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Multiple regulatory elements direct the complex expression pattern of the *Drosophila* segmentation gene *paired*

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Abstract

The *paired* (*prd*) gene of *Drosophila* belongs to the pair-rule class of segmentation genes involved in establishing the metameric organization of the *Drosophila* body plan. The complex expression pattern of *prd* has previously been shown to depend upon a number of segmentation genes, including gap and pair-rule genes. In an attempt to characterize and analyze the regulatory regions necessary and sufficient for *prd* expression, we have identified an 18-kb genomic fragment, consisting of the transcribed portion of *prd* and 10 kb of 5′- and 5 kb of 3′-flanking region, that is able to rescue *prd* mutant embryos to full viability. Analysis of a series of *prd-lacZ* fusion constructs containing progressively reduced lengths of *prd* 5′-flanking sequences delimits different cis-regulatory regions. The entire 5′-flanking region directs fusion gene expression in a pattern similar, but not identical, to the endogenous prd protein pattern. This 10-kb fragment contains both activator and repressor regions that mediate the establishment of the seven-stripe prd pattern, as well as the splitting into anterior and posterior stripes for the 14-stripe expression phase. The *prd* intron in combination with a minimal upstream region (0.15 kb) is able to direct low levels of *prd-lacZ* fusion gene expression in stripes. Information for expression of the anterior dorsal spot and of the early seven-stripe pattern is located downstream of the *prd* coding region. We propose that regulation of *prd* by pair-rule and gap gene products is mediated by upstream and downstream cis-regulatory elements. Regulation during separate but overlapping phases of expression by separable regulatory regions might be a general characteristic of segmentation genes.

Keywords: Drosophila; Gene regulation; *Paired*; Pair-rule gene; Segmentation

1. Introduction

Extensive genetic and molecular analyses of the fruit fly *Drosophila melanogaster* have identified a variety of regulatory genes that control early embryonic development. Each of these genes is expressed in a spatially and temporally restricted pattern that is tightly controlled. These patterns of gene expression, which evolve before any morphological differentiation of segments is apparent in these embryos, serve as prepatterns (Stern, 1954) that determine the future differentiation of the organism. These regulatory genes — maternally active coordinate genes and the gap, pair-rule and segment-polarity segmentation genes — divide the embryo into increasingly specified regions along the anteroposterior axis of the egg (Nüsslein-Volhard and Wieschaus, 1980). The endpoint of these processes is the establishment of the repeated metameric units — segments and parasegments — that underlie the basic body plan of the fly (for a review see Ingham and Martínez-Arias, 1992). The pair-rule genes are the first of the regulatory genes to be expressed in repetitive patterns in the embryo that preview and determine the repetitive segmental units in the larva and adult. Because each pair-rule gene is necessary for segment formation, considerable effort has gone into understanding the regulation of pair-rule gene expression. Ultimately, an understanding of the mechanisms underlying segmentation will require a dissection of the regulatory pathways governing the expression and function of each of these genes.

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The *paired* (*prd*) gene is one of eight pair-rule genes required to divide the embryo into repeated metameric units (Nüsslein-Volhard and Wieschaus, 1980; Kilchherr et al., 1986). In *prd* mutant embryos, the naked posterior part of even-numbered parasegments and the anterior denticle bands of odd-numbered parasegments are missing. The remaining segments are fused to form composite segments in these approximately half-sized embryos which die prior to hatching (Nüsslein-Volhard and Wieschaus, 1980). As predicted by the network hypothesis (Frigerio et al., 1986; Bopp et al., 1986), the *prd* gene contains several motifs that are conserved in other *Drosophila* regulatory genes: a *prd*-type homeodomain, a paired-domain and a his-pro or PRD repeat. The various domains assort independently in various *Drosophila* genes and have also been found in regulatory genes of other organisms, including zebra fish, frog, mouse and human (for a review, see Noll, 1993). The paired-domain and the *prd*-type homeodomain each bind DNA in a sequence-specific fashion (Czerny et al., 1993; Wilson et al., 1993). The prd protein thus contains two DNA binding domains that interact with different DNA sequences, suggesting the possibility that *prd* encodes a multifunctional transcriptional regulatory protein.

The expression pattern of *prd* has been examined at both RNA (Kilchherr et al., 1986; Baumgartner and Noll, 1990) and protein (Gutjahr et al., 1993) levels. The control of *prd* expression seems to occur at the transcriptional level since the RNA and protein patterns are indistinguishable except for a short lag required for protein synthesis between corresponding patterns. The *prd* gene is expressed in seven stripes by late syncytial blastoderm. These stripes arise differentially along the anteroposterior axis and appear first on the ventral side of the embryo while their extension around the embryo to the dorsal side is delayed. Since this delay is not observed in a subset of prd stripes in embryos mutant for some of the higher pair-rule genes, *prd* expression is controlled by factors that are differentially active along both anteroposterior and dorsoventral axes of the embryo and that interact with one another (Gutjahr et al., 1993). During cellularization and gastrulation, the seven stripes are converted into 14 stripes in a complex process during which an eighth stripe emerges and each of stripes 2–7 is divided into two thinner anterior (A) and posterior (P) stripes by a combination of activation and repression of *prd* expression. In addition to the striped expression, *prd* is expressed in an anterior dorsal spot during cellularization and germ band extension. Later, when the prd stripes have disappeared, prd is re-expressed in the head region and in specific cells of the developing central nervous system (Gutjahr et al., 1993).

Analysis of *prd* expression in embryos carrying mutations in all of the known maternal and segmentation genes has led to a detailed model of the pathways regulating the *prd* expression pattern (Baumgartner and Noll, 1990; Gutjahr et al., 1993). Mutations in any one of the pair-rule genes *eve*, *h*, *run*, *ftz*, *opa* and *odd* each have a dramatic effect on *prd* expression. However, in each case, a relatively normal seven-stripe pattern is established before abnormalities are observed. Only in embryos carrying mutations in the gap genes is the seven-stripe pattern itself disrupted. These observations led to the proposal that prd stripes are initially positioned in broad domains by the action of maternal and gap genes. The pair-rule genes then act to refine the crude seven-stripe pattern and to mediate the transition between the seven-stripe and 14-stripe phases of *prd* expression. In addition, the pair-rule genes appear to interact with dorsoventral factors accounting for the dorsal ventral differences observed during prd-stripe development. This model predicts that the *prd* gene contains cis-acting regulatory elements that mediate responses to gap, pair-rule and, possibly, dorsoventral genes.

To identify the cis-acting regulatory sequences that direct *prd* gene expression, we have analyzed the expression of a series of *prd-lacZ* fusion genes in developing embryos. The starting point for these constructs was an 18-kb genomic region that we show is sufficient to rescue the segmentation defects of *prd* mutant embryos. Multiple elements in the *prd* 5′-flanking region are necessary to direct fusion gene expression in stripes. Independent regions were required for A and P stripe expression, consistent with the regulation of these stripes by different pair-rule gene products. In addition, regions directing expression in the head, central nervous system and anterior dorsal spot were identified. A model for the regulation of various phases of *prd* gene expression by segmentation gene products through discrete cis-regulatory elements is presented.

2. Results

2.1. Expression of the endogenous paired protein

We have previously shown that the *prd* gene is expressed in a complex pattern during embryogenesis (Gutjahr et al., 1993). Initially, prd protein is detected in the anterior region of the embryo (Fig. 1A). This broad expression is soon restricted to a relatively narrow stripe (stripe 1–2) in which prd protein accumulates to almost final levels prior to the onset of cellularization (Fig. 1B). Early during cellularization, prd protein begins to be detectable in broad areas on the ventral side of the embryo. These broad areas soon resolve into individual, bell-shaped stripes (stripes 3–7), which expand in a dorsal direction. Characteristically, stripes 4 and 7 are expressed prior to stripes 3 and 6, while stripe 5 is the last to emerge. At this stage, the anterior stripe 1–2 begins to split on the dorsolateral sides of the embryo into two individual stripes. Subsequently, prd protein rises to

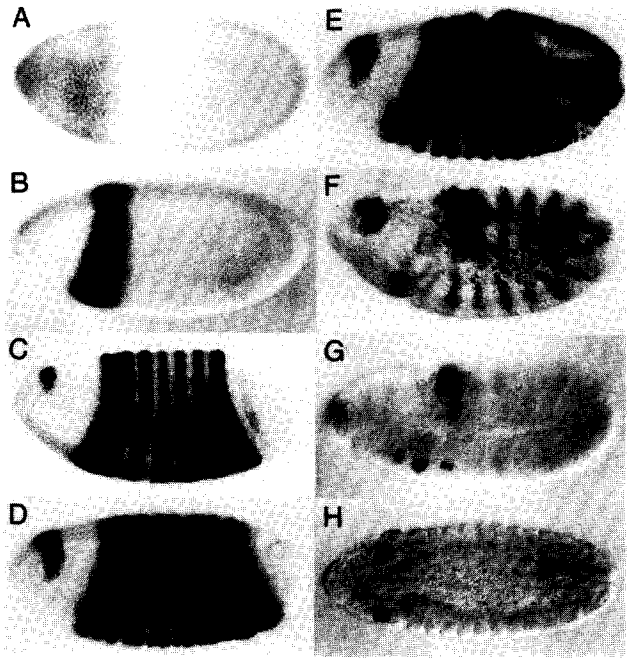

Fig. 1. Key phases in the expression of the endogenous *prd* gene in wild-type *Drosophila* embryos. Whole-mount preparations of wild-type embryos, stained with anti-prd antiserum and oriented with their anterior to the left, are shown at successive stages of embryogenesis, prior to the 13th nuclear division (A), at the onset (B) and mid of cellularization (C), at early (D) and mid-gastrulation (E), at late germ band extension (F), at the end of the extended germ band stage (G) and after germ band retraction (H). All panels show lateral or slightly dorso- or ventrolateral views (dorsal side up), except H which illustrates a ventral view. Panels A and E are from Gutjahr et al. (1993) with permission.

reach equal levels in all stripes by mid-cellularization. At the same time, the preferential accumulation of prd at the posterior margins generates a gradient-like prd distribution within each stripe (Fig. 1C). At gastrulation, prd protein is reduced in the middle of the stripes ('center cells'), whereas their anterior portions begin to accumulate prd protein (Fig. 1D). This 'splitting' into A and P stripes results in a doubling of the number of stripes towards the end of gastrulation (Kilchherr et al., 1986). The only exception is stripe 1 which, by repression of its anterior portion, is reduced in width but does not split. During the second half of cellularization, two additional regions begin to express *prd*, a patch of cells at the anterior dorsal end of the embryo and a posterior eighth stripe (Fig. 1C and D). The appearance of the eighth stripe generates the 14-stripe prd pattern (Fig. 1D and E) and, after the eighth stripe has split, the final 15-stripe pattern (Fig. 1F). Expression in the stripes fades during germ band elongation (Fig. 1F).

At the end of the extended germ band stage, when *prd* expression has decreased to nearly undetectable levels in the stripes, *prd* is re-expressed in the head region, most strongly in the maxillary lobe but also in the labial and mandibular lobes and in the clypeolabrum (Fig. 1G). In addition, prd protein has been detected in the developing central nervous system in two to three specific neurons per hemisegment (Fig. 1H). While prd is observed in the central nervous system only transiently and at low levels, it persists in the head segments until late embryogenesis.

2.2. Rescue of paired mutant phenotype

To test which sequences were necessary and sufficient to rescue the *prd*$^-$ phenotype and as a starting point for the following analysis of cis-regulatory regions, an 18-kb fragment of genomic DNA, consisting of the transcribed region as well as 10 kb of upstream and 5 kb of 3′ flanking sequences of *prd*, was subcloned into a P-element containing transformation vector (see Experimental procedures). This construct was used to establish two independent stable transformant lines. Both lines were tested for the rescue of combinations of the $prd^{2.45}$ null allele, an N-terminal insertion of a 1.1-kb fragment of repetitive DNA (Frigerio et al., 1986), with two different *prd* deficiencies, *Df(2L)Prl* and $Df(2L)prd^{1.7}$. In each case, flies carrying the transheterozygous combination of *prd* null alleles survived to adulthood only in the presence of a copy of the *prd* transgene. These adults gave rise to fertile offspring, suggesting that the *prd* transgene harbors all necessary elements needed for *prd* wild-type function.

This conclusion was confirmed by examining the expression of the *prd* transgene in embryos from $prd^{2.45}/Df(2L)Prl$; $P[prd^+;ry^+]$ ry^{506}/ry^{506} parents. As expected, no staining was detectable in about a quarter of the embryos (not shown), whereas the remaining embryos displayed expression patterns of the *prd* transgene (Fig. 2A–D) that are indistinguishable from those of the endogenous *prd* gene in wild-type embryos (Fig. 1; Gutjahr et al., 1993). In particular, the kinetics with which prd stripes 1–7 (Fig. 2A and B), stripe 8 and the dorsal spot appear (Fig. 2C) is the same as in wild type. Moreover, the narrowing of stripe 1, the splitting of stripes 2–7 (Fig. 2C) and the expression in the head and CNS (Fig. 2D) appear identical to the corresponding patterns of wild-type embryos. Consistent with this wild-type expression of the *prd* transgene, embryos that carry the *prd* transgene show a rescued wild-type (Fig. 2E) rather than *prd*$^-$ cuticular phenotype (Fig. 2F).

2.3. A 10-kb paired 5′-flanking region directs prd-lacZ expression in stripes

Since the 18-kb genomic fragment complemented the cuticular defects of *prd* mutant embryos and expressed the prd protein in patterns indistinguishable from those of wild-type embryos, this region must contain all cis-acting regulatory elements necessary for wild-type *prd* expression. As a first step, we analyzed the expression of *prd-lacZ* directed by 10 kb of *prd* 5′-flanking sequence

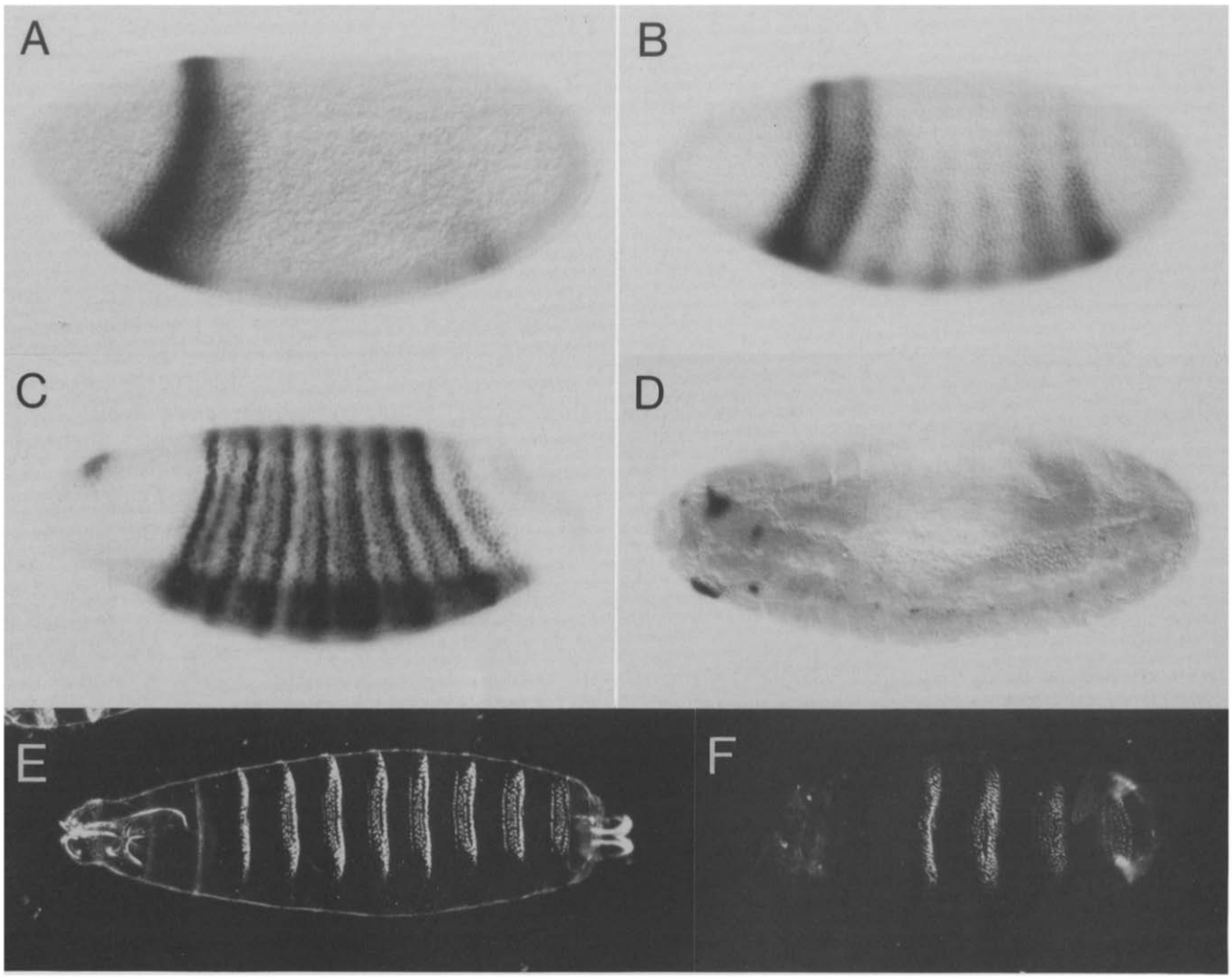

Fig. 2. Wild-type expression of a *prd* transgene in a *prd*⁻ background and rescue of the *prd* cuticular phenotype by the *prd* transgene. Embryos derived from $prd^{2.45}/Df(2L)Prl$; $P[prd^{+}; ry^{+}]$ ry^{506}/ry^{506} parents were stained with affinity purified anti-prd antiserum directed against the carboxy-terminal half of the prd protein (A–D). Whole-mount preparations of embryos carrying the 18-kb *prd* transgene are shown in lateral (A–C) or ventrolateral (D) views (anterior to the left, dorsal side up) at the onset of cellularization (A), during early cellularization (B), at early gastrulation (C) and at the beginning of head involution (D). Cuticle preparations of a rescued (E) and non-rescued *prd*⁻ embryo lacking the *prd* transgene (F) are shown as ventral views (anterior to the left) under dark-field illumination. Cuticles were prepared essentially as described (Wieschaus and Nüsslein-Volhard, 1986).

(*prdΔC* in Fig. 3). As shown in Fig. 4, fusion gene expression is monitored and compared to the patterns of the endogenous prd protein (Fig. 4A) at syncytial blastoderm (sb), gastrulation (g) and during germ band elongation (gbe). Although the fusion gene *prdΔC* is expressed in a pattern similar to that of the endogenous prd protein, a number of alterations are apparent (Fig. 4B). Most prominently, the anterior dorsal spot is completely missing. During gastrulation and germ band extension, *prdΔC* appears to be expressed in seven narrow rather than in 14 stripes and stripe 8 fails to emerge. This abnormal pattern results from the failure of *prd-lacZ* accumulation in the anterior portions of stripes 2–8 (A stripes). Furthermore, the initial stages of *prdΔC* expression at the onset of cellularization are altered in that stripe 1–2 is expressed at much lower levels, and stripes 3–7 emerge in a different order. Stripes 3–7 of the endogenous protein appear in the order of 7 and 4, followed by 6 and 3 and finally 5, whereas stripe 5 of *prdΔC* (and of *prd-lacZ* constructs with shorter *prd* 5′ regions) arises before stripes 3, 4, 6 and 7 (Fig. 4B). Another significant difference from the endogenous prd pattern is that the intensities of stripes 3–7 of *prdΔC* are clearly below that of stripe 1 at gastrulation.

We conclude that the 5 kb of 3′-flanking region that are missing in *prdΔC* but present in the *prd*-rescue construct contain cis-regulatory elements required for the early expression of *prd* in stripe 1–2 as well as the subse-

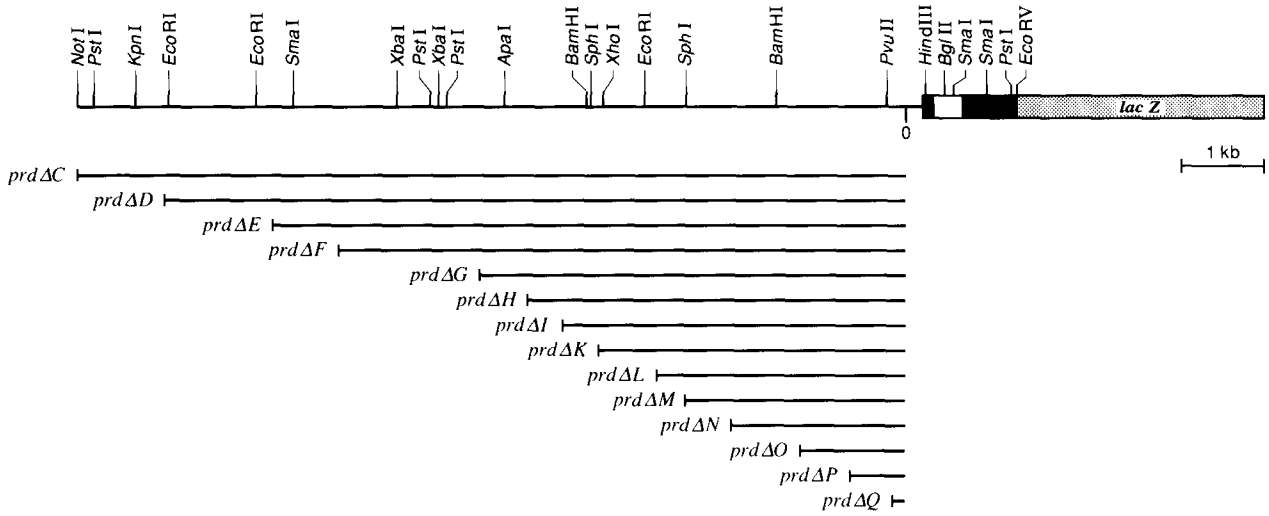

Fig. 3. Summary of *prd-lacZ* fusion constructs used for identification of *prd* upstream cis-regulatory elements. All *prd-lacZ* fusion genes, examined for *prd-lacZ* expression under the control of different lengths of the *prd* upstream region in transgenic embryos, are illustrated schematically. The constructs consist of decreasing lengths of the *prd* upstream region (between 10.0 kb of *prdΔC* and 0.15 kb of *prdΔQ*), the *prd* leader, intron (open box) and coding sequences (filled boxes), including the N-terminal paired-domain and beginning of the homeodomain (up to the *Eco*RV site), fused in frame to the *lacZ* reporter gene (stippled). For a detailed description of constructs and determination of the *prd* transcriptional start site, marked by 0, see Experimental procedures.

quent *prd* activation in the anterior dorsal spot and in the A stripes ('stripe-1–2 enhancer', 'dorsal-spot enhancer', and 'A-stripe enhancer'). Clearly, proper early activation of stripes 1–8 is impaired in the absence of the 3′-flanking region.

2.4. Analysis of 5′-deletion constructs

To analyze the *prd* upstream regulatory region in greater detail, a series of 5′ deletions of *prd-lacZ* fusion genes was constructed (Fig. 3) whose expression was studied in vivo (Fig. 4). A summary of the regulatory elements identified in this analysis is shown in Fig. 5. As shown in Fig. 4C, striking changes occcur in the *prd-lacZ* expression pattern as DNA is progressively deleted from −10 kb to −7 kb. Beginning with *prdΔD* (−9.0 kb) and continuing with *prdΔE* (−7.7 kb) (not shown), the A stripes and stripe 8 reappear in parallel until they reach similar levels as the P stripes in *prdΔF* (−6.9 kb) (Fig. 4C). Thus expression of *prd-lacZ* more closely resembles that of the endogenous prd protein in A and P stripes at gastrulation (Fig. 4C, g and gbe). These observations suggest that the *prd* 5′ region between −10.0 kb and −6.9 kb harbors an 'A-stripe repressor element' which is also responsible for the repression of stripe 8. The activity of this repressor element appears to be masked in the context of the endogenous *prd* gene and of the *prd* transgene which contains 5 kb of 3′ flanking sequence. We therefore assign an 'anti-A stripe repressor' to this region (see Fig. 5).

Further deletion of a region from −6.9 kb to −5.2 kb (*prdΔG*; data not shown) results in a significant decrease of the posterior portion of stripe 2 (2P) expression, suggesting that a stripe-2P specific element is located in this region ('stripe-2P enhancer').

The next major change occurs when the *prd* upstream region is reduced from −4.6 kb (*prdΔH*) to −4.2 kb (*prdΔI*). This evokes a drastic reduction of stripe 1 expression, which is first evident during syncytial blastoderm and persists throughout gastrulation and germ band extension (Fig. 4D). Thus, the 0.4-kb region between *prdΔH* and *prdΔI* contains sequences necessary for high levels of *prd* expression in stripe 1 ('stripe-1 enhancer').

Deletion of an additional 0.5 kb does not have any further dramatic effect on the pattern, whereas deletion of the next 0.7 kb (−3.7 kb to −3.0 kb, *prdΔK–prdΔL*) results in a reversal of the intensities of the A and P stripes, with the P stripes being significantly weaker than the A stripes (Fig. 4E, gbe). Further deletion of sequences between −2.7 kb and −2.1 kb (*prdΔM–prdΔN*) completely abolishes the P stripes during germ band extension (Fig. 4F, gbe). Hence, these regions contain elements required for high levels of *prd* expression in the P stripes ('P stripe enhancer').

For fusion gene *prdΔN*, expression of stripe 1 is no longer detectable (Fig. 4F, g and gbe), defining a second 'stripe-1 enhancer' between −2.7 kb and −2.1 kb. In fusion gene *prdΔO*, the A stripes were relatively broad because of the additional residual expression of *prd-lacZ* in the 'center cells' (data not shown, but cf. *prdΔQ* in Fig. 4G, gbe). This result suggests the presence of a 'center cell repressor element' between −2.1 kb and −1.3 kb.

Finally, the shortest fusion gene tested, *prdΔQ*, which contains 0.15 kb upstream of the *prd* transcription start

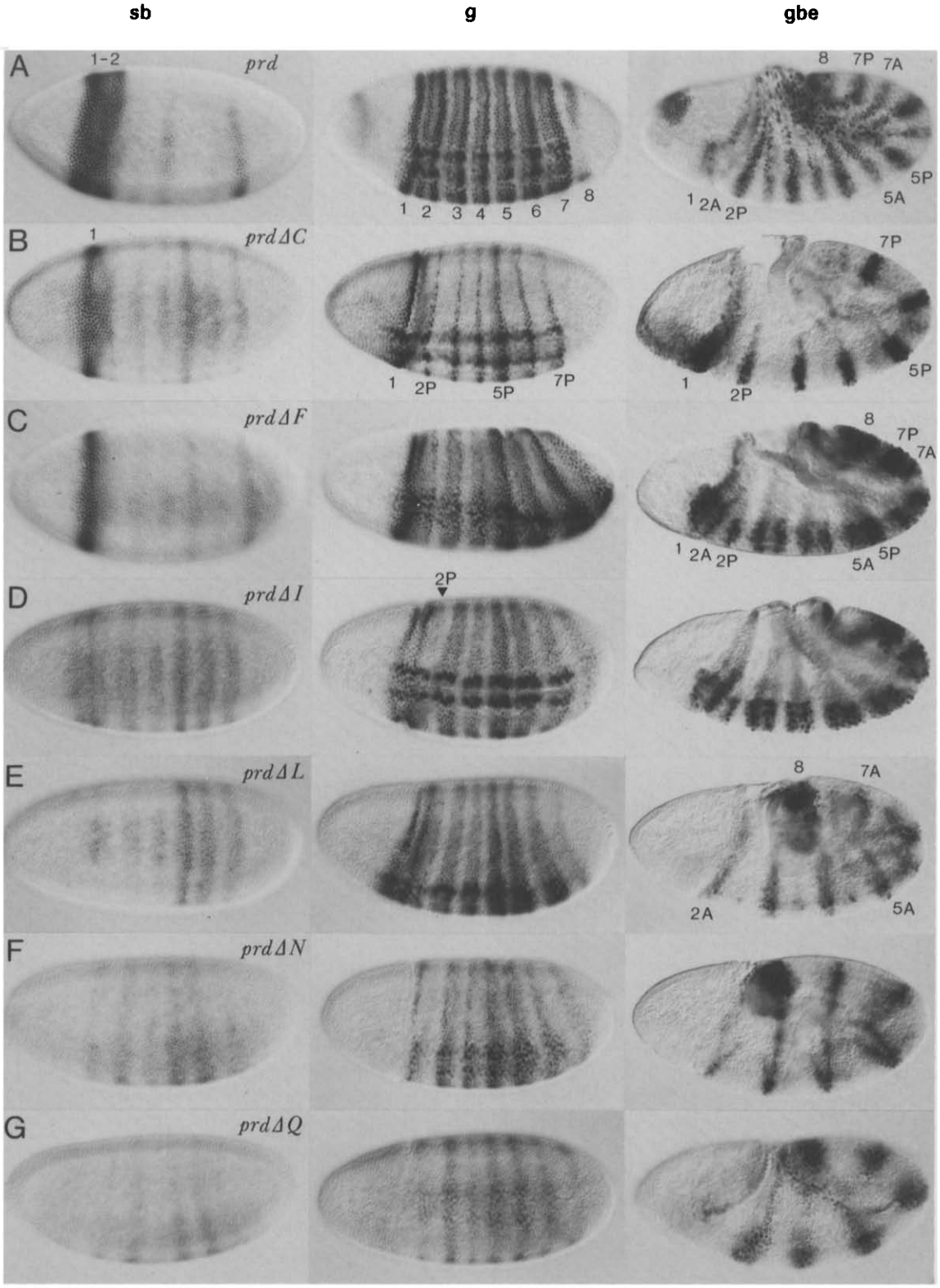

Fig. 4. Expression of *prd-lacZ* under the control of various lengths of *prd* upstream sequences. The expression patterns of *prd* (A) and of selected *prd-lacZ* fusion genes (B–G), detected immunohistochemically by the use of anti-prd or anti-β-galactosidase antibodies, are shown in whole-mount embryos at syncytial blastoderm (sb: left panels), gastrulation (g: middle panels) and germ band extension (gbe: right panels): *prd* (A), *prdΔC* (B), *prdΔF* (C), *prdΔI* (D), *prdΔL* (E), *prdΔN* (F) and *prdΔQ* (G). Embryos, oriented with their anterior to the left, are shown as lateral (gbe panels), ventrolateral (sb panels of A, F and G; and g panels of A–C, E and F) or ventral views (sb panels of B–E; and g panels of D and G). For convenience, some of the expression stripes are labeled.

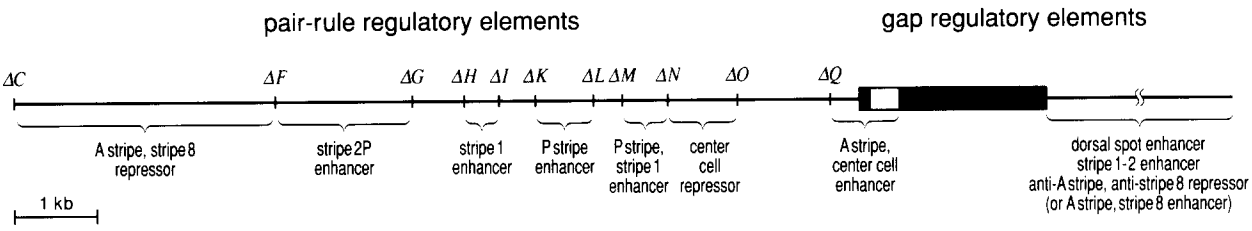

Fig. 5. A model for the interaction of *prd* cis-regulatory elements with segmentation gene products. A schematic depiction of the 18-kb genomic region including the entire *prd* gene is shown. Cis-regulatory elements identified in this study are indicated below the upstream and downstream regions. They are defined as minimal regions shown to be necessary for expression in a particular pattern by the 5′-deletion analysis and their limits are labeled with the corresponding *prd-lacZ* fusion gene constructs (Δ*C*–Δ*Q*, cf. Fig. 3). According to a model derived from detailed analyses of *prd* expression patterns in segmentation mutants (Baumgartner and Noll, 1990; Gutjahr et al., 1993), upstream cis-regulatory elements and probably the intron are predicted to interact with pair-rule proteins, whereas downstream cis-regulatory elements (and perhaps elements of the *prd*ΔQ construct) are expected to bind gap gene products and, possibly, run protein. For a detailed explanation see text.

site as well as the *prd* intron, is still expressed weakly in stripes 2–8 at early stages (Fig. 4G). During germ band extension, these seven stripes are wider than the endogenous prd stripes since this fusion gene is expressed both in A cells and in the center cells. Thus, the promoter proximal 0.15 kb and the *prd* intron contain sufficient information to direct *prd* expression in stripes at the blastoderm stage and hence include an 'A-stripe and center-cell enhancer' or '*prd* zebra element'.

In summary, the results of the progressive 5′ deletions suggest the presence of the following cis-regulatory elements: (i) an 'A-stripe and stripe-8 repressor element' between −10 kb and −6.9 kb; (ii) a 'stripe-2P enhancer' between −6.9 kb and −5.2 kb; (iii) a 'stripe-1 enhancer' between −4.6 kb and −4.2 kb; (iv) elements between −3.7 kb and −3.0 kb and between −2.7 kb and −2.1 kb necessary to direct P-stripe expression ('P-stripe enhancer'); (v) a second 'stripe-1 enhancer' between −2.7 kb and −2.1 kb; (vi) element(s) between −2.1 kb and −1.3 kb that mediate repression of *prd* in the 'center cells' during gastrulation and germ band extension ('center-cell repressor element'); and (vii) a '*prd* zebra element' delimited by a region between −0.15 kb and 1.34 kb that includes the *prd* intron (0.331 kb–0.686 kb).

Many of the observed effects are gradual, such as the reappearance of A stripes and stripe 8 when reducing the upstream region from −10 kb to −7 kb, suggesting that enhancers and repressors consist of several individual elements.

3. Discussion

The *prd* gene is expressed in a complex pattern during embryogenesis. Comparison of RNA and protein patterns of *prd* indicates that they are regulated entirely at the transcriptional level (Kilchherr et al., 1986; Gutjahr et al., 1993). We have rescued *prd* null mutants with an 18-kb genomic fragment including 10 kb of upstream and 5 kb of downstream flanking sequences of *prd*, which activate the *prd* transgene in the same pattern as the endogenous *prd* gene and thus comprise its entire cis-regulatory region. In order to delimit cis-regulators within the 10 kb of 5′-flanking region that activate different pattern elements of *prd* expression, we have examined the expression of a series of *prd-lacZ* fusion genes containing different 5′-flanking regions as well as the intron of *prd*. Our results indicate that 10 kb of the *prd* 5′-flanking sequence include most of the information necessary for *prd* expression in stripes, in the head and in the developing CNS.

Fig. 5 illustrates all identified *prd* cis-regulatory elements. A region including 0.15 kb of upstream sequences and the intron of *prd* may be defined as '*prd* zebra element', which directs *prd-lacZ* expression in seven broad stripes (2–8) consisting of A stripes and center cells. The next region, between −1.3 kb and −2.1 kb, contains a 'center-cell repressor' element, necessary for the splitting of A and P stripes during gastrulation. High levels of stripe 1 expression during syncytial blastoderm and gastrulation depends on two regions, a distal (−4.6 kb to −4.2 kb) and a more proximal (−2.7 kb to −2.1 kb) 'stripe-1 enhancer'.

Deletion analysis further identified two regions (−3.7 kb to −3.0 kb and −2.7 kb to −2.1 kb) that direct P-stripe expression ('P-stripe enhancers') as well as a region (−6.9 kb to −5.2 kb) required for high levels of stripe-2P expression ('stripe-2P enhancer'). Interestingly, these regions interdigitate with the stripe-1 enhancers (Fig. 5).

At the 5′ end of the *prd* upstream region (−10.0 kb to −6.9 kb), there is a sequence that represses expression in the A stripes and in stripe 8 ('A-stripe repressor'). In the endogenous *prd* gene, this repression is overridden by elements that are probably located within its 3′-downstream region. In addition, this 5-kb downstream region activates expression in the anterior dorsal spot ('dorsal-spot enhancer'), enhances the early expression of stripe 1–2 ('stripe-1–2 enhancer') and is required for proper early activation of the remaining stripes.

3.1. A model for paired regulation

Analysis of *prd* expression in embryos carrying mutations in all of the known maternal and segmentation genes has led to a detailed model of the pathways regulating the temporal and spatial patterns of prd protein (Baumgartner and Noll, 1990; Gutjahr et al., 1993). Mutations in any one of the pair-rule genes *h*, *eve*, *ftz*, *odd*, *opa* and *run* have a dramatic effect on *prd* expression. However, in each case, a relatively normal seven-stripe prd pattern is established before abnormalities are observed. Only in embryos carrying mutations in the gap genes is the seven-stripe pattern itself disrupted. These observations led to the proposal that prd stripes are initially positioned in broad domains by the action of maternal and gap genes. The pair-rule genes then act to refine the crude seven-stripe pattern and to mediate the transition between the seven and 14-stripe phases of *prd* expression (Baumgartner and Noll, 1990). The pair-rule genes *eve*, *h* and *run* are required to sharpen and modulate the initial seven stripes. Mutations in *odd*, *opa* or *run* lead to a loss of repression in the two center cells of the seven stripes that are required to generate 14 stripes. This suggests that each gene is required to decrease expression levels in the center cells of stripes 2–8. Four genes are required to maintain and/or increase expression levels in the A and P portions of the split stripes: *opa* is necessary for maintenance of high expression levels in the A stripes; *ftz*, *h* and *eve* maintain expression in the P stripes. This model predicts that the *prd* gene contains cis-regulatory elements that mediate the response to gap gene products and to the positive and negative regulation by several pair-rule genes.

Our analysis of the cis-regulatory elements directing *prd* expression fits well with the predictions of this model and allow us to assign putative regulatory elements through which trans-acting factors mediate their effects. First, the *prd* zebra element activates *prd* in the A portions of stripes 2–8 and hence appears to contain sites that respond to or interact with the *opa* product (Fig. 5). An independent regulatory element appears to be necessary for the splitting of A and P stripes, the center-cell repressor. This observation fits well with the genetic studies that defined three pair-rule gene products — opa, odd and run — as negative regulators of *prd* expression specifically in these cells. Thus, this 0.8-kb region between −1.3 kb and −2.1 kb is likely to contain one or more element(s) that are direct targets of at least some of these genes, or a complex thereof. A simple explanation would be if occupation of the center cell repressor with odd protein inhibited activation by opa via the zebra element or A-stripe enhancer while the effect of *run* is indirect (Baumgartner and Noll, 1990). It is important to note that our 5′-deletion analysis only suggests that the 0.8-kb center cell repressor is necessary, not sufficient, to inhibit activation by opa. It is possible that additional sites between −0.15 kb and −1.3 kb need to be occupied by odd for neutralizing opa and the A-stripe enhancer and that the center-cell repressor indeed extends further downstream than indicated in Fig. 5. Similarly, the A-stripe enhancer might extend upstream beyond −0.15 kb.

Upstream of the center-cell repressor and the A-stripe enhancer, there are enhancers for stripe 1, stripe 2P and the remaining P stripes (Fig. 5). The only gene product found to be specifically involved in P-stripe expression was the pair-rule gene *ftz*. However, expression of *prd* RNA was lost only in the anterior cell of the two-cell-wide P stripes in *ftz* mutant embryos. Thus, an additional gene product must be involved in the regulation of the posterior cell of the P stripes via either one or both of the identified P-stripe enhancers. Since the activation of all P stripes, including stripe 1, also depends on *eve* (Baumgartner and Noll, 1990; Gutjahr et al., 1993), these enhancers probably all respond to the *eve* product, at least indirectly. The *lacZ* patterns of the 5′-deletion constructs could easily be explained by such a model in that an increasing number of eve-binding elements are removed by the progressive deletions. According to this model, activation of stripe 2P would require more eve-response elements than that of stripe 1 or the other P stripes. Most of these eve-responsive enhancers are expected to be located within two relatively short regions of 0.4 kb ('distal stripe-1 enhancer') and 0.7 kb ('distal P-stripe enhancer'; Fig. 5).

Surprisingly, activation of the A stripes does not occur in the presence of the entire 10-kb upstream region and the intron, implying that the region between −6.9 kb (*prdΔF*) and −10.0 kb (*prdΔC*) contains an A-stripe repressor that overrides the A-stripe enhancer. The nature of the product(s) interacting with the A-stripe repressor is unclear. As low levels of odd protein are present in the regions of A stripes (Manoukian and Krause, 1993), it is conceivable that the A-stripe repressor consists of additional odd-binding sites which, if occupied with odd protein, inhibit activation by opa and the A-stripe enhancer. Since the A-stripe repressor is masked in wild-type *prd* genes, it must itself be antagonized by another A-stripe enhancer or by an anti-A-stripe repressor element within the 5-kb downstream region of *prd* (Fig. 5). As *run* is required for the activation of stripe 8 but not of the remaining A stripes (Baumgartner and Noll, 1990; Gutjahr et al., 1993), this enhancer or anti-repressor might consist of two parts separately regulating stripe 8 and the A stripes.

Although *eve* is required for the early activation of stripe 1, stripe-1 activation does not depend entirely on eve but, initially, also on the gap gene *giant* (*gt*) (Gutjahr et al., 1993). Since stripe 1 is not properly activated by the 10-kb upstream region of *prd*, a stripe-1 enhancer that responds to *gt* would have to be located in the 5-kb downstream region of *prd*. Similarly, the 10-kb upstream region and the intron fail to generate the cor-

rect pattern of the initial *prd* activation. Since this initial activation depends on gap gene products — such as on those of *hb*, *Kr*, *kni* and *gt* (Gutjahr et al., 1993) — and possibly on maternal proteins, additional enhancers that bind gap proteins (and maternal proteins) are located within the 5-kb downstream region of *prd*. Finally, this region is expected to contain enhancers for *prd* expression in the anterior-dorsal spot (Fig. 5).

It thus appears that cis-regulatory elements interacting with pair-rule proteins are located in the intron and upstream region of *prd* whereas gap proteins bind to elements well separated from the pair-rule control region in the *prd* 3′-flanking region. It is probably important that the two phases of *prd* regulation, its initial activation by gap gene products and subsequent control by pair-rule proteins, overlap in time and space to ensure its continued expression. Therefore, separate rather than interspersed cis-regulatory regions regulating the two phases of *prd* expression would avoid interference during the time of phase transition and have a selective advantage during evolution. An analogous argument has been proposed to explain that the *gsb* cis-regulatory elements GEE and GLE, activating *gsb* in response to pair-rule and segment-polarity gene products, are separable (Li et al., 1993). Thus, separation of control elements responding to products from different classes of segmentation genes might be a general property of segmentation genes.

4. Experimental procedures

4.1. Determination of paired transcriptional start site

The transcriptional start site of *prd* was determined by applying the RACE technique (Frohman, 1990) to 1 μg of poly(A)$^+$ RNA from 0–4-h-old embryos, using as gene-specific primers 5′-ATATCCATTGAAGAAGG-3′ and 5′-CATAGTTTCTGGAGGAG-3′ (301–317 bp and 250–266 bp from 5′ end of *prd* transcript). This method maps the *prd* transcriptional start site to position 234 of the published genomic sequence of *prd* (Frigerio et al., 1986).

4.2. Rescue of paired mutants

The construct for *prd* rescue (*prd*-SN20-rescue) was obtained by subcloning the 18-kb genomic *Not*I-*Sal*I fragment of the cosmid D7.3#2 into the corresponding sites of the P-element vector pHZ50pL (Hiromi and Gehring, 1987), which is derived from the Carnegie 20 vector and thus carries the ry^+ gene (Rubin and Spradling, 1983). The subcloning step removes all promoter elements and the *lacZ*-coding sequence from pHZ50pL, leaving only the heat shock trailer, the *ry* eye-color marker and P-element borders. After germ line transformation of ry^{506} embryos, two independent lines, $P[ry^+; prd^+]$, carrying the rescue construct on the first or third chromosome were established. Rescue of prd^- embryos was demonstrated by crossing $Df(2L)prd^{1.7}$, $b\ Adh^{n2}\ pr\ cn\ sca/SM1$; $P[ry^+;prd^+]$ with $prd^{2.45}\ b\ Adh^{n2}\ pr\ cn\ sca/SM1$; ry^{506} flies to obtain rescued $Df(2L)prd^{1.7}$, $b\ Adh^{n2}\ pr\ cn\ sca/prd^{2.45}\ b\ Adh^{n2}\ pr\ cn\ sca$; $P[ry^+;prd^+]/ry^{506}$ offspring. To improve the genetic background and establish a rescued stock, such males were crossed with $Df(2L)Prl/CyO;ry^{506}$ virgins and their $Df(2L)Prl/prd^{2.45}\ b\ Adh^{n2}\ pr\ cn\ sca$; $P[ry^+;\ prd^+]/ry^{506}$ offspring crossed *inter se*.

4.3. Construction of the 5′-deletion series of the paired-upstream region

Subcloning of the *prd*-upstream region and in-frame fusions of partial *prd* coding regions with the coding region of the *E. coli lacZ* gene necessitated the construction of several intermediate vectors.

Bluescript pKS$^+$-derived vectors. The pKSpL1 vector was derived from Bluescript pKS$^+$ by destroying its *Not*I site (cleavage and religation of blunt-ended site) and inserting eight base pairs (GCGGCCGC) into the cleaved *Eco*RV site of its polylinker to reintroduce a *Not*I site. The pKSpL2 vector was generated from pKSpL1 by removing a short stretch of its polylinker (between *Hind*III and *Xho*I; ligation of the filled-in sites restores the *Hind*III site). The pKSpL10 vector was also prepared from pKSpL1 by converting its *Bam*HI into an *Eco*RV site (after digestion with *Bam*HI, the 5′-protruding ends were partially copied with Klenow enzyme in the presence of dATP, dCTP and TTP, trimmed with S1 nuclease and religated). The pKSpL11 vector was derived from pKSpL10 by destroying its *Kpn*I site. Subsequently, blunt-end ligation of the 7.2-kb *Hind*III fragment of the *rosy* gene from HZ50PL (Hiromi and Gehring, 1987) into the filled-in *Eco*RI site of pKSpL11 produced ry-pKSpL11. Finally, prd-ry-pKSpL11 was obtained by ligating the genomic *Not*I fragment of clone prd#8 (see below) into the *Not*I site of the ry-pKSpL11 vector. In this construct, the genomic *Kpn*I site is unique.

Transformation vectors CZ.1 and CZ.2 containing the rosy gene. First, KS-lacZ was obtained by subcloning the 3-kb *Sal*I fragment of pFR21Sac into the *Xho*I site of pKS$^+$. The pFR21Sac plasmid was a gift from Mariann Bienz who constructed it from pFR109 (Shapira et al., 1983) by filling in the *Sac*I site of the polylinker and by modifying the 3′ end of *lacZ* (by the insertion of a 1.6-kb *Sac*I fragment from pGH2) to contain *hsp70* trailer sequences (Bienz et al., 1988; M. Bienz and Y. Hiromi, personal communication). CZ.1 was prepared by ligating the 3-kb *Kpn*I-*Sal*I fragment of KS-lacZ containing *lacZ* into the corresponding sites of pHZ50pL (Hiromi and Gehring, 1987). Finally, CZ.2 was obtained from CZ.1 by the removal of the 3′-protruding ends after cleavage with *Kpn*I and religation, eliminating its *Kpn*I site. A detailed description of these vectors is available on request.

Transformation vectors pWZ.1 and pWZ.2 containing the miniwhite gene. The transformation vectors pWZ.1 and pWZ.2 were generated by combining the 4-kb *Xba*I-*Hin*dIII fragment of CZ.1 or CZ.2, including *lacZ* and the *hsp70* 3′ end, with the *Xba*I-*Hin*dIII fragment of the pW6 vector that contains the *miniwhite* gene (Klemenz et al., 1987).

To subclone the 5′ region of *prd*, several steps were performed. First, a 9-kb *Xba*I fragment of genomic *prd* DNA from clone D7.11 (Kilchherr et al., 1986) was subcloned into pKSpL2 to generate prd#1. Subsequently, an *Xba*I-*Eco*RV fragment of prd#1 (consisting of 5.7 kb of 5′-upstream sequences and 1.3 kb of transcribed sequences of *prd*) was ligated into pKSpL2 cleaved with *Eco*RI (and protruding ends filled in) and *Xba*I to generate prd#9. Finally, prd#8 was obtained by ligating three contiguous *Eco*RI *prd* upstream fragments (−3.2 kb to −10.6 kb of *prd*), prepared from a partial *Eco*RI digest of phage D8.1 DNA (Kilchherr et al., 1986), into the single *Eco*RI site of prd#9, located 3.2 kb upstream of the transcriptional start site of *prd*.

Deletions of the *prd* 5′-flanking region were obtained by partial *Bal*31 digestions of prd-ry-pKSpL11 DNA linearized with *Kpn*I. After release of the remaining *ry* fragment by cleavage with *Eco*RV, the partial digests were recircularized and the resulting *prd* deletion series was transferred as *Spe*I-*Not*I or *Xba*I-*Not*I fragments into the *Xba*I and *Not*I sites of pWZ.2. Fusion gene *prdΔC* was obtained by subcloning the *Not*I fragment of prd#8 into the P-element vector CZ.2.

4.4. Generation of transgenic flies and immunostaining of embryos

The final constructs were injected into either ry^{506} or w^{1118} embryos (Rubin and Spradling, 1982) and homozygous transgenic stocks were established for the series of deletions shown in Fig. 3. Initially, more than eight transgenic lines were obtained for several constructs. Typically, five lines were kept and analyzed. Embryos were collected and stained with a rabbit antiserum against β-galactosidase (or prd protein; Gutjahr et al., 1993) according to standard procedures. Photographs were taken on a Zeiss Axiophot, using Nomarski optics and Kodak T-MAX 100 film.

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