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The Notch ligand, X-Delta-2, mediates segmentation of the paraxial mesoderm in *Xenopus* embryos

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SUMMARY

Segmentation of the vertebrate embryo begins when the paraxial mesoderm is subdivided into somites, through a process that remains poorly understood. To study this process, we have characterized *X-Delta-2*, which encodes the second *Xenopus* homolog of *Drosophila Delta*. Strikingly, *X-Delta-2* is expressed within the presomitic mesoderm in a set of stripes that corresponds to prospective somitic boundaries, suggesting that Notch signaling within this region establishes a segmental prepattern prior to somitogenesis. To test this idea, we introduced antimorphic forms of X-Delta-2 and *Xenopus* Suppressor of Hairless (X-Su(H)) into embryos, and assayed the effects of these antimorphs on somite formation. In embryos expressing these antimorphs, the paraxial mesoderm differentiated normally into somitic tissue, but failed to segment properly. Both antimorphs also disrupted the segmental expression of *X-Delta-2* and *Hairy2A*, a basic helix-loop-helix (bHLH) gene, within the presomitic mesoderm. These observations suggest that X-Delta-2, via X-Notch-1, plays a role in segmentation, by mediating cell-cell interactions that underlie the formation of a segmental prepattern prior to somitogenesis.

Key words: Delta, somites, segmentation, *Xenopus*

INTRODUCTION

Segmentation of the vertebrate embryo arises in embryonic development through a process which subdivides the paraxial mesoderm into repeating metameric units, called somites. Because the somites are the first segmented structures to form during embryogenesis, they lay the foundation for the segmentation of such tissues as the vertebrae and their associated muscles. Somites also play a critical role in the segmentation of the nervous system by influencing the migration of neural crest cells and outgrowth of the ventral nerve roots from the spinal cord (Keynes and Stern, 1984). Despite the fact that most segmented features of the vertebrate body plan are based on the pattern of segmentation first established during somitogenesis, how this pattern arises in the early embryo remains poorly understood.

In all vertebrate embryos, segmentation begins anteriorly, and progresses posteriorly along the length of the paraxial mesoderm. Thus in any given embryo, the different stages of segmentation are conveniently laid out spatially along the anterior-posterior (A-P) axis in their temporal order. At late stages of the segmentation process, the paraxial mesoderm undergoes the elaborate morphogenetic changes which underlie somitogenesis (see Fig. 1). These changes in a mouse or chick embryo entail a mesenchymal/epithelial transition: a somite forms when a group of mesenchymal cells at the anterior end of the paraxial mesoderm segregate and coalesce into an undifferentiated, epithelial ball. Somites then lose their epithelial morphology, while further subdividing into a myotome, sclerotome and dermatome. By contrast, in *Xenopus* embryos, a somite consists entirely of differentiated, mononucleated myotomal cells, which span each segmental unit (Youn and Malacinski, 1981; see Fig. 1). These somites form without going through an epithelial intermediate, but rather by a morphological process in which cells segregate from the paraxial mesoderm, and rotate 90 degrees (Hamilton, 1969). Thus, vertebrate species differ greatly during somitogenesis in terms of tissue morphology, the cell types which make up somites, and the morphogenetic processes underlying their formation. Despite these variations, different vertebrates may nonetheless use similar mechanisms to pattern the paraxial mesoderm into segmental units. Based on a morphological feature apparent in scanning electron micrographs (SEM), the presomitic mesoderm of all vertebrate embryos appears to be prepatterned into metameric segments called somitomeres (Jacobson and Meier, 1986; Meier, 1979). If somitomeres are indeed the segmental precursors to somites, as suggested by several lines of evidence, then vertebrates may use similar mechanisms to establish a segmental prepattern within the presomitic mesoderm, but then diverge in the types of segmental structures that are generated during somitogenesis.

Among the mechanisms that underlie segmentation in vertebrate embryos could be ones that instruct cells in terms of their relative position by local cell-cell interactions. One form of such interactions is mediated by the Notch family of receptors, which regulate cell fate decisions in a variety of different organisms (reviewed by Artavanis-Tsakonas et al., 1995). The Notch-related receptors have an extracellular domain that is largely made up of tandem copies of EGF-like repeats, as well as tandem copies of a second cysteine-rich repeat, the lin-12/Notch motif, while the intracellular domain contains a series of ankyrin repeats. The extracellular EGF repeats are thought to bind a conserved family of ligands, collectively referred to as DSL proteins, which then leads to activation and transduction of a Notch signal via a conserved transcription factor called Suppressor of Hairless (Su(H); Bailey and Posakony, 1995; Fortini and Artavanis-Tsakonas, 1994; Christensen et al, 1996; Wettstein et al., 1997). The Notch-related receptors and their ligands were first shown to mediate a local inhibitory, cell-cell interaction called lateral inhibition, or specification (reviewed by Campos-Ortega, 1994). This mechanism is ubiquitously used in different organisms to generate a fine-grain pattern, by allowing neighboring cells in an equipotential field to take on alternative fates (Chitnis et al., 1995; Posakony, 1994; Wilkinson et al., 1994). However, not all cases where Notch and its ligands are found to regulate cell fate involve the selection of individual cells. In the developing *Drosophila* wing imaginal disc, two DSL ligands, Delta and Serrate, appear to signal reciprocally across the boundary between the dorsal and ventral compartments to specify the prospective wing margin (Couso et al., 1995; de Celis et al., 1996; Doherty et al., 1996; Kim et al., 1995). Thus these more recent studies have shown that Notch and its ligands also mediate a patterning event in which a boundary is specified between two developmental compartments.

Recent evidence suggests that the Notch receptors may play a role in segmentation of the mouse embryo. At least three Notch-like genes have been identified in the mouse, and two of these, *Notch-1* and *Notch-2*, are expressed in the paraxial mesoderm in the area where somites form (Del Amo et al., 1992; Lardelli and Lendahl, 1993; Reaume et al., 1992). At least two mouse genes have been identified which encode DSL ligands, and one of these, *Dll-1*, is also expressed in the presomitic mesoderm, where it localizes to an anterior domain just prior to somitogenesis (Bettenhausen et al., 1995; Lindsell et al., 1995). The most compelling evidence that Notch-1 is required for segmentation comes from the analysis of mice which are mutant for *Notch-1* via gene targeting (Conlon et al., 1995; Swiatek et al., 1994). One obvious developmental defect in these mutants is a somite abnormality, in that cell specification within the somites appears to be normal, but the somites vary in size and fail to align across the midline (Conlon et al., 1995). Similar somite defects have also been observed in the mouse knock out of *RBP-J*$_\kappa$ (Oka et al., 1995), in line with the idea that vertebrate homologs of the *Drosophila Suppressor of Hairless* (*Su(H)*) gene are required for Notch signaling (Bailey and Posakony, 1995; Christensen et al., 1996; Fortini and Artavanis-Tsakonas, 1994; Lecourtois and Schweisguth, 1995; Wettstein et al., 1997). While these observations suggest that Notch is required for the segmentation of the paraxial mesoderm, it is not known how Notch fulfills this role, nor when in the process of segmentation Notch acts.

Here we describe a new member of the vertebrate DSL ligands, called *X-Delta-2*, which is expressed along with *X-Notch-1* during segmentation of the *Xenopus* embryo. Specifically, *X-Delta-2* is expressed within the presomitic mesoderm in a set of stripes, that correspond to prospective somitic boundaries, suggesting that Notch signaling is required for the establishment of somitomeres. To test this idea, Notch signaling was inhibited in embryos by expressing antimorphic forms of X-Delta-2 and *Xenopus* Su(H). Both mutants did not have any detectable effect on the differentiation of the paraxial mesoderm into somitic tissue, in terms of myogenesis. Instead, both mutants produced profound defects in the ability of this tissue to segment properly. Furthermore, inhibition of Notch signaling perturbs the segmental expression of *X-Delta-2* as well as a bHLH gene, *Hairy2A*, within the presomitic mesoderm. Based on these observations, we propose that X-Delta-2, via X-Notch-1, mediates the establishment of somitomeres, perhaps by specifying segmental boundaries.

MATERIALS AND METHODS

Isolation of *X-Delta-2*

A λgt10 stage-17 *Xenopus* cDNA library (Kintner and Melton, 1987) was hybridized under low stringency conditions to a small *X-Delta-1* PCR fragment, isolated from stage 17 (neurulae) embryos, as described by Henrique et al. (1995) and Chitnis et al. (1995). This screen yielded a 2.86 kb cDNA clone, that was sequenced by dideoxynucleotide chain termination, using T7 DNA polymerase (USB). The DNA sequence of *X-Delta-2* has been submitted to GenBank (accession no. U70843).

Expression of synthetic *X-Delta-2* RNA in embryos

For generating synthetic RNA in vitro, the coding region of the *X-Delta-2* cDNA was inserted into the CS2$^+$ vector (Turner and Weintraub, 1994), producing a construct in which both the 5′ and 3′ untranslated regions of the *X-Delta-2* cDNA were deleted. To measure expression of *X-Delta-2* from injected RNA, a construct was also generated in which six myc-epitope tags were added to the carboxy terminus (Turner and Weintraub, 1994). The generation of constructs entailed producing the appropriate DNA fragment using PCR, and the sequence of all constructs was confirmed by DNA sequencing. The myc-tagged version of *X-Delta-2* was used to show by immunohistochemistry that protein expression from injected RNA persisted into late neurulae stages. Both the Myc-tagged version of X-Delta-2, as well as the version of *X-Delta-2* RNA lacking 5′ and 3′ untranslated sequences gave identical phenotypes and were used interchangeably. A truncated version of *X-Delta-2*, encoding all but the 88 amino acids at the carboxyl end of the protein, was generated by introducing a stop codon and a *Stu*I site after the 15th amino acid beyond the transmembrane domain.

RNA was transcribed in vitro using SP6 polymerase, in the presence of GpppG, from templates linearized with *Not*I as described previously (Chitnis et al., 1995). The templates for the synthesis of nuclear *nlacZ* RNA (Turner and Weintraub, 1994), and for *XSu(H)*^{DBM} (referred to as X-Su(H)1^{DBM} in Wettstein et al., 1997) were described previously. Capped RNAs were phenol/chloroform extracted, ethanol precipitated, and resuspended in a final concentration of at least 40 ng/μl. Integrity of the resulting mRNA was assayed by formaldehyde-agarose gel electrophoresis. RNA was injected in a volume of 10-20 nl into the equatorial region of a single blastomere of a 2-cell embryo. For each set of experiments, *nlacZ* RNA alone was injected as negative control.

In situ hybridization

Whole-mount in situ hybridization was performed on staged embryos as described by Harland (1991). The probe for *X-Delta-2* encom-

passed the entire cDNA, while those for *cardiac actin*, *MyoD* and *Hairy2A* have been described previously (Hopwood et al., 1992; Kintner and Melton, 1987; Schmidt et al., 1995). To determine the relative spatial expression patterns of *X-Delta-2* and *Hairy2A*, double-labeled in situ hybridization was carried out using fluorescein-labeled *X-Delta-2* and digoxigenin-labeled *Hairy2A* riboprobes. Chromogenic reactions were performed on *Hairy2A* using BCIP (Molecular Probe), followed by reaction for *X-Delta-2* using Magenta-Phos (Molecular Probe). Stained embryos were stabilized in MEMFA (0.1 M MOPS pH7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde), and photographed with a Wild M33 microscope. For detection of *X-Delta-2* expression in *X-Delta-2^{tr}* RNA injected embryos, the in situ probe consisted of the *X-Delta-2* sequences that were removed from the 3′ end of *X-Delta-2^{tr}*.

Immunohistochemistry and histology

To score the effects of each RNA injection on somite formation, embryos were harvested at early tadpole stage (approx. stage 26) and fixed in MEMFA for 1.5 hours. β-galactosidase expression was detected by staining in 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal) as described previously (Chitnis et al., 1995). Embryos were then stained for gene expression using in situ hybridization with the appropriate probe, or for the formation of somitic tissue using the monoclonal antibody 12/101 (Kintner and Brockes, 1985), according to the procedure of Hemmati-Brivanlou and Harland (1989). For best antibody penetration into the paraxial mesoderm, embryos were incubated with a 1:10 dilution of a 12/101 culture supernatant, in the presence of blocking solution (20% goat serum, 0.1% Triton X-100, and 1× PBS) for 48 hours or longer; and a 1:250 dilution of HRP-conjugated goat anti-mouse secondary antibody for 36 hours or longer. Following staining in DAB, embryos were stabilized by postfixing in MEMFA, embedded in paraplast and sectioned at 10 μm.

RESULTS

Isolation of *X-Delta-2*

A cDNA clone encoding *X-Delta-2* was isolated by screening a *Xenopus* neurulae cDNA library with probes for *X-Delta-1* and *X-Serrate-1*. One cDNA clone, called 37, hybridized to both probes at low but not high stringency, and encoded a product whose predicted amino acid sequence was highly related to both *Drosophila* Delta as well to recently isolated vertebrate homologs from chicken (C-Delta-1; Henrique et al., 1995), mouse (Dll-1; Bettenhausen et al., 1995), and *Xenopus* (X-Delta-1; Chitnis et al., 1995) (Fig. 2). Like C-Delta-1, Dll-1 and X-Delta-1, the new *Xenopus* Delta protein contains 8, rather than the 9 EGF-repeats found in the extracellular domain of *Drosophila* Delta. The new *Xenopus* Delta protein also contains the DSL motif which is a structural hallmark of ligands for the Notch-related receptors (Tax et al., 1994). However, while C-Delta-1, Dll-1 and X-Delta-1 share approximately 72% similarity overall, they only share approximately 45% similarity with the new *Xenopus* Delta protein. Moreover, while C-Delta-1, Dll-1 and X-Delta-1 share about 65% similarity within the intracellular domain, they share less than 20% similarity with the intracellular domain of the new *Xenopus* Delta. Thus, based on these comparisons, we concluded that the 37 cDNA encodes a new member of the vertebrate Delta family, which we name X-Delta-2. The overall structure of X-Delta-2, including the presence of a DSL domain, suggests strongly that X-Delta-2 is a ligand for the Notch family of receptors.

Expression of *X-Delta-2* during early embryogenesis

We analyzed the expression of *X-Delta-2* in early *Xenopus* embryos by visualizing *X-Delta-2* transcripts using whole-mount in situ hybridization (Harland, 1991). By this analysis, one of the most prominent sites of *X-Delta-2* expression in early embryos is associated with segmentation of the paraxial mesoderm. After the completion of gastrulation, the expression of *X-Delta-2* RNA is first detected adjacent to the blastopore and in stripes of cells located at or near the anterior end of the paraxial mesoderm (Fig. 3A). As the embryo grows older, the position of *X-Delta-2* stripes shifts progressively towards the posterior of the paraxial mesoderm (Fig. 3B). Meanwhile, the expression adjacent to the blastopore, which now becomes the

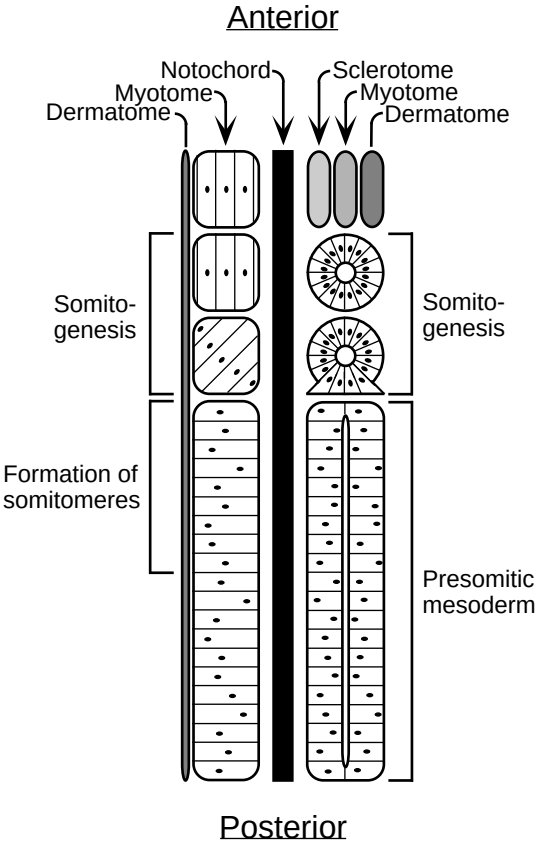

Fig. 1. Diagram illustrating the segmentation of the paraxial mesoderm in different vertebrate embryos. The paraxial mesoderm on the left side of the diagram represents somitogenesis in *Xenopus* embryos (Hamilton, 1969) while that on the right represents somitogenesis in higher vertebrates, such as in a chick or mouse embryo. In all vertebrates, anterior region of the presomitic mesoderm appears to be first pre-patterned into somitomeres (Jacobson and Meier, 1986; Meier, 1979). In *Xenopus*, however, a somite forms when a group of myotomal cells segregates, rotate 90°, and orients parallel to the A-P axis. Each somite consists entirely of mononucleated myotomal cells while the dermatome does not undergo segmentation or rotation (Hamilton, 1969). In mouse or chick embryos, each somite forms when a group of mesenchymal cells forms an epithelial ball, that then undergoes a further subdivision into sclerotome, myotome and dermatome. We have aligned the events of segmentation in the diagram by equating the segregation of myotomal cells in *Xenopus* to the formation of an epithelial ball in chick or mouse. However, what structures are analogous in the segmentation of different species is not known.

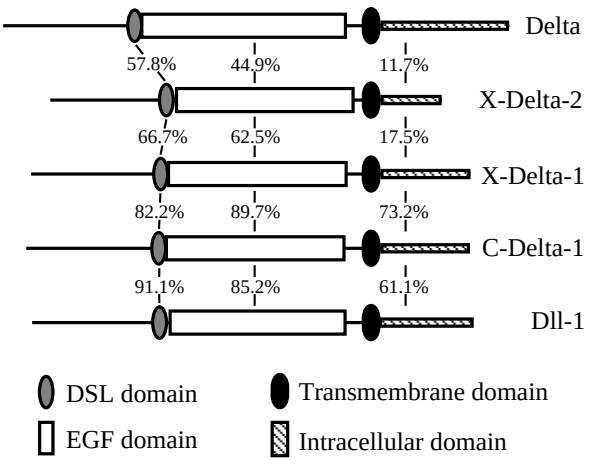

Fig. 2. The structure of X-Delta-2 from its predicted sequence, compared to that of Delta proteins previously isolated from *Drosophila* (Delta), mouse (Dll-1), Chick (C-Delta-1) and *Xenopus* (X-Delta-1). The percentage sequence similarity is for a pairwise comparison between proteins adjacent to each other in the diagram.

tailbud, is maintained. At tadpole stages, the stripes of cells expressing *X-Delta-2* RNA are localized to the paraxial mesoderm in the tail (Fig. 3C). Thus, these observations suggested that the expression pattern of *X-Delta-2* within the paraxial mesoderm is dynamic and appears to correlate with somitogenesis.

To examine this correlation further, embryos stained in whole-mount were sectioned along the longitudinal axis to determine where the cells expressing *X-Delta-2* RNA in the paraxial mesoderm were located relative to the events that occur during somitogenesis. Strikingly, expression of *X-Delta-2* RNA was not detected in somites but in the presomitic mesoderm. Moreover, despite the fact that the *X-Delta-2* is expressed prior to somitogenesis, its pattern of expression is nonetheless segmental, in that each stripe of *X-Delta-2*-expressing cells demarcates a domain in the paraxial mesoderm that corresponds to a prospective somite (Fig. 3D, for details see legend). Significantly, this segmental pattern of *X-Delta-2* expression also appears to undergo a refinement. Initially, *X-Delta-2* expression appears to fill most of a prospective segment, with higher expression in the anterior half than in the posterior half (Figs 3D, 4A). Expression of *X-Delta-2* then downregulates in a posterior to anterior progression within a prospective somite, resulting ultimately in a narrow stripe of *X-Delta-2*-expressing cells at the anterior edge of a prospective somite.

Since X-Delta-2 is likely to be a ligand for Notch-related receptors, we asked whether *X-Notch-1* expression overlaps that of *X-Delta-2* in the paraxial mesoderm. Whole-mount in situ hybridization with the *X-Notch-1* probe, resulted in staining of the somites, as well as the presomitic mesoderm (Fig. 3G). *X-Notch-1*, unlike *X-Delta-2*, is not expressed dynamically within the paraxial mesoderm, but shows a much more uniform pattern of expression (compare Fig. 3G with Fig. 4A).

Another *Xenopus* gene expressed in the presomitic mesoderm is *Hairy2A*, which encodes a bHLH transcription factor related to *Drosophila* Hairy (D. Turner, unpublished observations). *Hairy2A* is expressed transiently in one or two stripes approximately two prospective somites back from the most recently formed somite (Fig. 4B). The expression of *Hairy2A* relative to *X-Delta-2* was determined by double-

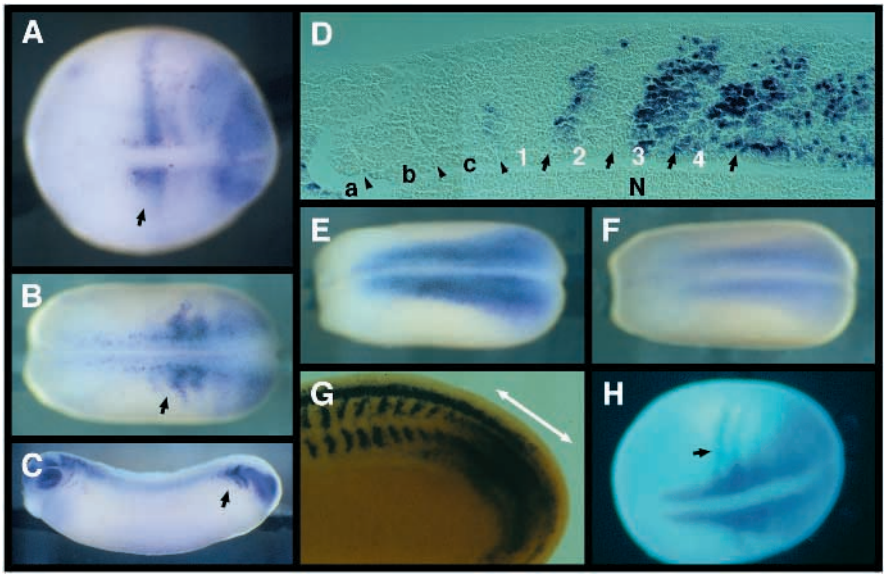

Fig. 3. Expression pattern of *X-Delta-2* transcripts in *Xenopus* embryos. In this and other figures, embryos and sections are oriented with anterior on the left. (A-C) Embryos stained in whole mount for the expression of *X-Delta-2* RNA at neural plate stage (A; dorsal view, stage 14), neurulae stage (B; dorsal view, stage 18), and tailbud stage (C; side view, stage 28). Note that *X-Delta-2* is expressed within the paraxial mesoderm within stripes of cells that are located progressively more posterior as development progresses (arrows). Note also that *X-Delta-2* expression occurs in a large domain in the tailbud. (D) Longitudinal section through neurulae embryos (stage 18), showing just one side of the paraxial mesoderm. The three somites that have formed (labeled a, b and c; demarcated by arrowheads) do not express *X-Delta-2* at detectable levels. Within the presomitic mesoderm, however, *X-Delta-2* RNA is expressed in stripes of cells, where the spacing between each strip (denoted by arrows) corresponds to about ten cells, and thus the width of a prospective somite, or somitomere (labeled 1-4; Jacobson and Meier, 1986). Note that *X-Delta-2* expression in the youngest somitomeres (labeled 3 and 4), is broad but then undergoes a refinement, localizing to the anterior edge of the first somitomere (labeled 1). (E,F) Dorsal view of myogenesis in the paraxial mesoderm of neural tube stage embryos (stage 18) as revealed by the expression of *cardiac actin* (E) and of *MyoD* (F). (G) Expression of *X-Notch-1* RNA in the presomitic mesoderm (double arrow) in a cleared, early tadpole embryo (stage 24), as shown by whole-mount in situ hybridization. (H) Dorsal view of an early neural plate stage embryo (stage 12.5), double-labeled for the expression of *cardiac actin* RNA (dark blue) and for *X-Delta-2* RNA (light blue). Note that the *X-Delta-2* staining (arrow) extends outside the myogenic domain marked by *cardiac actin* staining.

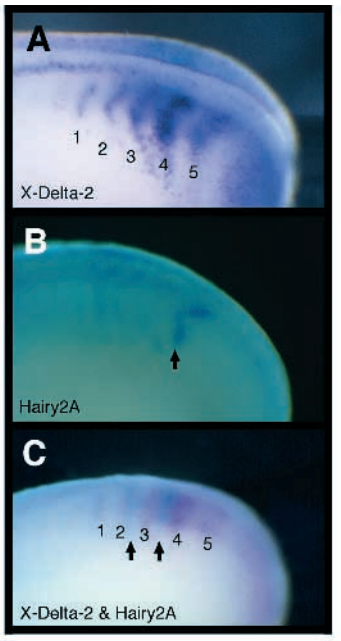

Fig. 4. Complementary and segmental expression of *X-Delta-2* and *Hairy2A* in the presomitic mesoderm. Shown are lateral, posterior views of neurulae stage embryos (stage 22) stained by whole-mount in situ hybridization for the expression of (A) *X-Delta-2*, (B) *Hairy2A*, or (C) both *X-Delta-2* (purple, numbers) and *Hairy2A* (light blue, arrows). In A, the stripes of *X-Delta-2* within somitomeres are numbered as in Fig. 3D. In C, note that *Hairy2A* is expressed in the posterior portion of the somitomere (arrows) where the expression of *X-Delta-2* is lost.

labelled, whole-mount in situ hybridization. The results show that the stripes of *Hairy2A* expression complement the stripes of *X-Delta-2* expression, such that as *X-Delta-2* expression is lost from each prospective somite, the expression of *Hairy2A* increases (Fig. 4C).

In sum, *X-Delta-2* is expressed in the presomitic mesoderm in a dynamic pattern of stripes which demarcates the prospective somites (i.e. somitomeres). The first stripe of *X-Delta-2* expression, which appears to emerge from a broad posterior domain of *X-Delta-2* expression in the tailbud region, initially fills a somitomere with higher levels of expression at the anterior than the posterior end. Expression of *X-Delta-2* within the somitomeres then undergoes a refinement where expression is lost in the posterior part of each somitomere, eventually localizing to the cells at the anterior side of each somitomere. During this refinement, *Hairy2A* is transiently expressed in the posterior half of each somitomere where *X-Delta-2* expression is lost.

Expression of *X-Delta-2* does not correspond to myogenic differentiation

Ligands for the Notch receptors have been shown to play a role in regulating cell differentiation in a variety of different tissues, including in the mesoderm. In cultured myogenic cells lines, for example, Notch ligands can regulate muscle differentiation (Lindsell et al., 1995). To examine whether *X-Delta-2* plays a role in myogenic differentiation, we asked if the expression of *X-Delta-2* corresponded in some way to the pattern of myogenesis within the paraxial mesoderm. To examine this pattern, we stained embryos using whole-mount in situ hybridization, for the expression of *MyoD*, which encodes a determinative, myogenic bHLH transcription factor (Frank and Harland, 1991; Harvey, 1992; Hopwood et al., 1992), and for *cardiac actin*, which encodes a muscle structural protein (Gurdon et al., 1985). As shown previously, both genes are expressed following gastrulation in a large dorsal domain, which includes both the somites and the presomitic mesoderm (Fig. 3E,F). The pattern of myogenesis revealed by these markers does not correspond to *X-Delta-2* expression. For example, while *X-Delta-2* is expressed in a set of stripes, *MyoD* and *cardiac actin* are broadly and uniformly expressed in all paraxial mesoderm (compare B to E and F in Fig. 3). In addition, cells expressing *X-Delta-2* extend outside the domain of paraxial mesoderm where the myogenic genes are expressed, indicating that

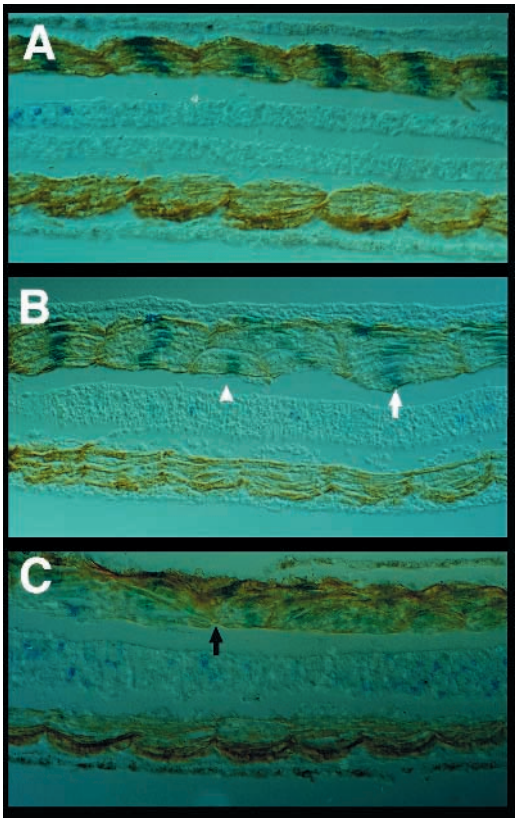

Fig. 5. Expression of *X-Delta-2*^{tr} alters the pattern of segmentation in *Xenopus* embryos. Embryos injected on one side, at the two-cell stage, with (A) *nlacZ* RNA or (B,C) with a mixture of *X-Delta-2*^{tr} and *nlacZ* RNAs. At early tadpole stages, embryos were fixed and reacted in whole-mount with X-gal, which stains the nuclei blue, and with the 12/101 antibody using HRP immunohistochemistry, which stains the muscle cells brown. Shown are representative longitudinal sections. (A) Embryo injected with just the *nlacZ* tracer. Note that each somite consists of mononucleated muscle cells, whose nuclei line up in a row at the center of each somite. (B) Embryo expressing *X-Delta-2*^{tr} RNA with a 'mild' phenotype. Note that some of the myotomal cells have formed somites which are shorter (arrowhead) or longer than normal (arrow). 12/101 staining on both injected and uninjected sides is incomplete due to penetration artifacts. In subsequent experiments using more stringent staining protocols, the expression of 12/101 was found to be uniform throughout the width of the somite. (C) Embryo expressing *X-Delta-2*^{tr} RNA with a 'strong' phenotype. Arrow marks the formation of intramyotomal junction.

expression of *X-Delta-2* also extends into the lateral plate mesoderm (Fig. 3H). Thus, the expression of *X-Delta-2* in the paraxial mesoderm does not appear to correlate with the differentiation of mesodermal derivatives.

Inhibition of X-Delta-2 activity produces segmentation defects

The expression pattern of *X-Delta-2* suggests that it mediates cell-cell interactions within the presomitic mesoderm during the process of segmentation. To determine if X-Delta-2 indeed plays a role in segmentation, we inhibited its activity in embryos using a dominant-negative form of X-Delta-2, called X-Delta-2^{tr}, which was generated by deleting the intracellular domain. We predicted that X-Delta-2^{tr} would behave as an antimorph that inhibits X-Delta-2 activity, since identical forms of X-Delta-1, or *Drosophila* Delta or Serrate are potent inhibitors of Notch signaling (Chitnis et al., 1995; Sun and Artavanis-Tsakonas, 1996). Synthetic *X-Delta-2^{tr}* RNA was directed into the prospective mesoderm by microinjection into the marginal zone of two-cell stage embryos, leading to ectopic expression of X-Delta-2^{tr} protein in one half of the embryo; the other half served as an uninjected control. To mark the injected side, embryos were co-injected with *nlacZ* transcripts (which encodes a nuclear-localized form of β-galactosidase) and stained for β-galactosidase activity using X-gal (Chitnis et al., 1995).

Embryos injected with *X-Delta-2^{tr}* transcripts were first examined by whole-mount in situ hybridization with markers for myotomal determination and differentiation to determine whether the inhibition of X-Delta-2 activity led to alterations in mesodermal cell fates, in which case the expression of myogenic markers should be perturbed. However, embryos injected with *X-Delta-2^{tr}* RNA transcripts showed no obvious effect on myogenic differentiation when scored by the expression of *cardiac actin* or *MyoD* RNA (data not shown), suggesting that the specification of myogenic fates was not affected. Myogenic differentiation was also assessed by analyzing the injected embryos for staining with the 12/101 monoclonal antibody, which recognizes an epitope present on the surface of differentiated muscle cells (Kintner and Brockes, 1985). In embryos ectopically expressing *X-Delta-2^{tr}*, 12/101 staining appeared to be the same on both the injected and control sides, suggesting that the inhibition of X-Delta-2 activity does not affect cell fate in the paraxial mesoderm, at least in terms of myotomal differentiation (data not shown).

While *X-Delta-2^{tr}* did not appear to have an effect on myogenesis, it did have a profound effect on the pattern of segmentation, which was clearly apparent when injected embryos were sectioned longitudinally, after staining in whole-mount with X-gal and the 12/101 antibody. In tissue sections, the segmental organization of myotomal cells can be readily visualized by the arrangement of myotomal nuclei, which stain blue with X-gal when they express nuclear-β-galactosidase (Fig. 5A). In *nlacZ* RNA injected embryos, the myotomal nuclei line up, reflecting each somitic unit (Fig. 5A). In contrast, in embryos injected with both *X-Delta-2^{tr}* and *nlacZ* transcripts, the position of the somitic nuclei were more chaotic, reflecting the fact that the myotomal cells are not segmented properly (Fig. 5B,C). While 51 out of 51 embryos injected with *X-Delta-2^{tr}* RNA showed segmentation defects, no such defects were observed in 29 embryos injected with *nlacZ* RNA. Interestingly, the degree of the segmentation defects varied, presumably depending on the amounts of *X-Delta-2^{tr}* RNA expressed. Thus, in embryos with a mild effect, the appearance of somitic tissue was largely normal, but the segmental pattern contained minor perturbations where some segments were longer and other shorter than normal (Fig. 5B). In embryos with more severe effects, the somitic tissue showed a near total loss of a segmental pattern (Fig. 5C). Staining with 12/101 antibody indicated that except for the improper segmental organization, somitic tissue appeared to have differentiated normally in that myotomal cells extended, grouped together and formed intramyotomal junctions. Thus, while X-Delta-2^{tr} did not inhibit the formation of somitic tissue, it did inhibit the pattern of segmentation.

Segmentation defects produced by a DNA binding mutant of Su(H)

The results above suggest that X-Delta-2 activity is required for the segmentation of the paraxial mesoderm. Because X-Delta-2 is likely to be acting as a ligand for a Notch family receptor, we asked whether similar segmentation defects would occur in embyros, following inhibition of the Notch signaling pathway. One component of this pathway is a DNA binding protein that has been isolated from mouse (RBP-J$_\kappa$), *Drosophila* (Suppressor of Hairless), human (CBF1), and *C. elegans* (LAG-1; Amakawa et al., 1993; Christensen et al., 1996; Matsunami et al., 1989; Schweisguth and Posakony, 1992). These proteins are thought to associate with a transactivator, perhaps the Notch intracellular domain, and activate transcription of target genes, following stimulation of the Notch pathway (Jarriault et al., 1995). We have generated a DNA binding mutant of *Xenopus* Suppressor of Hairless, called XSu(H)^{DBM}, that acts as a dominant negative mutant in that it blocks transcriptional activation of a gene normally upregulated by Notch signaling (referred to as XSu(H)1^{DBM} in Wettstein et al., 1997). Thus, to block Notch signaling in the paraxial mesoderm, XSu(H)^{DBM} was directed into the mesoderm by injecting synthetic *XSu(H)^{DBM}* RNA into the marginal zone of two-cell stage embryos. The effects of *XSu(H)^{DBM}* expression on the development of the paraxial mesoderm were then examined by whole-mount staining with X-gal and the 12/101 antibody, followed by tissue sectioning.

In embryos expressing *XSu(H)^{DBM}*, segmentation of the paraxial mesoderm was severely disrupted (21/21 embryos). As with *X-Delta-2^{tr}*-injected embryos, *XSu(H)^{DBM}* did not appear to affect myogenesis, as indicated by the onset of 12/101 expression within the presomitic mesoderm (Fig. 6B, right side), or the appearance of 12/101 staining on somitic tissue (Fig. 6B, left side). At the very onset of somitogenesis, however, intramyotomal junctions were abnormally formed at ectopic locations, suggesting that the paraxial mesoderm on the side expressing *XSu(H)^{DBM}* RNA failed to form clear segmental boundaries, resulting in an extremely abnormal somite organization at later stages (Fig. 6B, left side). Notably, even though segmentation was severely disturbed, the somitic tissue appeared to have organized cells into groups and form intramyotomal junctions. Thus, the results with *XSu(H)^{DBM}* confirm those obtained with *X-Delta-2^{tr}*, by showing that the Notch signaling pathway is not required for the formation of somitic tissue, but rather for the organization of this tissue into a segmental pattern.

Inhibition of Notch signaling alters the segmental expression of *X-Delta-2* and *Hairy 2A*

The defects in segmentation produced by *X-Delta-2^{tr}* and *XSu(H)^{DBM}* suggest that X-Delta-2, along with X-Notch-1, mediates cell-cell interactions required for segmentation of the paraxial mesoderm. Moreover, the fact that *X-Delta-2* is expressed within the presomitic mesoderm, suggests that it is required for segmentation prior to somitogenesis. Therefore one model consistent with these results is that X-Delta-2 mediates the formation of somitomeres within the paraxial mesoderm. To test this model, we asked whether *X-Delta-2^{tr}* and *XSu(H)^{DBM}* alter the formation of somitomeres as revealed by the expression of X-*Delta-2* or *Hairy 2A*.

Embryos were injected with *X-Su(H)^{DBM}* transcripts and then *X-Delta-2* expression was determined at neurulae stages. As described above, the expression of *X-Delta-2* normally has a posterior limit where it is upregulated, an area of refinement where expression is lost in the posterior part of each somitomere but retained in the anterior part, and then an anterior limit where expression is lost when somites form (Figs 3D, 4A). When embryos express *X-Su(H)^{DBM}*, the anterior and posterior limits of *X-Delta-2* expression did not appear to change (compare Fig. 7B with A). However, *X-Su(H)^{DBM}* blocked the refinement of *X-Delta-2* expression within the somitomeres, thus preventing *X-Delta-2* expression from localizing to the anterior side of each somitomere (Fig. 7B). This effect was observed in 120 out of 123 embryos injected with *X-Su(H)^{DBM}* while 85 out of 87 embryos injected with *nlacZ* RNA appeared normal in terms of *X-Delta-2* expression. Embryos were also analyzed for *X-Delta-2* expression after injection with *X-Delta-2^{tr}* RNA, but this necessitated the use of a short probe to distinguish between the endogenous and injected *X-Delta-2* RNA, resulting in weaker in situ staining. Nonetheless, expression of *X-Delta-2*^{tr} appears to have the same effect on *X-Delta-2* expression as seen in embryos expressing *X-Su(H)^{DBM}*; the expression of *X-Delta-2* appeared to be upregulated normally, but then failed to refine into a segmental pattern of expression (32/32 embryos; Fig. 7C,D). Thus, the results obtained with both *X-Delta-2^{tr}* and X-*Su(H)^{DBM}* are consistent with the idea that Notch signaling is required for the establishment of a segmental prepattern.

To extend these results, we also analyzed the effects of *X-Su(H)^{DBM}* and *X-Delta-2^{tr}* on the expression pattern of *Hairy 2A*. *Hairy 2A* is normally expressed in one or two stripes within the presomitic mesoderm (Fig. 8A,C,E). However, this striped expression is lost in embryos injected with *X-Su(H)^{DBM}* (21/25 embryos; Fig. 8D) or *X-Delta-2^{tr}* RNA (68/75 embryos; Fig. 8F), but not lost in those injected with *nlacZ* RNA (82/83 embryos; Fig. 8B). Thus segmental expression of *Hairy2A* within the presomitic mesoderm also appears to depend on X-Delta-2 and Notch function.

Ectopic expression of *X-Delta-2* also inhibits segmentation

The results described above indicate that X-Delta-2 mediates the formation of a segmental prepattern, during which its expression is refined to the anterior border of each somitomere. To determine if localized expression of *X-Delta-2* is required for segmentation, *X-Delta-2* was expressed ectopically within the paraxial mesoderm by RNA injections. Analysis of these embryos showed myogenic differentiation appeared to be normal as assessed by *cardiac actin* expression (data not shown) and by 12/101 staining (Fig. 6C). However, ectopic expression of *X-Delta-2* also produced defects in segmentation (14/14 embryos), indicating that localized *X-Delta-2* expression is required for the establishment of somitomeres.

DISCUSSION

Although segmentation of the vertebrate embryo is first evident when somites form, Meier and colleagues suggested that the paraxial mesoderm is already prepatterned into segments before somitogenesis by the formation of somitomeres, which they defined on the basis of a subtle but distinct morphology apparent in SEM images (Jacobson and Meier, 1986). The idea of a segmental prepattern is also supported by experiments in which segmentation defects were generated in amphibian embryos using a brief heat shock. Such defects follow heat shock with a delay, during which 4-6 somites form normally, suggesting that heat shock disrupts a component of the segmentation process that acts around the time of somitomere formation (Pearson and Elsdale, 1979). Thus, these observations suggest that segmentation begins with the establishment of somitomeres, during which cells in the paraxial mesoderm gain their segmental identity, presumably via patterning mechanisms that provide such positional information as the location of segmental boundaries or the A-P polarity of each segmental unit. To analyze these mechanisms, we have characterized the expression pattern of *X-Delta-2*, and examined how X-Delta-2 might contribute via Notch signaling to the establishment of a segmental prepattern.

Expression of *X-Delta-2* and *Hairy 2A* mark a segmental prepattern

Expression of *X-Delta-2* is dynamically regulated within the paraxial mesoderm, rapidly evolving into a series of 4-5 stripes which pass wave-like down the axis of the embryo (Fig. 3). The spacing between each stripe corresponds to a prospective somite, suggesting strongly that each stripe of *X-Delta-2* expression demarcates a somitomere. Moreover, the expression of *X-Delta-2* undergoes a refinement within each somitomere, apparently reflecting the establishment of an A-P polarity. Thus, an early broad stripe of *X-Delta-2* expression appears to fill a somitomere, but in a graded fashion along its A-P axis. This graded expression of *X-Delta-2* appears to undergo further refinement, ultimately localizing to the anterior boundary of each somitomere. Importantly, *Hairy2A* expression increases in the posterior half of each somitomere as expression of *X-Delta-2* is lost, suggesting a role for *Hairy 2A* in this refinement process. Finally, expression of *X-Delta-2* appears to be downregulated by the time somites form, suggesting that X-Delta-2 functions prior to somitogenesis.

Inhibition of Notch signaling alters segmentation but not myogenesis

To examine the role of X-Delta-2 in segmentation, we expressed dominant-negative forms of X-Delta-2 and XSu(H) in the paraxial mesoderm. Expression of *X-Delta-2^{tr}* or *X-Su(H)^{DBM}* produced similar segmentation defects, apparently without affecting the timing or extent of myotomal differentiation. In addition, both *X-Delta-2^{tr}* or *X-Su(H)^{DBM}* disrupted the formation of a segmental prepattern within the presomitic mesoderm as

marked by *X-Delta-2* and *Hairy 2A* expression. These antimorphs inhibited the stripes of *Hairy2A* expression, and blocked the refinement of *X-Delta-2* expression within each somitomere. Because two independent approaches that block Notch signaling give similar effects, our contention is that both antimorphs are acting as dominant-negative mutants, as also suggested strongly by the effects of both mutants on lateral inhibition during neurogenesis (Chitnis et al., 1995; Wettstein et al., 1997). Gain-of-function experiments such as the ectopic expression of *X-Delta-2* (Fig. 6) or *X-Su(H)* (data not shown) also give segmentation defects that at the present time cannot be distinguished from those produced by purported loss-of-function experiments. Thus, we cannot show that the phenotypes produced by *X-Delta-2*^{tr} or *X-Su(H)*^{DBM} can be rescued by co-injection of wild-type RNAs. Nonetheless, the simplest interpretation of our data at present is that *X-Delta-2*^{tr} or *X-Su(H)*^{DBM} inhibit X-Delta-2 and X-Notch-1 function, and that this prevents the formation of a segmental prepattern within the presomitic mesoderm.

Model for Notch function during segmentation

How might X-Delta-2 and Notch contribute to the formation of somitomeres? One finding that is likely to bear on this question is that X-Delta-2 appears to regulate its own expression within each somitomere. This regulation is evident from the fact that in embryos expressing either *X-Delta-2*^{tr} or *Su(H)*^{DBM}, the expression of *X-Delta-2* within the somitomeres is not striped but continuous. Rapid changes in expression, particularly via a negative feedback loop, is a characteristic of DSL ligands in other developmental processes. For instance, during development of the hermaphrodite gonad in *C. elegans*, the DSL ligand, lag-2, and the Notch-related receptor, lin-12, determine which of two equipotential cells will give rise to a presumptive anchor cell (AC), versus a presumptive uterine precursor (VU). During this decision, *lag-2* is initially expressed in both cells, and then rapidly changes in expression, by upregulating in the presumptive AC and downregulating in its neighbor, the presumptive VU (Wilkinson et al., 1994). In this example, the dynamic regulation of expression of the DSL ligand is thought to be due to negative feedback between signaling and responding cells: cells whose receptors are activated express less of the DSL ligand, and vice versa. The important developmental consequence of such a mechanism is that by amplifying small differences between cells, it enhances, or even generates de novo, a particular pattern of differentiation among cells that are initially equi-potential, or nearly so. In a similar way, a difference in the expression of *X-Delta-2* along the A-P axis of each somitomere appears to be regulated by a negative-feedback loop. In principle, these local cell-cell interactions could restrict expression of *X-Delta-2* to the anterior end of each somitomere, thereby amplifying an initial difference between cells in the anterior and posterior half of each somitomere.

The consequence of this A-P difference in *X-Delta-2* expression is unclear although it is apparently a requirement for proper segmentation, because segmental defects occur in embryos when *X-Delta-2* expression is de-localized by ectopic expression. One possibility, based on analogy to the role of Notch signaling at the dorsal/ventral boundary of the *Drosophila* imaginal wing disc, is that the differential expression of *X-Delta-2* in the paraxial mesoderm selects cells with special properties at the boundary between each segment (Couso et al., 1995; de Celis et al., 1996; Doherty et al., 1996; Kim et al., 1995). Thus, one possibility is that these cells are not specified properly when *X-Delta-2* expression is not localized within a somitomere, and that this can occur experimentally either by blocking Notch signaling, which is

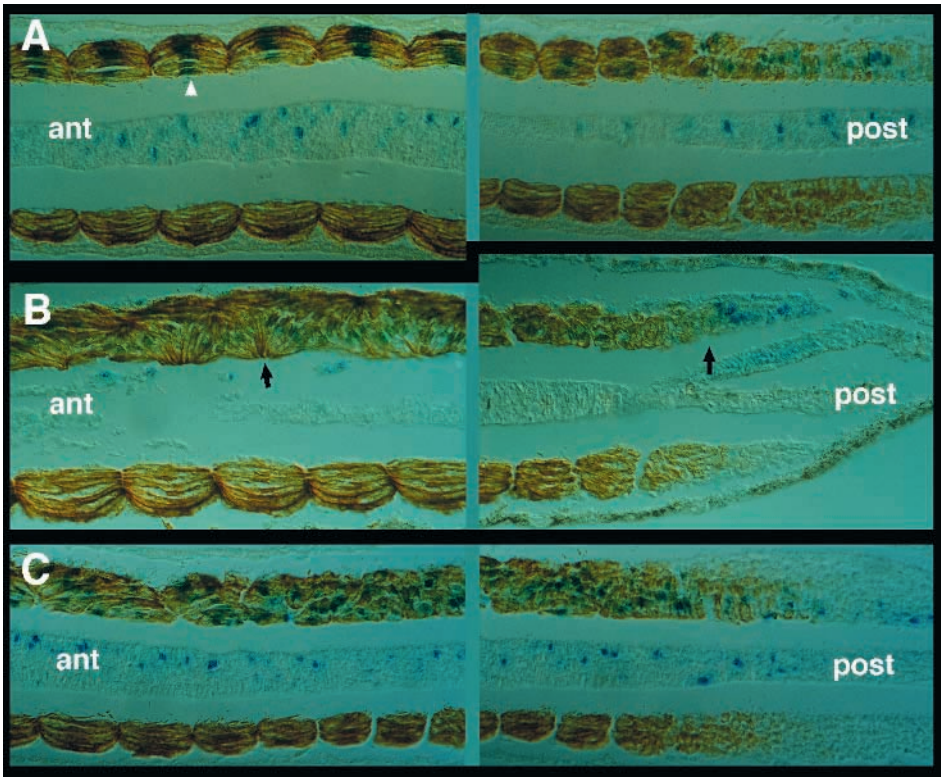

Fig. 6. Expression of *XSu(H)*^{DBM} and *X-Delta-2* alters the pattern of segmentation in *Xenopus* embryos. Embryos were injected on one side with (A) *nlacZ* RNA, (B) a mixture of *nlacZ* and *XSu(H)*^{DBM} RNAs, or (C) a mixture of *nlacZ* and *X-Delta-2* RNAs. Embryos were processed for X-gal and 12/101 staining at early tadpole stages as described in the legend of Fig. 5. In each panel, the left side shows the anterior region of the tissue section where mature somites are located, while the right side shows a posterior view where somites begin to form. (A) Negative control showing the pattern of segmentation is not affected by injection of *nlacZ* RNA. The nuclei within each somite are normally aligned (arrowhead). (B) Embryo injected with *XSu(H)*^{DBM} RNA. Note that the onset of 12/101 expression is normal, as shown in the right panel (arrow). However, as somites begin to form, the arrangement of somitic tissue is chaotic, failing to segment properly as shown in the left panel. Intramyotomal junctions appear to form (arrow in left panel) as shown by dense 12/101 staining, but the position and number of these junctions are abnormal. (C) Embryo injected with RNA encoding *X-Delta-2*.

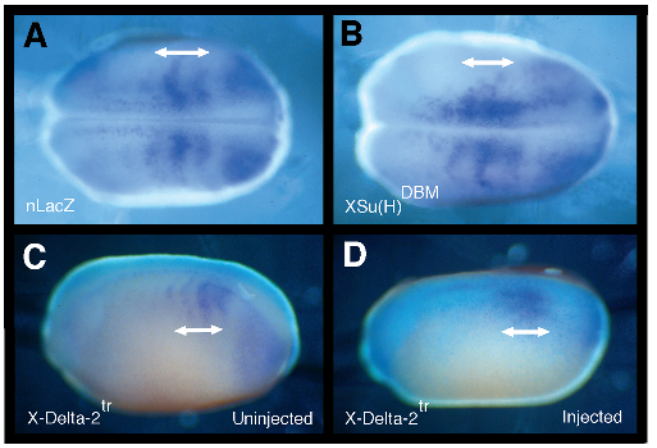

Fig. 7. The segmental prepattern as marked by the expression of *X-Delta-2* is altered by *XSu(H)^{DBM}* and *X-Delta-2^{tr}*. Embryos injected with (A) *nlacZ* RNA, (B) a mixture of *nlacZ* and *XSu(H)^{DBM}* RNAs or (C,D) a mixture of *nlacZ* and *X-Delta-2^{tr}* RNAs. Embryos were fixed at early neurulae stages, stained for both *nlacZ* expression with X-gal (light blue), and for *X-Delta-2* expression using whole-mount, in situ hybridization (dark blue-purple). (A) Dorsal view of an embryos injected with just *nlacZ* RNA, with the injected side oriented to the top of the panel. Note the expression pattern of *X-Delta-2* demarcated by a double arrow is the same on both sides. (B) Dorsal view of an embryo injected with both *nlacZ* and *XSu(H)^{DBM}* RNAs, with the injected side up. Note that on the injected side the expression of *X-Delta-2* (demarcated with a double arrow) is not refined into a segmental prepattern, but is uniform within the somitomeric region. (C,D) Embryo injected with a mixture of *nlacZ* and *X-Delta-2^{tr}* RNAs. C shows a lateral view of the uninjected side, while D shows a lateral view of the injected side of the same embryo. Double arrow indicates the expression domain of *X-Delta-2.*

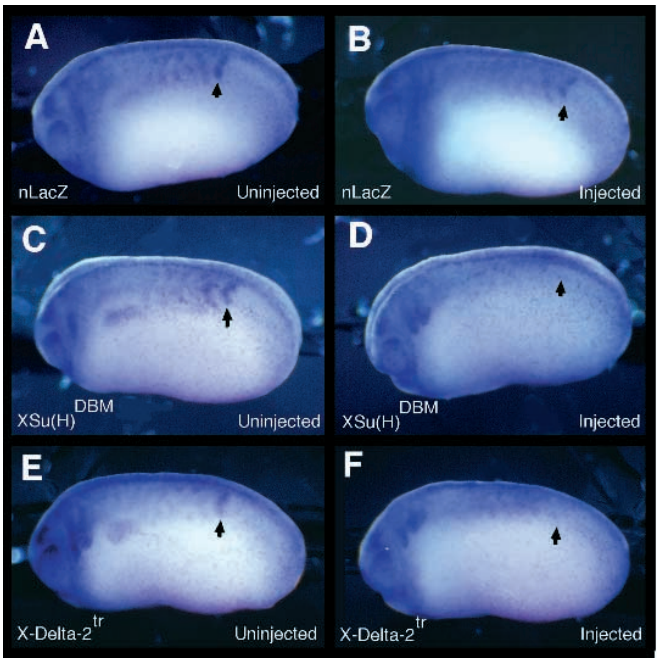

Fig. 8. The segmental prepattern as marked by the expression of *Hairy2A* is altered by *XSu(H)^{DBM}* and *X-Delta-2$^{tr.}$* Embryos injected with (A,B) *nlacZ* RNA, (C,D) a mixture of *nlacZ* and *XSu(H)^{DBM}* RNAs, or (E,F) a mixture of *nlacZ* and *X-Delta-2^{tr}* RNAs. Embryos were fixed at neurulae stages, stained for both *nlacZ* expression with X-gal, and for *Hairy 2A* expression using whole-mount, in situ hybridization. (A,C,E) Lateral views of the uninjected side. (B,D,F) Lateral views of the injected side of the same embryo. Note that *nlacZ* expression alone has no effect on *Hairy2A* expression while expression of *XSu(H)^{DBM}* or *X-Delta-2^{tr}* suppresses the striped expression of *Hairy2A* (arrow).

required for this localization, or by the ectopic expression of *X-Delta-2*.

In sum, our current model for X-Delta-2's role in segmentation is based on the observation that, during other developmental processes, Notch and its ligands often appear to act to refine rather than to initiate a particular patterning event. In this model, a mechanism other than one involving X-Delta-2 generates the asymmetry that is required for forming a repeating, segmental pattern within the paraxial mesoderm. This initial phase of segmentation may involve the use of long-range morphogens as in a reaction-diffusion model, or through the use of the cell cycle (Meinhardt, 1986; Stern et al., 1988). In addition, in zebrafish, a bHLH protein related to *Drosophila* Hairy, called HER-1, which is distinct from Hairy2A, shows a pair-rule like expression pattern within the presomitic mesoderm (Müller et al., 1996), suggesting that the early phases of segmentation may involve a similar use of gap and pair-rule genes as during segmentation of the *Drosophila* embryo (St Johnston and Nüsslein-Volhard, 1992). Whatever the mechanism that initiates the process of segmentation, we imagine that the initial pattern would be imprecise, and that cell-cell interactions mediated by X-Delta-2 would refine this pattern further, by determining for example where segmental boundaries will form or where cells lie along the A-P axis. We suggest that X-Delta-2, by activating the Notch signaling pathway, regulates its own expression via a negative feedback loop involving *Hairy2A*, thus localizing *X-Delta-2* to the anterior boundary of each somitomere.

Segmentation of the vertebrate embryo may involve conserved mechanisms

Somitogenesis involves morphogenetic processes that vary in nature among the different vertebrate species (see Introduction and Fig. 1). Despite these differences, however, the defects in segmentation that we have observed in embryos when Notch signaling is blocked by X-Delta-2^{tr} or XSu(H)^{DBM} are very similar to those that occur in mice which are mutant for Notch or for the mouse homolog of Su(H). Rossant and colleagues have presented a model based on their analysis of the Notch-1 mutant which emphasizes a role for Notch in the process of somitogenesis (Conlon et al., 1995). We suggest that X-Delta-2 and Notch are required in the presomitic mesoderm to establish a segmental prepattern during the formation of somitomeres. Thus, we propose that vertebrates use a conserved mechanism involving Notch for patterning the paraxial mesoderm into segments, but that these segments develop differently during somitogenesis to generate the morphologically diverse segmental structures characteristic of different vertebrate embryos.

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