Functional dissection of the *paired* segmentation gene in *Drosophila* embryos

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An ectopic expression assay in *Drosophila* embryos was used to investigate the roles of pair-rule segmentation genes in the spatial regulation of the segment-polarity gene, *engrailed* (*en*). It is hypothesized that the regions of overlap in expression of two genes, *paired* (*prd*) and *even-skipped* (*eve*), define the odd-numbered *en* expression stripes. Consistent with this combinatorial model, ectopic expression of *prd* caused these *en* stripes to be expanded posteriorly. Surprisingly, however, ectopic expression of a *prd* gene with a deletion of the conserved paired box resulted in loss of these odd-numbered *en* stripes. This dominant negative effect is a phenocopy of *en* expression in *prd*⁻ embryos and suggests that the paired box is necessary for normal *prd* function. A similar deletion of odd-numbered *en* stripes was also observed after ectopic expression of a chimeric *fushi tarazu* (*ftz*) gene containing a substituted *prd* gene homeo box; in addition, in these embryos, the even-numbered *en* stripes were expanded anteriorly, as observed when the unaltered *ftz* gene is ectopically expressed. These effects suggest that the chimeric protein may have DNA or protein targets of both the normal Ftz and Prd proteins.

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Precise and orderly spatial regulation of segmentation genes is critical for coding different developmental pathways in the *Drosophila* embryo. The zygotic segmentation genes are organized in a regulatory cascade (gap genes → pair-rule genes → segment-polarity genes), reflecting the progressive increase in refinement of their spatial patterns of RNA and protein expression (Akam 1987; Ingham 1988). Hence, within the cascade, the gap genes, which are expressed in broad patches, are upstream of the pair-rule genes, which are expressed in striped patterns with two-segment periodicity; these, in turn, are upstream of the segment-polarity genes, which are expressed in stripes with one-segment periodicity. We have focused on the transition in this cascade between the pair-rule and segment-polarity genes, using as a model problem, the regulation of the segment-polarity gene *engrailed* (*en*) by the pair-rule genes *fushi tarazu* (*ftz*), *even-skipped* (*eve*), and *paired* (*prd*).

Two general models have been suggested for the regulation of segment-polarity genes such as *en*. Gergen et al. (1986) were first to suggest that the segment-polarity genes may be regulated by combinatorial mechanisms; that is, specific combinations of pair-rule gene products are required to define the expression stripes of the segment-polarity genes. Combinatorial regulation is supported as a possible mechanism, both by genetic studies indicating that mutations in many pair-rule genes alter the RNA and protein expression patterns of segment-

polarity genes and by the observation that pair-rule genes are expressed in complex arrays of overlapping patterns that in principle could define the segment-polarity gene expression domains (see below). A contrasting model, proposed by Lawrence et al. (1987), emphasizes the importance of the expression boundaries of the pair-rule genes (*ftz* and *eve*) in the specification of the *en* stripes. They observed that in germ-band-elongated embryos, the anterior borders of *lacZ* expression stripes driven by *ftz* or *eve* promoters coincide cell by cell with the anterior borders of *en* stripes in even- and odd-numbered parasegments, respectively, suggesting that *ftz* and *eve* may define the anterior edges of the respective *en* stripes.

Elements of both these models have been incorporated into more recent discussions of *en* (DiNardo and O'Farrell 1987; Ingham et al. 1988; Weir et al. 1988). Figure 1 illustrates our current view of *en* regulation. The essence of this "boundary-combinatorial" model is that the *en* stripes are defined by the overlapping expression of several pair-rule genes. The model proposes that expression of *en* RNA by nuclei in odd-numbered stripes (within odd-numbered parasegments) requires the presence of two positive regulators, the products of *eve* and *prd* (Eve and Prd proteins). Expression of *en* in even-numbered stripes requires the presence of the positive regulator Ftz, and the presence or absence of a positive or negative regulator that is yet to be identified. Consistent with this, loss-of-function mutations in any of these pos-

Figure 1. The regulation of *en*. (A) Boundary specification. Illustrated is the general hypothesis that the boundaries of expression of a gene can be specified by either positive or negative regulators with the same expression boundaries (Weir et al. 1988; Carroll 1990). (B) A boundary-combinatorial model for *en* regulation. This regulatory scheme, based on previous models (DiNardo and O'Farrell 1987; Ingham et al. 1988; Weir et al. 1988), proposes that the anterior and posterior borders of the odd-numbered *en* stripes are defined by *eve* and *prd*, respectively. The anterior borders of the even-numbered *en* stripes are defined by *ftz*; whether the posterior borders of these stripes are defined by a positive or negative regulator is unknown. The relative expression domains of *en* and its regulators support the model (see text and Fig. 2). Also consistent with the model, loss-of-function mutations in *ftz* or *prd* result in the absence of even- or odd-numbered *en* stripes, respectively (Howard and Ingham 1986; DiNardo and O'Farrell 1987; Ingham et al. 1988). In addition to lacking odd-numbered *en* stripes, *eve* mutants also lack even-numbered stripes, probably because the *ftz* expression domains are shifted in *eve* embryos (Harding et al. 1986; MacDonald et al. 1986; Frasch et al. 1988). Because the pair-rule regulators define specific *en* boundaries, these boundaries should be respecified when the pair-rule gene is expressed ectopically. As predicted, ectopic *ftz* causes the anterior border of even-numbered *en* stripes to be respecified, as illustrated (Ish-Horowitz et al. 1989). Ectopic *prd* causes the posterior border of odd-numbered stripes to be shifted posteriorly (this study). Also illustrated are the effects of ectopic expression of two constructs: a *prd* gene with a deletion of the paired box (*hs-prdΔPB*) and a *ftz* gene with a *prd* gene homeo domain (*hs-ftz/prdHD/ftz*).

itive regulators results in the absence of the appropriate *en* stripes as illustrated (Fig. 1). We describe the model as a boundary-combinatorial model because the pair-rule regulators effectively define the boundaries of *en* expression (Fig. 1A). Thus, the postulated spatial relationship between the expression of *en* and its regulators implies that the anterior edges of the odd- and even-numbered *en* stripes are specified by *eve* and *ftz*, respectively, and the posterior edges of odd-numbered stripes are defined by *prd* (Fig. 1B). This is supported by expression studies as described above (also see Results).

In this study we investigated the role of the *prd* gene in the regulation of *en*. As with *ftz* and *eve*, the *prd* gene has a homeo box that is thought to encode DNA-binding activity in the protein product (Laughon and Scott 1984; McGinnis et al. 1984; Hoey and Levine 1988; for review, see Scott et al. 1989). In vitro studies (Treisman et al. 1989) suggest that the binding specificity of the *Prd* homeo domain may depend in part on the identity of the ninth residue of the homeo domain recognition helix (the second helix of the helix-turn-helix). In addition to the homeo domain, the *prd* gene also encodes an evolutionarily conserved domain of 128 amino acids, the paired-box domain, which is also thought to have a DNA-binding activity different from that of the *Prd* homeo domain (Bopp et al. 1986; Burri et al. 1989; Treisman et al. 1991). The functional significance of these putative dual binding activities within the same *Prd* protein is presently unknown: For example, does the combinatorial regulation of *en* rely on both of these binding activities? As a first step in dissecting the in vivo function of the *Prd* protein, we expressed the *prd* gene ectopically using a heat-inducible *hsp70* promoter. This resulted in characteristic changes in embryonic expression of *en* that were consistent with the boundary-combinatorial model (Fig. 1). Because these changes were distinct from those observed after ectopic expression of *ftz*, we decided to use this ectopic expression assay to test two altered versions of *prd* and *ftz*: a *prd* gene with a deletion of the paired box, and a *ftz* gene with a substituted *prd* homeo box. The product of the paired-box deletion gene appeared to have a dominant negative effect on the function of the resident endogenous *prd* gene, providing evidence that the paired box is important for the functioning of the *Drosophila prd* gene. The *ftz-prd* chimera also appeared to have a similar dominant negative effect on *prd* function and also had aspects of *ftz* function. These observations suggest that regions both inside and outside of the homeo domain may be important in the specification of targeting of these homeo domain proteins.

Results

Comparison of expression domains of *en*, *ftz*, and *prd*

As a prelude to the ectopic expression studies, we wished to verify that the normal spatial expression of the *prd* gene is consistent with that predicted by the boundary-combinatorial model for *en* regulation. According to the model, the posterior borders of odd-numbered *en* stripes

should coincide with the posterior borders of *prd* stripes (Fig. 1B). Hence, as illustrated in Figure 2a, superimposition of the expression domains of *prd* and *ftz* should reveal interbands the same width as the *en* interbands in odd-numbered parasegments. To test this prediction, we hybridized embryos with *prd* and *ftz* probes simultaneously. Examination of embryos at gastrulation, the time when the array of *en* expression stripes is first detected (DiNardo et al. 1985; Weir and Kornberg 1985), revealed interbands lacking *prd* and *ftz* expression that were two to three cells wide, the same width as the corresponding *en* interbands (cf. square brackets in Fig. 2a–c; see also Kilchherr et al. 1986). This result is consistent with the hypothesis that *prd* gene-expression boundaries

coincide with and specify the posterior borders of odd-numbered *en* stripes. Supporting this conclusion, direct comparison of En and Prd protein distributions revealed a coincidence of the posterior borders of odd-numbered En stripes and Prd stripes (T. Gutjahr and M. Noll, pers. comm.).

Ectopic expression of *prd*

To further investigate the putative role of the *prd* gene in the regulation of *en*, we constructed germ-line transformant lines containing an extra copy of the *prd* gene under the control of an *hsp70* promoter (Fig. 3A). These transformants were tested for ectopic expression of *prd* by heat-treating embryos in nuclear cycle 14 [170–200 min after egg deposition (AED) at 25°C] for 5 min at 37°C. In situ hybridization with a *prd* probe revealed high levels of RNA expression throughout these embryos at all stages in cycle 14. Significant levels of ectopic *prd* RNA were observed at 5 and 20 min after the end of heat shock (see Fig. 6c, below) and somewhat lower levels were detected after 40 min.

To test whether the introduced *prd* gene had biological activity, we examined the cuticle patterns of larvae that were heat treated during embryogenesis in the latter part of cycle 14 (170–200 min AED at 25°C), the time when the pair-rule genes are thought to regulate spatially the segment-polarity genes. We observed a phenotypic series of cuticle patterns (Fig. 4a–c) that were altered compared with wild type (Fig. 4d), indicating that the introduced *prd* gene has biological activity. Patterns ranged from complete or almost complete deletion of all denticle belts (Fig. 4a) to complete (Fig. 4b) or partial (Fig. 4c) deletion of every other denticle belt. Similar patterns were observed in embryos that were heat treated earlier in cycle 14 (not shown). Comparison of the weaker *hs-prd* phenotypes (Fig. 4c) with weaker *prd*[−] phenotypes (Nüsslein-Volhard and Wieschaus 1980) suggested that the *hs-prd* and *prd*[−] phenotypes are qualitatively different: *hs-prd* embryos with less pronounced deletions have partial deletions of even-numbered abdominal denticle belts, whereas weaker *prd*[−] phenotypes have partial pairwise fusions of even-numbered denticle belts with their anterior neighboring denticle belt. As summarized in Figure 4e, this suggests that in *hs-prd* embryos, the centers of deletions lie over the even-numbered denticle belts; whereas in *prd*[−] embryos, the centers are over the naked cuticle anterior of these denticle belts. Interestingly, analogous partially overlapping deletions were observed in heat shock *ftz* and *ftz*[−] embryos (Struhl 1985).

To assay more directly the regulatory effect of the ectopic *prd*, we examined the patterns of *en* RNA in heat-treated *hs-prd* embryos. Unlike wild-type embryos, which have evenly spaced stripes of approximately equal width (Fig. 5a), the spacing and widths of the *en* stripes in *hs-prd* embryos were reproducibly altered. In germ-band-elongating embryos, the odd-numbered stripes were broader, and the interbands behind these stripes were narrower than in wild type (Fig. 5b–d). This indicates that the posterior borders of the odd-numbered

Figure 2. The definition of *en* interbands in odd parasegments. (a) Summary diagram illustrating the correspondence of expression domains of *en*, *prd*, and *ftz*. (b,c) In both images, regions of darker staining correspond to regions of gene expression (RNA or protein). (b) Immunostaining of En protein (see Karr et al. 1989) in an early gastrula illustrating expression stripes (dark) one to two cells wide separated by interbands two to three cells wide. (c) Whole-mount in situ hybridization with *prd* and *ftz* probes simultaneously reveals interbands the same width as the *en* interbands in odd parasegments (two to three cells wide; brackets in a, b, and c). This is consistent with there being a coincidence of the posterior borders of *prd* and odd-numbered *en* stripes (Fig. 1). b and c are both lateral views of similarly aged embryos shortly after gastrulation, visualized using simultaneous bright-field and DAPI optics. Bar, 25 μ m.

Figure 3. Maps of heat shock constructs. (A) The *hs-prd* construct. Illustrated is the *hs-prd* construct, which consists of a *Hind*III–*Eco*RI fragment of *prd* cDNA (c7340.6; Frigerio et al. 1986) cloned between *hsp70* promoter and polyadenylation sequences. The *prd* sequence includes only 32 bp upstream of the AUG translation start codon, which includes the *prd* cDNA polyadenylation signal (AATAAA) but does not include the *prd* gene polyadenylation site. (B) *hs-prd* Δ PB is similar to *hs-prd* except that DNA encoding 125 of 128 amino acids of the paired-box domain has been deleted. (C) The *hs-ftz/prd*HD/*ftz* construct has an in-frame substitution encoding residues 3–61 of the Prd homeo domain substituting for the equivalent residues of Ftz. The *ftz* sequence includes ~90 bp upstream of the translation start codon and 421 bp downstream of the stop codon, which does not include the *ftz* polyadenylation signal or site. Arrows in B and C illustrate PCR primers used for the constructions. The constructs in B and C include polyadenylation sequences from the *Adh* gene instead of *hsp70*. (PB) Paired box; (HB) homeo box.

stripes are shifted posteriorly, as predicted by the boundary–combinatorial model (see Fig. 1B). In younger embryos just after gastrulation, low levels of extra *en* expression were detected throughout the interbands on the posterior side of the odd-numbered *en* stripes (not shown). The altered *en* patterns were observed at 40 or 60 min after heat shock but were not obvious at 20 min after the end of heat shock. The new patterns were not observed in some of the heat-treated *hs-prd* embryos,

possibly because the period of heat shock was short (5–10 min; Fig. 5). However, longer periods of heat shock were not used in our expression studies because they cause significant disruption of nuclear cycling in both wild-type and *hs-prd* embryos (M. Weir, unpubl.).

In the *hs-prd* embryos, the odd-numbered *en* stripes were often more intense than their even-numbered neighbors (Fig. 5b,c). This is in contrast to expression in wild-type embryos in which the even-numbered stripes develop earlier and are transiently more pronounced than their odd-numbered neighbors (DiNardo et al. 1985; Weir and Kornberg 1985). This result is consistent with observations that *prd* RNA stripes generally develop later than the corresponding *eve* stripes in normal development (L. Waldman and M. Weir, unpubl.). Hence, ectopic expression of *prd* before its normal expression would be expected to result in abnormally early *en* expression as observed.

Our observation that ectopic *prd* causes the posterior borders of odd-numbered *en* stripes to be shifted posteriorly parallels the effects of ectopic *ftz* (Struhl 1985), which causes the anterior borders of even-numbered *en* stripes to be shifted anteriorly (Ish-Horowitz et al. 1989) as predicted. In this case, the expression stripes of the endogenous *ftz* gene are also expanded one to two cells anteriorly; therefore, it is not known whether the expanded *en* expression is caused by the heat-induced or expanded endogenous *ftz* expression. Given that expression of endogenous *ftz* is altered in heat-treated *hs-ftz* embryos, we decided to examine endogenous *prd* expression in *hs-prd* embryos. Because the introduced *prd* gene lacked most of the untranslated leader sequence of the endogenous transcript, we were able to use a 140-bp leader sequence probe to measure specifically the endogenous *prd* expression. At 5, 20, and 40 min after the end of heat shock, endogenous *prd* was expressed in striped patterns similar to those observed in wild-type embryos (cf. Fig. 6a with b). Hence, it is likely that the broadened *en* stripes observed in *hs-prd* embryos are a consequence of regulation by the heat-induced ectopic (Fig. 6c) rather than endogenous (Fig. 6b) *prd* expression.

According to the boundary–combinatorial model (see Fig. 1B), the broadened *en* stripes in heat-treated *hs-prd* embryos would be explained by a broadened region of overlap between *prd* and *eve* expression during specification of the *en* stripes. In normal development *eve* stripes gradually become narrower during cycle 14, reaching a width of about two cells at the time of gastrulation (Harding et al. 1986; Macdonald et al. 1986). Our comparison of *eve* RNA expression in wild-type and *hs-prd* embryos at 20 and 40 min after heat shock revealed similar patterns of *eve* stripes (data not shown), indicating that ectopic *prd* does not affect the *eve* expression at this stage in development. This is consistent with the hypothesis that the expanded overlapping expression of *eve* and *prd* is responsible for the broadened *en* stripes. The combinatorial model also predicts that in the absence of *eve* function, broadened odd-numbered *en* stripes should not be observed. It has been shown previously that in *eve*[−] embryos during germ-band elonga-

Figure 4. Cuticle patterns in *hs-prd* embryos. (a–c) Phenotypic series showing altered cuticle patterns in heat-treated *hs-prd* embryos. (a) Strong phenotype: partial or complete deletion of all denticle belts. (b) Intermediate pair-rule phenotype: complete deletion of even-numbered abdominal denticle belts. (c) Weak phenotype: partial deletion of even-numbered denticle bands. (d) Cuticle pattern in wild-type embryo. Embryos were heat treated for 20 min (a,b) or 10 min (c) starting at 170–200 min AED. Embryos in a and b are from a homozygous infertile line; those in c are from a homozygous fertile line; however, the same range of patterns was observed in both lines. (e) Summary diagram illustrating the partially overlapping deletion domains observed in *hs-prd* and *prd*[−] cuticle patterns. (A1–A4) Abdominal denticle belts. Weaker *hs-prd* or *prd*[−] phenotypes have deletions of only the centers of the deletion domains (darker shading). Hence, weak *hs-prd* embryos lack parts of even-numbered abdominal denticle belts (c), whereas *prd*[−] embryos lack some naked cuticle between odd-numbered and their posterior neighboring denticle belts, resulting in partial fusions of these belts (Nüsslein-Volhard and Wieschaus 1980). Bar, 50 μ m.

tion, there is a deletion of all *en* stripes except for limited expression in the head region (Harding et al. 1986; MacDonald et al. 1986). We observed the same *en* pattern in *hs-prd* embryos homozygous for *eve*^{1.27} (a small chromosomal deletion removing the *eve* gene) fixed at 40 min after heat shock (not shown). This indicates that *eve* function is necessary for the broadened odd-numbered *en* stripes in *hs-prd* embryos. Taken together, these data support a combinatorial role for *prd* and *eve* in the regulation of *en*.

Deletion of the paired box

Because the ectopic expression of *prd* caused characteristic changes in the expression of *en*, we decided to use this *in vivo* assay to test the ectopic function of altered versions of the *prd* gene. To test this approach, we constructed a *prd* gene lacking the conserved paired box, normally located upstream of the *prd* homeo box (*hs-prd* Δ PB; see Fig. 3B). Heat treatment of *hs-prd* Δ PB embryos with two 5-min pulses during cycle 14 resulted in an altered cuticle pattern, suggesting that the *hs-prd* Δ PB gene has biological activity. In embryos with strong phenotypes, much of the naked cuticle between denticle belts was substituted with a reverse-polarity, mirror-symmetric pattern of denticles (Fig. 7a), a phenotype similar to that observed in the segment-polarity mutant *gooseberry* (Fig. 7b; Nüsslein-Volhard and Wieschaus 1980). In *hs-prd* Δ PB embryos with weaker phenotypes, there was less deletion of the naked cuticle between denticle belts and not all denticle belts had mirror-image patterns (not shown). These cuticle phenotypes were observed in two independent transformant lines.

Heat treatment of *hs-prd* Δ PB embryos had an unexpected dominant negative effect on the expression of *en*. The odd-numbered *en* stripes were often partially or completely deleted (Fig. 7c); the even-numbered expression stripes were of normal strength and width. This deletion of odd-numbered *en* stripes is a phenocopy of *en* expression in *prd*[−] embryos. However, the effect of *hs-prd* Δ PB is not caused by an insertion mutation into the *prd* gene, which is on the second chromosome, as one of the two tested transformant lines has an insertion on the third chromosome. Consistent with this, expression of endogenous *prd* RNA was not detectably altered in *hs-prd* Δ PB embryos (not shown). To explain the deletion of odd-numbered *en* stripes in *hs-prd* Δ PB embryos, we suggest instead that the Prd Δ PB protein may poison the function of the endogenous Prd protein (see Discussion).

Swapping homeo boxes

To examine the relative contributions of regions inside and outside the homeo box to the functioning of *ftz* and *prd*, we decided to use ectopic expression to test a chimeric *ftz* gene with a *prd* homeo box. The chimera (*hs-ftz/prdHD/ftz*; see Fig. 3C) was introduced into flies by germ-line transformation. To verify that a chimeric gene had indeed been introduced, we performed *in situ* hybridizations with two probes, a *ftz* probe and a *prd* homeo box probe, and examined RNA levels at 5 min after the end of heat shock treatment. For both probes significant levels of uniform expression were observed, consistent with the ectopic expression of the chimeric gene (not shown).

Figure 5. Expression of *en* RNA in wild-type and *hs-prd* embryos. (a) Wild-type *en* pattern shortly after the beginning of germ-band elongation: *en* is expressed in 14 stripes of equal width separated by interbands of equal width. (b–d) Heat-treated *hs-prd* embryos: *en* expression is altered compared to wild type. The odd-numbered stripes are broader than normal, and the interbands posterior of these stripes are narrower. In younger embryos early in germ-band elongation (b,c), odd-numbered stripes are often more pronounced than their even-numbered neighbors (e.g., stripes 9, 11, 13) (see text). In older embryos further into germ-band elongation (d), the broadening of odd-numbered *en* stripes is most pronounced toward the posterior (stripes 9, 11, 13). Embryos b–d are homozygous for the *hs-prd* insertion. Embryos b and c were heat treated with two 5-min pulses separated by 30 min, and fixed 40 min after the end of the second pulse. Embryo d was heat treated for 5 min and fixed 40 min later. Embryos were photographed with bright-field and DAPI epifluorescence simultaneously. (a,b,d) Lateral views, ventral down; (c) lateral-ventral oblique view. Bar, 50 μ m.

A “pair-rule-like” cuticle phenotype was observed in *hs-ftz/prdHD/ftz* embryos that were heat treated in cycle 14 with two 5-min pulses (Fig. 8a), suggesting that the chimeric gene has biological function. Embryos with a strong pair-rule phenotype had half the normal number of denticle belts, whereas embryos with weaker phenotypes had partial deletions of even-numbered abdominal denticle belts or partial pairwise fusions of even-numbered belts with their more posterior neighbors. These

phenotypes suggest that the regions of cuticle deleted in these embryos included the even-numbered belts and the naked cuticle posterior of these belts (Fig. 8b). This phenotype differs from that of *hs-prd* in that although the same denticle belts were deleted, pairwise fusions were observed in *hs-ftz/prdHD/ftz* but not *hs-prd* embryos, suggesting that the regions of cuticle deleted lie slightly more posteriorly in *hs-ftz/prdHD/ftz* (Fig. 8b). However, the regions of cuticle deleted in *hs-ftz/prdHD/ftz* embryos appear to be the same as those deleted in *hs-ftz* embryos (Fig. 8b; Struhl 1985), consistent with the possibility that the *hs-ftz/prdHD/ftz* chimera has aspects of function of ectopic *ftz*.

The expression of *en* was altered in two ways in heat-treated *hs-ftz/prdHD/ftz* embryos. First, the odd-numbered *en* stripes were completely (Fig. 8c) or partially deleted (Fig. 8d), as observed in *hs-prd* Δ PB embryos. Hence, the Ftz/PrdHD/Ftz chimera, like the Prd Δ PB protein, appears to poison the normal specification of the odd-numbered *en* stripes. A second effect was observed. The even-numbered *en* stripes were expanded anteriorly (Fig. 8d,e), as was observed in *hs-ftz* embryos (see Fig.

Figure 6. Expression of *prd* RNA in wild-type and *hs-prd* embryos. (a) Wild-type expression of *prd* RNA late in cycle 14. Lateral view, ventral down (b). Expression of endogenous *prd* RNA in a homozygous, heat-treated *hs-prd* embryo. Only endogenous *prd* is detected because the DNA probe consisted of 140 bp of leader sequence that was present in the endogenous *prd* RNA but absent from the ectopically expressed *prd* RNA. The pattern of endogenous *prd* appears normal. The embryo was heat treated with two 5-min pulses separated by 30 min and fixed 20 min later (ventral view). (c) General expression of ectopic *prd* RNA at 20 min after the end of 5-min heat treatment (ventral view). Bar, 50 μ m.

Figure 7. Analysis of *hs-prdΔPB* embryos. (a) Cuticle patterns in *hs-prdΔPB* embryos. Illustrated is an embryo in which much of the naked cuticle between each denticle belt was substituted with a mirror-image duplication of the denticle band. This phenotype is similar to that of the segment-polarity mutant *gooseberry*. (b) Summary diagram showing the approximate domains that are substituted with mirror-image duplications of the remaining pattern in *hs-prdΔPB* and *gooseberry*⁻ embryos. (c) Expression of *en* RNA in *hs-prdΔPB* embryos. Ectopic expression of *hs-prdΔPB* caused complete or partial deletion of odd-numbered *en* stripes as shown. Even-numbered *en* stripes were of normal width and strength. Embryos shown here and in Figs. 8 and 9 were heat treated with two 5-min pulses separated by 30 min and either fixed 40 min later for in situ analysis or incubated at 18°C for cuticle preparations. Bars, 50 μm.

1B), suggesting that the chimera may function like the unaltered Ftz protein, despite having a *prd* gene homeo domain.

We observed three classes of *hs-ftz/prdHD/ftz* embryos with respect to deletions of odd-numbered stripes and expansions of even-numbered stripes: (1) deletions alone (Fig. 8c); (2) deletions and expansions (Fig. 8d); and (3) expansions alone (Fig. 8e). Within a given sample of heat-treated *hs-ftz/prdHD/ftz* embryos there was a tendency for the younger embryos to fall in the first class (deletions alone), intermediate ages in the second (deletion and expansion), and older embryos in the third (expansion alone). Because the older embryos did not have deletions, this suggests that the specification of odd-numbered stripes in these embryos may have passed beyond a period of sensitivity to ectopic expression of the chimera.

Given that the chimeric gene caused expansion of even-numbered *en* stripes, just as observed in *hs-ftz* embryos, it was of interest to determine whether the chimera was also able to “autoregulate” the endogenous *ftz* gene. As described earlier, ectopic *ftz* causes the endogenous *ftz* stripes to be expanded approximately one cell anteriorly (Ish-Horowicz et al. 1989). Examination of *ftz*

expression in *hs-ftz/prdHD/ftz* embryos shortly after gastrulation at 40 min after the end of heat treatment revealed that the *ftz* expression stripes are also broader than in wild-type embryos. At this age, *ftz* stripes in wild-type embryos are normally two to three cells wide (Fig. 9a); whereas in *hs-ftz/prdHD/ftz* embryos, the stripes are three to four cells wide, approximately the same width as the interbands (Fig. 9b). This result is consistent with the suggestion that ectopic chimera (*hs-ftz/prdHD/ftz*), as with ectopic *ftz*, is capable of autoregulating the endogenous *ftz* gene (Fig. 9c).

Discussion

Regulation of *en* requires boundary specification and combinatorial regulation

Ectopic expression of the *prd* gene caused the odd-numbered *en* stripes to be expanded posteriorly. This result supports a model for *en* regulation in which the *prd* gene acts in combination with another gene, probably *eve*, to specify the posterior and anterior boundaries, respec-

Figure 8. Analysis of *hs-ftz/prdHD/ftz* embryos. (a) Cuticle patterns in heat-treated *hs-ftz/prdHD/ftz* embryos. A pair-rule-like phenotype was observed with half the normal number of abdominal denticle belts. We observed complete (e.g., A4) or partial (e.g., A2) deletions of even-numbered denticle bands and partial fusions of these bands with their posterior neighboring bands (e.g., A6). (b) Summary diagram of deleted domains. The same domains are deleted in *hs-ftz/prdHD/ftz* and *hs-ftz* embryos. However, the deletion domains in *hs-prd* embryos are a little more anterior. (c–e) *en* expression in *hs-ftz/prdHD/ftz* embryos. Ectopic expression of *hs-ftz/prdHD/ftz* had two effects: expansion of even-numbered *en* stripes and complete or partial deletion of odd-numbered *en* stripes. Three classes of embryos were observed with respect to these two effects: (c) deletions only; (d) deletions and expansions; and (e) expansions only. Bars, 50 μm.

Figure 9. Autoregulation of *ftz* by *hs-ftz/prdHD/ftz*. (a,b) *ftz* RNA in embryos shortly after the beginning of germ-band elongation. (a) Wild-type embryo. *ftz* stripes are two to three cells wide. (b) *hs-ftz/prdHD/ftz* embryo. *ftz* stripes are three to four cells wide, separated by interbands of the same width. Although not demonstrated in this study, it is likely that the broadening of *ftz* stripes is toward the anterior, as has been demonstrated in *hs-ftz* embryos (Ish-Horowicz et al. 1989). (c) Summary diagram illustrating this interpretation of these expression patterns. Bar, 50 μ m.

tively, of each odd-numbered *en* stripe (boundary—combinatorial model; see Fig. 1) According to this model, *en* is activated in those regions where both *prd* and *eve* are expressed. Hence, the ectopic expression of *prd* is expected to cause the regions of overlapping expression of *prd* and *eve* to be expanded (to encompass the *eve* stripes); hence, the regions of *en* expression are also expanded (see Fig. 1). The result supports the importance of both combinatorial regulation and boundary specification in the regulation of *en*.

Deletion of the paired box alters *prd* function

Given that the observed changes in *en* expression in *hs-*

prd embryos are as would be predicted for *prd* function at ectopic sites, this result provides an *in vivo* assay system to dissect which regions of the Prd protein are necessary for *prd* function. In addition to the homeo domain, the Prd protein contains another evolutionarily conserved motif, the paired-box domain, which, as it is conserved, is also expected to have some functional importance. Indeed, a point mutation in the homologous paired box of the mouse *pax-1* gene (the *undulated* mutation) results in characteristic disruption of the mouse vertebral column (Balling et al. 1988). Furthermore, recent *in vitro* DNA-binding studies suggest that the paired-box domain of *Drosophila* Prd may have a DNA-binding activity that is distinct from that of the Prd homeo domain (Treisman et al. 1991).

To test *in vivo* the importance of the paired box in the *Drosophila prd* gene, we constructed a *prd* gene with an in-frame deletion of 125 of 128 amino acids of the paired-box domain. Ectopic expression of this deletion construct (*hs-prd Δ PB*) did not cause expansion of odd-numbered *en* stripes, as observed with ectopic *prd*. Instead, the odd-numbered *en* stripes were completely or partially deleted; the even-numbered *en* stripes were unaffected.

Formally, this result suggests that the Prd Δ PB protein acts as a repressor of *en*. However, it is not a general repressor of *en*, as only the odd-numbered stripes are deleted. These data suggest that the presence of Prd Δ PB is able to poison the specification of only the odd-numbered stripes. As discussed earlier, the specification of odd-numbered stripes is thought to depend on the combination of *prd* and *eve* function. Indeed, the poisoning of odd-numbered stripes by Prd Δ PB mimics the phenotypes of loss-of-function mutants in *prd* and *eve* in which odd-numbered *en* stripes are deleted (Harding et al. 1986; Macdonald et al. 1986; DiNardo and O'Farrell 1987) (*eve* mutants also lack even-numbered *en* stripes, possibly due to indirect effects on *ftz* expression; Frasch et al. 1988). This dominant negative effect of Prd Δ PB occurs despite the presence of wild-type *prd* and *eve* in *hs-prd Δ PB* embryos.

To explain this dominant negative effect, we propose that Prd Δ PB competes for ligands with wild-type Prd, blocking its normal function. However, as Prd Δ PB lacks part of the Prd protein, it may not function successfully and, instead, interferes with and poisons the function of the resident wild-type Prd protein. The Prd Δ PB could poison the binding of Prd either to itself or to other proteins or it may interfere with Prd binding to DNA. Consistent with this interpretation of the dominant negative phenotype, expression of the endogenous *prd* and *eve* transcripts is not detectably altered in heat-treated *hs-prd Δ PB* embryos (not shown). Our observation that deletion of the paired box converts *prd* into a dominant negative regulator provides the first indication that the paired box is important for normal *prd* function in the *Drosophila* embryo. However, it is possible that the paired-box deletion might have caused conformational changes in other parts of the Prd protein that are responsible for the change in *prd* function.

Like the homeo box, the paired box has been postulated to encode a DNA-binding activity (Burri et al. 1989; Treisman et al. 1991). Perhaps the function of the normal Prd protein requires DNA (or protein) targeting by both the homeo domain and paired-box domain of the protein, and the ectopic Prd Δ PB protein does not function successfully because it lacks one of these two binding activities.

Interestingly, ectopic expression of Prd Δ PB resulted in an altered cuticle pattern similar to that observed in *gooseberry*⁻ embryos (Nüsslein-Volhard and Wieschaus 1980). It seems unlikely that the *gooseberry*⁻ phenocopy is an indirect consequence of the poisoning of endogenous *prd*, as the striped expression of *gooseberry* RNA (*BSH9*; Baumgartner et al. 1987) is not detectably diminished or altered in heat-treated *hs-prd Δ PB* embryos (not shown). However, as the *gooseberry* gene contains a paired box and a homeo box encoding very similar protein sequences to those in the homologous domains of the *prd* gene (Bopp et al. 1986), it is possible that in addition to poisoning endogenous *prd*, the ectopic Prd Δ PB may also poison the function of endogenous *gooseberry* by binding its ligands, thereby explaining the *gooseberry*⁻ phenocopy.

A Ftz/Prd chimera also deletes odd-numbered en stripes

The effects of ectopic *ftz* and ectopic *prd* are distinguishable in that ectopic *ftz* broadens even-numbered *en* stripes, whereas ectopic *prd* broadens odd-numbered *en* stripes (see Fig. 1). To determine whether these different ectopic functions depend on regions of the protein inside or outside the homeo domain, we constructed a chimeric gene coding for a Ftz protein with residues 3–61 of the Ftz homeo domain substituted with the equivalent residues of the *prd* gene homeo domain (Ftz/PrdHD/Ftz). Previous homeo domain swapping experiments involving homeotic genes (Kuziora and McGinnis 1989, 1991; Gibson et al. 1990; Mann and Hogness 1990; for review, see Hayashi and Scott 1990) have suggested that the DNA (or possibly protein) targeting of the chimeras depends on regions within the homeo domain, whereas the quality of gene regulation by the chimera (whether it is positive or negative) depends on regions outside the homeo domain.

Ectopic expression of the Ftz/PrdHD/Ftz protein caused partial or complete deletion of odd-numbered *en* stripes, similar to that observed with ectopic Prd Δ PB. Using parallel reasoning to that above, we propose that the Ftz/PrdHD/Ftz chimera, like Prd Δ PB, binds to ligands of the Prd protein and thereby poisons the function of the endogenous *prd* gene. Presumably, the chimera would bind to targets of Prd because it has a Prd homeo domain, but it does not function successfully like ectopic Prd because it lacks the rest of the Prd protein. Hence, this result is consistent with the proposal that regions within the homeo domain are important in determining the specification of targeting of these proteins. This interpretation is supported by the observation that

ectopic Ftz, unlike Ftz/PrdHD/Ftz, does not cause deletion of odd-numbered *en* stripes, presumably because ectopic Ftz does not bind targets of Prd.

Implicit in this interpretation is the idea that within the chimera, the Ftz sequences flanking the Prd homeo domain do not substitute for the absent Prd-flanking sequences. Perhaps, Ftz/PrdHD/Ftz lacks Prd domains necessary for combinatorial mechanisms involving protein–protein or protein–DNA interactions, an idea consistent with our *hs-prd Δ PB* result. Our observation of poisoning as a consequence of putative Prd-like targeting may reflect the complexity of combinatorial regulation of segmentation genes, structural components of which are not simply additive or interchangeable. This is in contrast to the results of previous homeo domain swapping experiments with homeotic genes in which poisoning has not been observed (Kuziora and McGinnis 1989, 1991; Gibson et al. 1990; Mann and Hogness 1990). Instead, the combined heterologous structural domains have been additive in their net functions, with the targeting specificity of the chimera appearing to be that of the homeo domain and the regulation quality (whether positive or negative) reflecting normal regulation of the gene contributing the flanking sequences (Kuziora and McGinnis 1989, 1991).

Our chimera result extends the *hs-prd Δ PB* result to suggest that the Prd homeo domain is sufficient to mediate the observed poisoning effect. Because the homeo domain is thought to have DNA-binding activity, the poisoning could be due to binding to normal DNA targets of Prd. However, it is also possible that the poisoning is a consequence of protein–protein interactions mediated by the Prd homeo domain. Indeed, there is a precedent for this possibility: The homeo domain of Oct-1 (a POU-domain protein) has been shown to bind the herpes simplex virus *trans*-activator, VP16, through residues in the first helix and turn in the helix–turn–helix motif of the Oct-1 homeo domain (Stern et al. 1989). A second case of protein–protein interactions between homeo domain proteins has been documented for two other POU-domain proteins, I-POU and CF1a, although in this case the interaction occurs through the POU domains of both proteins (located upstream of their homeo domains) (Treacy et al. 1991). Interestingly, when these two proteins form a heterodimer, the CF1a protein loses its ability to bind DNA and no longer activates the *Drosophila dopa-decarboxylase* gene. It is an intriguing possibility that this inactivation of one homeo domain protein by another might be analogous to the poisoning effects of Prd Δ PB and Ftz/PrdHD/Ftz.

Ftz/PrdHD/Ftz has ectopic Ftz function

In addition to deleting the odd-numbered *en* stripes, ectopic expression of Ftz/PrdHD/Ftz also caused broadening of even-numbered *en* stripes, as observed when unaltered Ftz protein is ectopically expressed (Ish-Horowitz et al. 1989). This result is rather unexpected because it suggests that the Ftz/PrdHD/Ftz chimera can

bind to normal DNA (or protein) targets of Ftz protein, despite having a Prd homeo domain and despite apparently being able to bind targets of Prd (see above). Although it is formally possible that the chimera binds to targets of the Prd protein, but still manages to provide an ectopic effect such as that of *ftz*, this seems unlikely, especially given that the chimera also autoregulates the endogenous *ftz* gene and causes an altered cuticle pattern as observed with ectopic *ftz*. These results are explained most easily assuming that the chimera binds to targets of the unaltered Ftz protein.

It is conceivable that this ectopic *ftz*-like function does not depend on targeting by the chimera's Prd homeo domain but, instead, depends on protein or DNA interactions by Ftz regions outside the homeo domain. However, it seems more likely that the *ftz*-like effect is mediated by the chimera's Prd homeo domain. Assuming this second interpretation, and given that it is unlikely that the Prd homeo domain in its normal context (the Prd protein) binds to targets of the Ftz protein, the proposal that the Ftz/PrdHD/Ftz chimera can bind these (Ftz) targets suggests that regions outside the homeo domain are important in conferring this targeting property to the chimera's homeo domain. Either the presence of Ftz sequences outside the homeo domain or the absence of Prd flanking sequences might be responsible for this effect. The possible importance in DNA targeting of regions flanking the homeo domain has been suggested previously by structural studies of the homeo domains of *en* (Kissinger et al. 1990) and *Antennapedia* (Otting et al. 1990), which indicate that residues very near the amino-terminal end within the homeo domain make specific contacts with target DNA. This suggested that regions of the protein outside the homeo domain might be important in positioning these residues for correct DNA binding. It is interesting to note that within subfamilies of homeo domain proteins, there is often significant conservation of regions immediately upstream or downstream of the homeo box [e.g., *engrailed/invected* (Coleman et al. 1987), *paired/gooseberry-proximal/gooseberry-distal* (Bopp et al. 1986), *Deformed*-like genes (see Scott et al. 1989)]. Because they are conserved, these regions would be expected to have some functional importance. Furthermore, we note that our Ftz/PrdHD/Ftz chimera was constructed with substitution of only residues 3–61 of the homeo domain, and this chimera apparently retained targeting of Ftz; whereas previous homeo box swaps by others have included the homeo domain plus several residues upstream or downstream of the homeo domain, and these chimeras apparently did not retain targeting specificity normally associated with the homeo domain that was replaced (Kuziora and McGinnis 1989, 1991). However, the significance of this correlation remains to be tested.

In summary, the dual effects of the Ftz/PrdHD/Ftz protein on *en* expression (deletion of odd-numbered stripes and expansion of even-numbered stripes) suggest that the chimeric protein may be able to bind to targets of both the Ftz and Prd proteins. This indicates that sequences both inside and outside of the homeo domain

may be important in determining the specificity of targeting of these homeo domain proteins.

Materials and methods

Construction of *hs-prd* embryos

A *HindIII*–*EcoRI* fragment of *prd* cDNA (c7340.6; Frigerio et al. 1986) was ligated, with *KpnI* linkers, into the *KpnI* site of the vector *pHT4* (Schneuwly et al. 1987). This vector contains both *hsp70* promoter and polyadenylation sequences, allowing for heat-inducible expression of the inserted gene (Fig. 3A). The *pHT4/prd* construct (*hs-prd*) was coinjected (Rubin and Spradling 1982) with the disabled P-element vector *pπ25.7wc* (Laski et al. 1986) into *rosy*⁵⁰⁶ embryos. Three independent *rosy*⁺ transformant lines were obtained, none of which were homozygous fertile. However, mobilization of one of these *hs-prd* insertions to new chromosomal locations with Δ2–3 (Cooley et al. 1988) resulted in several homozygous fertile lines. For our studies we used two transformant lines, one homozygous fertile, and the other a balanced homozygous infertile line; equivalent results were obtained with both lines.

Construction of *hs-ftz/prdHD/ftz* and *hs-prdΔPB* embryos

To replace the *ftz* homeo box with that from the *prd* gene, a *prd* homeo box fragment was generated by polymerase chain reaction (PCR), containing a *HinfI* site at its 5' end and a blunt 3' end (Fig. 3C). The *prd* homeo box fragment was blunt-end ligated to a *ftz* 3'-arm fragment, which was also generated by PCR with *ftz* *HR1.9* as template [a *ftz* genomic clone that includes a substitution of *ftz* cDNA removing the intron (Struhl 1985), and an introduced *HindIII* site ~90 bp upstream of the translation start site (G. Winslow, pers. comm.)]. The ligation product of the two PCR fragments was gel-isolated and ligated to the *ftz* 5' arm (*HindIII*–*HinfI*) fragment of *ftz* *HR1.9*. The complete chimeric gene was subcloned into pBluescript and the PCR-generated regions were sequenced to ensure complete fidelity of the *Taq* polymerase.

Deletion of the paired box was also accomplished by using PCR, making use of an *EagI* site at the 3' end of the paired box (Fig. 3B). PCR was used to generate a fragment extending from the *HindIII* site of *prd* to the 5' end of the paired box, to which an *EagI* site was incorporated. This PCR fragment, which lacked the paired box, was used to replace, by ligation, an equivalent *HindIII*–*EagI* fragment of *prd* cDNA in pGEM-2. The PCR-generated region was then sequenced.

The *HindIII*–*EcoRI* fragment of both the *ftz/prdHD/ftz* chimera and the paired-box deletion were subcloned into the polylinker of the vector *pHSB* (Malicki et al. 1990), which is flanked on one side by the *hsp70* promoter and on the other side by polyadenylation signals from the *Adh* gene. *NotI* fragments from these subclones, containing the modified gene and regulatory sequences on either side, were subcloned into the *NotI* site of the P-element vector *pW8* (Klemenz et al. 1987) that contains the *white*⁺ gene. These constructs were injected with helper plasmid (*pπ25.7wc*) into *Df(1)w^{67c23y}* embryos, and *white*⁺ transformants selected. Seven transformant lines were obtained for the *hs-ftz/prdHD/ftz* chimera, and seven lines for *hs-prdΔPB*. Two homozygous fertile lines for each construct were analyzed for cuticle and expression studies, and equivalent results were obtained with the different insertion sites for each construct.

Identification of mutant embryos

To identify embryos homozygous for *eve*^{1,27}, we used a mutant

stock containing a balancer chromosome (*CyO*) onto which we had jumped a *lacZ* gene driven by a *hunchback* promoter (Driever et al. 1989). Embryos containing this balancer have *lacZ* expression throughout the anterior half of the embryo, a pattern easily distinguished from that of *en*. In situ hybridization was performed with *en* and *lacZ* probes simultaneously, and homozygous *eve*^{1,27} embryos were distinguished by their lack of the *hunchback-lacZ* pattern.

Heat treatment of embryos

Embryos collected (30 min) on agar plates (seeded with glacial acetic acid and yeast) were incubated at 25°C until 10–20 min before heat treatment. Embryos were rinsed from the agar plates with water, rinsed with 0.7% NaCl and 0.04% Triton X-100, dechorionated in 50% Clorox for 2.5 min, and placed in a watch glass with NaCl–Triton solution at 25°C. Heat treatment was performed by submerging the embryos in NaCl–Triton solution at 37°C with either (1) one pulse of 5, 10, or 20 min at 170–200 min AED, or (2) two 5-min pulses, the first at 140–170 min AED and the second 30 min later. After heat treatment, embryos were returned immediately to NaCl–Triton solution at 25°C. Embryos were processed either for cuticle preparations or in situ hybridization (see below). For larval cuticle analysis, embryos were incubated for 30–40 hr after heat treatment, fixed for 1 hr at 60°C in glycerol–glacial acetic acid (1 : 4), and mounted in Hoyer's medium (Wieschaus and Nüsslein-Volhard 1986).

In situ hybridization

Our in situ hybridization method to detect mRNA was based on methods of Tautze and Pfeifle (1989) with modifications according to S. DiNardo, B. Edgar, and C. Oh (pers. comm.). After dechorionation, heat shock treatment, and incubation at 25°C [see above], embryos were fixed for 20 min in 4 ml of heptane, 4 ml of 1.5× PEM with 10% formaldehyde [1× PEM is 100 mM PIPES (pH 6.8), 2 mM EGTA, 1 mM MgCl₂]. Devitellinization was performed according to Mitchison and Sedat (1983) by removing the aqueous layer, adding 6 ml of MetOH, and shaking vigorously for 5 min. After devitellinization, embryos were rinsed three times in MetOH and three times in 95% EtOH, 5 min each. The embryos were stored up to 6 months in EtOH at –20°C.

Fixed embryos stored in EtOH were rinsed for 5 min in MetOH and for 5 min in MetOH–PBT with 4% formaldehyde (PBTf) (1 : 1) and fixed for 20 min in PBTf [PBT is PBS (130 mM NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄) with 0.1% Tween 20]. Rinses and fixes were performed in 5-ml culture tubes with 4.5 ml of solution. After fixing, the embryos were rinsed three times for 5 min in PBT. The embryos were then digested with 50 mg/ml of proteinase K (Boehringer) for 6 min. Digestion was stopped by rinsing twice for 30 sec in PBT with 2 mg/ml of glycine. The embryos were then rinsed twice for 5 min in PBT and postfixed for 20 min in PBTf. The fixative was removed with five rinses, 5 min each, in PBT. The embryos were moved to 1.5-ml silanized Eppendorf tubes for hybridization. After 10 min at 48°C in 100 µl of PBT–hybridization solution (HS) (1 : 1), followed by 100 µl of HS, the embryos were prehybridized for 1 hr in HS (HS is 50% deionized formamide, 5× SSC, 100 µg/ml of sonicated boiled salmon sperm, 100 µg/ml of tRNA, 50 µg/ml of heparin, 0.1% Tween 20, stored at –70°C for ≤60 days). The embryos were hybridized overnight at 48°C in 50 µl of HS with 5–10 ng of the probes.

Probes were prepared from isolated fragments of *en*, *ftz*, *prd*, *eve* and *BSH9* cDNAs. DNA (50–100 ng) and 0.1-µg random primers [pd(N)6, Pharmacia] (14 µl total volume) was denatured at 95°C for 5 min and quick-chilled on ice. Two microliters of

10× Vogel buffer [1 M PIPES (pH 6.8), 50 mM MgCl₂, 100 mM 2-mercaptoethanol], 2.0 µl of deoxynucleotide mix containing digoxigenin–dUTP (Genius kit, Boehringer), and 2 units of Klenow were added. This incorporation reaction was incubated overnight at 14°C and then for 4 hr at room temperature after adding 2 units more of Klenow. The reaction was stopped by adding 0.5 M EDTA to 50 mM and incubating at 65°C for 10 min. The probe was precipitated at –70°C after the addition of 80 µl of H₂O, 50 µg of tRNA, 3 M NaOAc to 300 mM, and 300 µl of EtOH. The probe was resuspended in 50 µl of HS and applied to the embryos after being diluted further in HS (1 : 10), denatured for 3 min at 95°C, and quick-chilled on ice.

After probe hybridization to embryos overnight, excess probe was removed by rinsing at 48°C in 500 µl of HS, 500 µl of HS–PBT (1 : 1), and rinsing five times in 1 ml of PBT (the last three rinses were at room temperature). The embryos were then incubated for 1 hr at room temperature in anti-digoxigenin alkaline phosphatase-conjugated antibodies (Genius kit, Boehringer) in PBT (1 : 2000). The excess antibodies were removed with four washes, 20 min each, in PBT. The embryos were rinsed three times for 5 min in X-phosphate buffer [100 mM Tris (pH 9.5), 100 mM NaCl, 50 mM MgCl₂, 1 mM levamisole, and 0.1% Tween 20]. To the third rinse 4.5 µl of nitroblue tetrazolium (NBT) and 3.5 µl of X-phosphate (Genius kit, Boehringer) were added for color reaction (performed in the dark). The reaction was stopped after 1–40 hr by rinsing three times for 20 min in PBT. The embryos were left at 4°C in PBT for 2–10 days before staining with DAPI (0.5 µg/ml; Sigma) and mounting in Aqua-Poly/Mount (Polysciences).

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