3′ UTR is sufficient for the PSM stripe, we compared this domain with the UTRs of *Xenopus hairy2b* and the *hairy2* orthologs zebrafish *hairy1*, *c-hairy1*, and human *HES4*. There is a 25 bp motif within the 90 bp domain that is identical in the *hairy2b* UTR, highly similar in the zebrafish UTR, and for which there are statistically significant matches in the chicken and human UTRs (Figure 8A; Bailey and Elkan, 1994). To test a requirement for this motif, it was replaced with a scrambled version (mut, Figure 8A). When this mutant motif is used in the 90 bp domain (mut3U1-90), or the entire *hairy2a* 3′ UTR (mut3U), PSM stripe expression is abolished (Figure 5; Figures 7D and 7E). We tested sufficiency of the motif by including it, and six nonconserved flanking base pairs on each side, as the only 3′ UTR (3U48-72). In this case, the PSM stripe is present; however, the number of positive embryos is reduced, and the expression level is weaker than with the 3U1-90 or full-length UTRs (Figure 5; Figures 7F and 7G). This suggests that the motif is the major UTR element required for the PSM stripe, although it functions more efficiently within the 3U1-90 or full-length 3′ UTRs.

**The *hairy2a* 3′ UTR Confers Instability on a Heterologous RNA**

The rapid disappearance of *hairy2a* RNA anterior to the PSM stripe could be explained, in part, by rapid RNA degradation. Because the *hairy2a* 3′ UTR can reduce transgene RNA levels, we asked if the UTR can target RNA for rapid turnover. Equimolar amounts of synthetic RNA for eGFP alone, or eGFP fused to different 3′ UTRs, were injected into one cell of four-cell stage embryos. Assessment of the remaining RNA was performed by RT-PCR of total RNA from stage 18 embryos (Figure 8B). eGFP RNAs containing the *hairy2a* and *esr5* 3′ UTRs were undetectable, compared to the injected RNAs with the control 3′ UTRs. Thus, RNAs containing the *hairy2a* or *esr5* 3′ UTRs have significantly decreased stability. These results suggest that reduced expression levels for transgenes that contain a correctly positioned *hairy2a* 3′ UTR are a consequence of an increased rate of RNA degradation.

**Discussion**

In vertebrate segmentation, anterior structures form first, while posterior ones form by regularly timed, sequential subdivision. The translation of this periodic activity into a spatial pattern has long suggested an underlying clock-like mechanism (Cooke, 1998; Stern and...
The dynamic expression of hairy genes suggests that they are a readout of this “segmentation clock” (Palmeirim et al., 1997; Pourquie, 1999, 2000a, 2000b). By uncovering the necessary and sufficient cis regulatory sequences that drive the Xenopus hairy2a segmental pattern, we can now describe the gene regulatory targets of this putative clock in molecular terms.

The hairy2a PSM pattern depends on two separate processes. The first is transcriptional and requires the hairy2a promoter paired Su(H) motif. The second involves an unexpected modulation of RNA levels by the hairy2a 3’ UTR. The hairy2a promoter alone can drive most of the hairy2a pattern, but not the anterior PSM stripe. Nontissue-specific promoters, such as sCMV or EF1α, that drive transgenes containing the hairy2a 3’ UTR express everywhere, but with reduced levels compared to those without the UTR. Combined, the hairy2a promoter and 3’ UTR reconstitute the anterior PSM stripe.

The hairy2a Promoter

The complex pattern of hairy2a expression in the PSM and elsewhere initially suggested potentially complex regulation at the level of transcription (i.e., by multiple inputs acting on multiple cis regulatory elements). It is remarkable that transcriptional activation of hairy2a in the PSM and most other tissues is controlled solely by the paired Su(H) motif. The motif consists of three simple components required for the PSM stripe. Two of these are the Su(H) binding sites (Bailey and Posakony, 1995; Nellesen et al., 1999). The third is the intervening sequence hexamer. Although previous characterization of the hexamer has relied on sequence conservation, here we demonstrate a functional requirement for the hexamer, suggesting that other DNA binding proteins may act on the paired Su(H) motif through the hexamer.

A single Su(H) binding site suffices for the neuroectoderm pattern, while the paired Su(H) motif is required for the PSM pattern, supporting the notion that single Su(H) binding sites and paired Su(H) motifs are functionally distinct. Such cell-type or tissue-type distinctions between these DNA elements had not been readily discernible in cell culture assays (Jarriault et al., 1995). It is known that dominant interfering forms of Xenopus Delta2 or Su(H) can abolish the hairy2a PSM stripe, demonstrating that hairy2a expression is dependent on Notch signaling in the PSM (Jen et al., 1997). While Notch
signaling is a primary input for transcription of hairy2a, the required architecture of the paired Su(H) motif indicates that there is more to hairy2a transcription than just activation of Su(H) protein by Notch.

The hairy2a 3’ UTR
To function in PSM expression, the UTR must be in its normal position and orientation in the transcript of the transgene, a requirement consistent with a modulator of RNA levels after transcription initiation, possibly entirely at the posttranscriptional level. That 3’ UTR function can be reduced to a discrete 25 bp motif suggests this motif is the target for trans-acting factors controlling PSM expression. The role for this sequence could in principle affect a nuclear or a cytoplasmic event required for hairy2a RNA levels to accumulate in anterior PSM cells. The hairy2a 3’ UTR also increases the rate of RNA turnover. Based on these findings, one plausible mechanism for UTR function is to both destabilize RNA in general and to provide a specific sequence for factors that transiently stabilize the RNA in the anterior PSM. To produce a transient stripe, these factors must themselves be rapidly inactivated soon after hairy2a expression and before somite formation. Our results do not rule out a role for the UTR in transcriptional control of hairy2a, although such a role would likely occur after transcription initiation. Because neither the promoter, nor the 3’ UTR, alone yield an obvious subpattern of the PSM stripe, a more speculative interpretation of these results would posit a molecular interaction between the promoter and 3’ UTR through bound trans-acting factors.

The substitution of the hairy2a 3’ UTR with UTRs from other segmentally expressed genes, even from other species, suggests a common mechanism controlling the segmental expression of these other genes. The c-hairy1 and mouse HES1 3’ UTRs generated a somewhat broader, more diffuse PSM stripe than the hairy2a or other Xenopus 3’ UTRs. This suggests to us that while there is some functional recognition of these UTRs, they do not show the more focused spatial specificity of the natural 3’ UTR. Possibly, Xenopus 3’ UTR binding factors may have higher affinity, or slower turnover, on the c-hairy1 and mouse HES1 UTRs, and thus widen the domain of cells where the transgene RNA accumulates. Consistent with this broader PSM expression, the cardiac actin promoter transgene with the hairy2a 3’ UTR is expressed in a much wider PSM domain, as is the endogenous cardiac actin gene, but is still appreciably downregulated in newly formed somites and remains at low levels in more anterior somites. This suggests that factors that recognize the hairy2a 3’ UTR are, in fact, distributed over a much wider domain of the PSM than is hairy2a RNA, whose expression is further restricted to the anterior PSM by the hairy2a promoter. Taken together, these results support a role for the 3’ UTR in controlling the segmental pattern of Notch target genes.

A similar situation may exist in Drosophila, where Posakony and colleagues have shown that the 3’ UTRs of E(spl) genes and the bearded class genes (which coincidentally negatively regulate Notch activity) can negatively regulate their respective expression levels and patterns (Lai et al., 1998; Leviten et al., 1997). This negative regulation affects neural tissue patterning controlled by these genes, while no 3’ UTR-dependent regulation of hairy has been described for segmentation in Drosophila. It is possible that the 3’ UTR function has been coopted in evolution for regulating vertebrate segmentation genes (based on the conservation of Notch signaling), or perhaps lost in the evolution of segmentation in Drosophila and other long germ-band insects.

hairy2a and the Mechanisms of Segmentation
The hairy2a PSM pattern shows two types of temporal asynchrony. First, expression is consistently activated first in the dorsal half of the PSM stripe. A similar asynchrony exists in the anterior PSM stripes of esr5, esr4, and BC9, as well as the stripe patterns of Delta1 and Delta2 (Figure 8C, and data not shown). It seems reasonable to suggest that this aspect of hairy2a expression, as well as that of the esr genes, may be driven, in part, by the similar pattern of Delta1 or Delta2 (i.e., timing of Notch activation). Second, the hairy2a pattern is consistently phase-advanced on the right side of the embryo (as are the esr, Delta1, and Delta2 stripes, data not shown). Such bilateral asynchrony has not been described in other vertebrates, though its observation in the frog suggests that there is no obligate bilateral synchronization in vertebrate segmentation. It may be that genes involved in left-right asymmetry, such as Xenopus nodal-related 1 (Lustig et al., 1996), cause a phase shift in segmentation on the two sides of the embryo.

Rather than oscillating over the whole PSM, the hairy2a pattern behaves as a single wavefront traversing through the anterior PSM and prefiguring new somite formation. In fact, none of the genes whose orthologs are reported to oscillate in the fish, chick, or mouse PSM do so in Xenopus, including hairy2a, hairy1 (orthologous to mouse and human HES1 and c-hairy2 [Jouve et al., 2000], lunatic fringe, or esr5 (related to her-1 in zebrafish [Holley et al., 2000; Jen et al., 1999, and our unpublished results]). This suggests that cycling in the PSM, exemplified by c-hairy1, is not an invariant property of vertebrate segmentation. Though there are some differences in the exact temporal pattern of hairy and E(spl)-related genes in the PSM of different vertebrates, it is likely that the basic timing mechanisms and the readout of these timing mechanisms are very similar. The pattern differences are probably a function of upstream events, such as activation of Su(H) by regulation of Notch signaling. We predict that the c-hairy1 promoter will be very similar to the Xenopus hairy2a and human HES4 promoters, while the c-hairy2 promoter will be very similar to that of the mouse and human HES1 promoters.

In Figure 8C, we present a schematic of the molecular targets that control the hairy2a pattern, and, by implication, the targets of the segmentation clock. There are at least three types of cis regulatory inputs. The first one is represented by the two Su(H) binding sites, which are presumably bound by a Su(H) containing complex that represses the hairy2a promoter, until Notch signaling switches Su(H) to an activator (Kao et al., 1998). The second one is represented by the conserved hexamer between the Su(H) binding sites. Although mutation of the hexamer lowered expression levels in tissues other
than the PSM, we cannot rule out the possibility that the hexamer has a specific function in modulating the paired Su(H) motif function in the PSM. The central role of the paired Su(H) motif for transcriptional activation of hairy2a suggests that Notch signaling must be a fundamental component of the segmentation clock. The third input occurs through the 3' UTR, in particular through the 25 bp motif. The 3' UTR confers global instability on the hairy2a RNA, but apparently local stability in the anterior PSM. How inputs through these three types of small sequences are integrated to control the dynamic hairy2a PSM pattern is a major question for understanding the molecular mechanisms of segmentation.

Experimental Procedures

Xenopus Embryos, Transgenesis, and Injections
X. laevis frogs (NASCO) were used to obtain eggs for transgenesis or in vitro fertilization. Embryos were staged according to Nieuwkoop and Faber (1967). Transgenesis was performed using the nuclear transplantation technique as described (Amaya and Kroll, 1999). Embryos were fixed in MEMFA, generally at stages 26–31, for whole-mount in situ hybridization (Sive et al., 2000). Injection of synthetic mRNA or expression plasmids was performed by standard procedures (Sive et al., 2000).

Cloning the hairy2a Promoter
PCR primers based on conserved amino acids in the bHLH domain of the Drosophila hairy and E(spl) proteins (forward primer heb1, coding amino acids RARM/IN: GAGCTCMGNGCNMGNATNAA; reverse primer heh21, coding amino acids KAD/EI: MCGAATTCA RNATNCGCYYTT) were used to isolate fragments of related Xenopus mRNAs by RT-PCR of stage 14–15 embryo RNA, as previously described (Turner and Weintraub, 1994). PCR products were recovered corresponding to three hairy-like genes, as well as several E(spl)-related genes. Full-length cDNAs for X. laevis hairy2a, 2b, and 1 were isolated from a Xenopus stage 23 embryo lambda gt10 cDNA library (Sive et al., 1989) using pooled probes derived from the hairy-like PCR products.

In Xenopus, the two hairy homologs are named hairy2 and hairy1. We indicate the hairy2 and hairy1 vertebrate orthologs, based on phylogenetic analysis, and their GenBank accessions (if available) as follows: (a) hairy-like genes are Xenopus hairy2a (AF383159) and 2b (AF383160), zebrafish hairy1 (AF301264), c-hairy1 (AF038268), and human HES4 (AB046791); and (b) hairy1-like genes are Xenopus hairy1 (US26194), zebrafish hairy6 (X87733), c-hairy2, mouse HES1 (D16464), and human HES1/HRY (NM-005524). The human HES4 gene is on chromosome 1 (GenBank accession NT-004384).

A Xenopus genomic library in Lambda FIX II (Stratagene) was screened, and five clones, from approximately 4 × 108 screened, were isolated and amplified by long-range PCR (rTh XL, Perkin-Elmer). We used a vector-specific primer (modified T3 or T7) and a primer that overlapped the amino-terminal coding and 5' UTR sequences of hairy2a (mutant for the start codon), and oriented toward the 5' UTR to obtain a 12 Kb product. Sequencing of the 5' UTR of one genomic clone showed a 102/103 bp match to the 5' UTR of the hairy2a cDNA. The 5' UTR sequences of hairy2b and hairy1 are completely different. The GenBank accession number for the hairy2a proximal promoter sequence is AY037926.

Expression Plasmids
Most plasmids for transgenesis were based on RARE-3E, a promoterless derivative of CS2 (Rupp et al., 1994; Turner and Weintraub, 1994). In RARE-3E, the scMV/E94 promoter has been replaced with a polynucleotide for restriction enzymes with 8 bp recognition sites, 3' of this polynucleotides is the eGFP (Clontech) coding sequence, followed by the SV40 virus late polyadenylation signal.

The standard hairy2a promoter in RARE-3E was from 294 bp to the TATA box, through the entire 5' UTR. Most transgenes also have the Xenopus laevis larval type I p-globin gene intron-1 inserted after eGFP (from PCR of genomic DNA) (Meyerhof et al., 1984). This intron increases expression in transgenic embryos but otherwise has no effect on spatial or temporal control of gene expression. In one instance, we replaced the SV40 polyadenylation signal with that of the larval type I p-globin gene, also obtained by PCR of genomic DNA. RARE-3ESy, used for the Su(H)Sy constructs, contains a minimal promoter with an inverted CCAAT box, TATA box, and transcription initiation region from human adenovirus MLP transcription unit sequences (Braselmann et al., 1993).

3' UTRs were cloned between eGFP and the globin intron. Xenopus hairy2a, hairy1, and MyoD (Hopwood et al., 1989) 3' UTRs were cloned by PCR from their respective cDNAs. 3' UTRs of e5s (Jen et al., 1999) and B9 (Gawantka et al., 1998) were cloned by PCR of a Xenopus st. 11.5–15 cDNA library in CS2. 3' UTRs of the Xenopus cardiac actin gene (Mohn et al., 1986) and β-major globin gene (Patient et al., 1983) were cloned by PCR of genomic DNA. The c-hairy1 3' UTR (Palmeirim et al., 1997) was cloned by 3' RACE (SMART RACE, Clontech) of total RNA prepared from somite and segmental plate explants of E3 chick embryos (generously supplied by Charlie Murtaugh). The 3' UTRs of mouse HES1, human HES1/HRY, and human HES4 (Bessho et al., 2001; Fedor et al., 1994; Takebayashi et al., 1994) were amplified by PCR of LI.M.A.G.E. clones 456976, 722572, and 2782501, respectively (Research Genetics). Other construct variants, such as insertion, deletion, and point mutants, were generated by PCR-based methods and sequenced for verification. Additional plasmids included CS2, XeXS2 (substituting the CS2 cMV promoter with the Xenopus EP1s promoter [Johnson and Krieg, 1994]) or CAR (Xenopus cardiac actin promoter [Kroll and Amaya, 1996]) driving eGFP, with or without the hairy2a 3' UTR. The Su(H) binding site point mutations were CCGTGCAAA for the 5' site and TTGACAGC for the 3' site, mutation underlined (Bailey and Posakony, 1995). Mutation of the conserved hexamer was to CGCTTA. Mutation of the hairy2a promoter and 5' UTR N boxes was as follows: CACAGG to CACAGG (Sasai et al., 1992), where N is degenerate.

In Situ Hybridization and Imaging
Embryos were processed for in situ hybridization by standard protocols (Sive et al., 2000). Staining was performed generally with a NBT/BCIP combination (yielding a purple/brown color), although in some cases, BCIP alone (yielding a cyan color) or magenta-phos alone (yielding a magenta/purple color) were used. We conclude that a hairy2a promoter transgene does not express the PSM stripe, if, in embryos stained to saturation, the stripe is never observed and neuroectoderm expression levels are comparable to the endogenous hairy2a gene (based on rates of staining) in at least 50% of the transgenic embryos. Some hairy2a promoter transgene mutants abolish or severely attenuate neuroectoderm expression, and these mutants never express the PSM stripe. As a rule, we do not stain to saturation embryos with transgenes that do show a PSM stripe (in contrast to those with constructs that do not), so as to observe subtle pattern differences between embryos. Therefore, the PSM stripe may not be detected in all embryos, with constructs that do show the stripe, because it is below the sensitivity of in situ hybridization detection in a subset of embryos. Embryos were imaged either on a Zeiss Stemi SV111 stereooscope, or with low power optics on a Zeiss Axiohot, using either a Sony color CCD video camera, or a Hamamatsu ORCA-1 cooled CCD digital camera, respectively. Images were acquired using Open Lab software (Improvision).

RT-PCR
Embryos were injected with synthetic mRNAs, encoding eGFP with or without different 3' UTRs. RT-PCR was performed on total RNA isolated from neural (stage 18) embryos. Although the 3' UTRs have varying length, none are longer than 450 bases (for comparison, hairy2a is 317 bases, while MyoD is 428 bases), and reverse-transcription was performed for 1 hr to ensure full-length cDNA synthesis from these RNAs. Nonradioactively labeled PCR products were analyzed by agarose gel electrophoresis. All experimental and control PCR products were tested for reverse-transcriptase dependence.
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References


Accession Numbers

The GenBank accession numbers reported in this paper are as follows: Xenopus hairy2a, AF383159; Xenopus hairy2b, AF383160; Xenopus hairy2a proximal promoter, AY037926.