**Complex regulation of early *paired* expression: initial activation by gap genes and pattern modulation by pair-rule genes**

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**SUMMARY**

The *paired* gene is one of approximately 30 zygotic segmentation genes responsible for establishing the segmented body plan of *Drosophila melanogaster*. To gain insight into the mechanism by which the *paired* gene is expressed in a complex temporal and spatial pattern, we have examined *paired* protein expression in wild-type and mutant embryos. In wild-type embryos, *paired* protein is expressed in several phases. Initial expression in broad domains evolves into a pair-rule pattern of eight stripes during cellularization. Subsequently, a segment-polarity-like pattern of fourteen stripes emerges. Later, at mid-embryogenesis, *paired* is expressed in specific regions of the head and in specific cells of the central nervous system. Analysis of the initial *paired* expression in the primary pair-rule mutants *even-skipped*, *runt* and *hairy*, and in all gap mutants suggests that the products of the gap genes *hunchback*, *Krüppel*, *knirps* and *giant* activate *paired* expression in stripes. With the exception of stripe 1, which is activated by *even-skipped*, and stripe 8, which depends upon *runt*, the primary pair-rule proteins are required for subsequent modulation rather than activation of the *paired* stripes. The factors activating *paired* expression in the pair-rule mode appear to interact with those activating it along the dorsoventral axis.

Key words: *Drosophila* segmentation, *paired* gene regulation, gap genes, primary pair-rule genes, CNS expression

**INTRODUCTION**

A striking feature of the *Drosophila* body plan is its metameric organization. The molecular basis for segmentation is laid down early in embryogenesis during cellularization of the syncytial blastoderm (reviewed by Akam, 1987; Ingham, 1988). After cellularization, gastrulation ensues followed by complex morphogenetic movements, including extension and retraction of the germ band. During germ band extension, the nervous system begins to develop and the individual segments acquire their characteristic identities.

In a genetic screen aimed at identifying the genes involved in metamORIZATION, two groups of genes were found, the maternal and the segmentation genes (reviewed by Pankratz and Jäckle, 1990; Ingham and Martinez-Arias, 1992; St Johnston and Nüsslein-Volhard, 1992). Mutations in these genes are usually lethal and exhibit characteristic alterations in the cuticle of the dying embryo. Based on the phenotypes of these cuticular pattern defects, the segmentation genes were grouped into three classes, gap, pair-rule and segment-polarity genes (Nüsslein-Volhard and Wieschaus, 1980). In most cases, the regions affected in mutant embryos derive from the primordia where the corresponding segmentation gene is expressed at high levels in wild-type embryos (Coulter and Wieschaus, 1986). The maternal and gap genes are expressed nonperiodically in domains encompassing several segment anlagen whereas the pair-rule and segment-polarity genes are expressed in patterns exhibiting double- or single-segment periodicity. Analysis of the expression of the segmentation genes in mutant backgrounds has demonstrated complex hierarchical regulatory interactions among these genes. In general, maternal genes regulate the gap genes which again control the pair-rule genes. The pair-rule genes have been further subdivided into primary and secondary pair-rule genes based on the proposal that the primary pair-rule genes serve to mediate the transition from the non-periodic expression of the gap genes to the periodic striped expression of the other pair-rule genes (Ingham and Martinez-Arias, 1986; Ingham, 1988).

The activities of the segmentation genes seem not to be confined to metamORIZATION, since most of them are reexpressed in the developing central nervous system (CNS) (Doe et al., 1988a,b; Patel et al., 1989). Assessing their functions in the CNS, however, is difficult because of the lack of mutations affecting only the CNS and due to the complex architecture of the CNS. The first problem was overcome by inactivating the product of a temperature-sensitive *even-skipped* (*eve*) allele during neurogenesis (Doe et
al., 1988b) or through the analysis of transgenic flies carrying a copy of the \textit{fiz} gene lacking the CNS-specific regulatory element in a \textit{fiz} background (Doe et al., 1988a). The second problem can be partially relieved by the use of antibodies that recognize distinct subsets of neurons in the CNS and hence serve as markers (e.g. Doe et al., 1988b; Patel et al., 1989).

The pair-rule gene \textit{paired} (\textit{prd}) seems to be exceptional in several ways. In addition to a homeodomain, it contains another highly conserved domain, the paired-domain (Bopp et al., 1986, 1989; Dressler et al., 1988; Burri et al., 1989), which might be a second DNA-binding domain (Treisman et al., 1991) as previously proposed (Burri et al., 1989; Bopp et al., 1989). Furthermore, \textit{prd} is expressed in an unusually dynamic manner, showing both pair-rule and segment-polarity transcript patterns (Kilchherr et al., 1986). Finally, \textit{prd} has recently been shown to be at the bottom of the regulatory cascade of the pair-rule genes and hence has been designated as a tertiary pair-rule gene ( Baumgartner and Noll, 1990).

In this report, an affinity-purified anti-prd antisemur was used in a detailed study of \textit{prd} protein expression throughout embryogenesis. To clarify whether the pair-rule pattern of \textit{prd} results from activation by primary pair-rule proteins or gap gene products, \textit{prd} expression was examined in all gap mutants and in embryos deficient for any one of the primary pair-rule genes \textit{eve}, \textit{haairy} (\textit{h}) or \textit{runt} (\textit{run}). Our results suggest that the initial activation of \textit{prd} in a pair-rule pattern of eight stripes occurs through the products of the gap genes \textit{hunchback} (\textit{hb}), \textit{Krüppel} (\textit{Kr}), \textit{knirps} (\textit{knirps}) and \textit{giant} (\textit{gt}). The primary pair-rule proteins assist the activation of stripes 1 and 8 but act primarily as modulators of the pair-rule pattern. The relative positions of \textit{prd}- and \textit{en-grailed} (\textit{en})-expressing cells was determined with single cell resolution. Finally, we have shown that \textit{prd} protein is expressed in specific cells of the developing head and CNS.

**MATERIALS AND METHODS**

**Construction of expression plasmids and purification of induced proteins**

A plasmid expressing the full-length \textit{prd} protein was constructed by cloning a 1.95 kb \textit{Bam}HI fragment, generated by partial digestion of a c7340.4 \textit{prd} cDNA (Frigerio et al., 1986) subclone in pTRB0 (Bürglin and De Robertis, 1987), into the vector pAR3038 (Studier and Moffat, 1986). The pTRB0 subclone had been obtained by ligating a 2.3 kb \textit{Hind}III fragment from a c7340.4 \textit{prd} cDNA subclone in pGEM-2 into pTRB0. A plasmid expressing the ‘box-less’ \textit{prd} protein (C-terminal half of \textit{prd} protein without N-terminal paired- and homeodomain) was constructed by ligating a 1.3 kb \textit{Pvu}II-\textit{Smal} fragment, obtained from a c7340.6 \textit{prd} cDNA subclone in pGEM-2, into the blunt-ended \textit{Bam}HI site of the vector pAR3039. Expression of the plasmids in \textit{E. coli} BL21(DE3) cells was essentially as described (Studier and Moffat, 1986) and the IPTG-induced proteins were purified as follows. Cells from 200 ml cultures were harvested and resuspended in 10 ml of buffer A (20 mM Tris-\textit{HCl}, pH 8.0, 50 mM NaCl). To enrich the expressed proteins, the resuspended cells were sonicated on ice for 2 to 3 minutes, and the suspension was pelleted at 4°C for 10 minutes at 10 000 revs/minute in a Sorvall SS-34 rotor. The resulting pellet containing the induced protein in inclusion bodies was resuspended in 10 ml of the same buffer, sonicated for 3 minutes on ice and centrifuged as before. This step was sequentially repeated with buffers (20 mM Tris-\textit{HCl}, pH 8.0) containing 1 M NaCl, 3 M NaCl, 50 mM NaCl, 1% Triton X-100; and finally in H\textsubscript{2}O. The final pellets were dissolved in 10 ml of buffer A containing 8 M urea and stored frozen at −20°C.

**Preparation, purification and test of antisemur**

White New Zealand rabbits were immunized with the full-length \textit{prd} protein (150 µg per injection) dialyzed against buffer A containing 2 M urea. The crude anti-prd antisemur was first batch-adsorbed with crude bacterial protein extract (from induced bacteria containing the pAR3038 vector without an insert) coupled to CNBr-activated Sepharose 4B (Pharmacia) as recommended by the manufacturer (negative adsorption). The supernatant was subsequently batch-adsorbed overnight to a resin to which the ‘box-less’ \textit{prd} protein had been coupled (positive adsorption). The resin was packed into a column and the bound antibodies were eluted with 50 mM NaCl, 0.2 M glycine, pH 2.2, directly into a beaker containing 1 M Tris-\textit{HCl}, pH 8.5 to neutralize the eluate. The eluate was dialyzed against PBS. Antibodies from the 50 ml dialyze were concentrated by ammonium sulfate precipitation (44% saturated at 4°C), dissolved in 5 ml of PBS, dialyzed overnight twice against 2 liters of 0.2x PBS, stabilized by the addition of 0.1 mg/ml BSA, and stored frozen in small aliquots at −80°C. After thawing, aliquots were stored at 4°C.

**Immunocytochemistry and immunofluorescence**

Embryos were collected and fixed essentially as described (Bopp et al., 1989). Embryos could be kept in methanol for several months at −20°C prior to use. Before incubation with antisemur, the embryos (20 µl to 100 µl in an Eppendorf tube) were rehydrated by three rinses with 1.2 ml to 1.5 ml of PBST (10 mM Na\textsubscript{2}HPO\textsubscript{4}/NaH\textsubscript{2}PO\textsubscript{4}, pH 7.8, 100 mM NaCl, 5 mM MgCl\textsubscript{2}, 0.05% Tween-20). The affinity-purified antisemur was preabsorbed for at least 4 hours at room temperature at a concentration of 1:1000 with 0.1 volume of fixed, 0-15 hours old wild-type embryos in PBST. Nine volumes (or at least 180 µl if less than 20 µl of packed embryos were used) of the preabsorbed, affinity-purified antisemur were then incubated with 1 volume of fixed embryos of the desired stages at room temperature overnight. The embryos were rinsed briefly three times with PBST and washed for 1.5 hours with three changes of PBST. The secondary antibody (Vectastain goat anti-rabbit antibody conjugated to biotin, preabsorbed overnight at a final concentration of 1:200 as before) was added and reacted with the embryos for 2 hours. After the embryos were rinsed and washed as before, the preformed AB complex (Vectastain) was added and allowed to react for 1.5 hours. After another cycle of rinses and washes, the embryos were suspended in 0.5 mg/ml DAB in 0.1 M Tris-\textit{HCl}, pH 7.1, 0.2% NiCl\textsubscript{2}, 0.003% H\textsubscript{2}O\textsubscript{2}. The reaction was stopped with three rinses of PBST. Embryos were mounted in about 30 µl of PBS and covered with a 20 mm × 20 mm coverslip, which flattened the embryos to some extent. Further flattening of the embryos without damaging them could be achieved by draining some PBS from the sides of the coverslip with a filter paper. This procedure results in satisfactory morphology for embryos up to germ band extension but not for visualization of neuroblasts or inner cell layers.

Photographs were taken using Nomarski (differential interference contrast) optics on a Zeiss Axioptot. Kodak black and white T-MAX 100 film was developed for lowest contrast using Microdot-X developer (Kodak). Immunocytochemical double-labeling was performed according to a protocol devised by Nipam Patel. For photography, Kodak EPY-50 Ektachrome professional film (tungsten) was used for color slides.

Immunofluorescence double-labeling of wild-type embryos was
performed essentially as described above, except that the anti-prd antiserum was reacted with embryos at a concentration of 1:100 simultaneously with anti-en asci fluid at a concentration of 1:200. Subsequently, preabsorbed secondary anti-mouse antiserum coupled to biotin (Vectastain) was applied at a concentration of 1:200 for 2 hours at room temperature. After rinses and washes, preabsorbed anti-rabbit antiserum coupled to rhodamine (TRITC, 1:400; Dakopatts) and fluorescein (FITC) coupled to avidine (0.25 μg/ml; Vectastain; preabsorbed at 5 mg/ml as above), were added for 2 hours at room temperature. Finally, the embryos were mounted in 95% glycerol, 50 mM Tris-HCl, pH 7.5, 0.5% n-propyl gallate. Photographs were taken on a Zeiss Axiophot using Kodak EPT-160 Ektachrome professional (tungsten) film.

Fly stocks
The following gap and pair-rule mutant stocks (all of which are strong alleles) were used: cn bw sp Kr+/SM1; kni+/TM3, Sb; tll+/TM3, Sb; st hh+/CyO; Df(1)run1.7; Df(2R)prd1.7 by gift of H. Jäckle). The allele used to test the specificity of the prd antiserum was Df(2L)prd+/CyO; Df(2R)prd1.7/CyO. The specificity of the affinity-purified antibodies was further demonstrated by their failure to bind to fixed embryos deficient for the prd antiserum was Df(2L)prd+/CyO.

RESULTS
Production of anti-paired antiserum
To obtain a prd-specific antiserum, rabbits were immunized with bacterially expressed and partially purified full-length prd protein. The resulting antiserum was first batch adsorbed with a crude bacterial extract and then affinity-purified over a column to which bacterially expressed truncated prd protein (‘box-less’ paired protein, see Materials and methods) was bound. The affinity-purified antibodies were specific for the ‘domainless’ portion of the prd protein since cross-reactivity to one of the groovey proteins, gsb-BSH9, was lost after positive absorption with ‘box-less’ paired protein (not shown). The specificity of the affinity-purified antibodies was further demonstrated by their failure to bind to fixed embryos deficient for the prd gene (not shown).

Several phases of paired expression during embryogenesis
During embryonic development, prd is expressed in two main phases. The first phase (phase A), during which the prd protein is expressed in transverse stripes, may be further subdivided into four distinct, partially overlapping sub-phases (A1.1 to A1.3 and A2) including nuclear cycle 13, cellularization, gastrulation and germ band extension. During the second phase (phase B), which includes all following stages of embryogenesis, prd is expressed in a region-specific manner in the gnathal segments of the prospective head region, the clypeolabrum and the central nervous system. At all stages, the prd protein is detected exclusively in the nucleus.

Expression of paired protein in stripes
As shown in Fig. 1A, prd protein is first detectable at extremely low levels in the anterior region of the embryo towards the end of nuclear cycle 13 (Foe and Alberts, 1983). This region resembles a ‘cap’, extending from 100% egg length (EL) to approximately 60% EL (0% EL is at the posterior pole of the embryo). The distribution of prd protein along the anteroposterior axis resembles a shallow gradient with a maximum close to the posterior margin, while protein levels appear to be quite homogeneous along the circumferenece (Fig. 1A). By the end of the 13th nuclear division (Fig. 1B), this region has become restricted to a bell-shaped stripe, stripe 1-2, which comprises about 16 nuclei on the ventral and approximately 8 nuclei on the dorsal side. Between the end of the 13th nuclear division and the onset of cellularization (Fig. 1C), prd protein accumulates to high levels in stripe 1-2 and expands dorsally to a width of about 10 nuclei (end of phase A1.1).

At the same time, prd protein becomes detectable in two abutting broad domains exclusively on the ventral side of the embryo. These domains are located between about 60% and 25% EL and correspond to the future stripes 3 and 4, and 5 to 7 (start of phase A1.2; Fig. 1D). Levels of prd protein increase in specific regions within these broad areas, resulting in the appearance of five individual stripes, stripes 3 to 7 (Fig. 1E-G). The order of their appearance parallels a preceding shallow gradient of prd expression in the two broad domains (Fig. 1D), i.e. stripe 7 precedes stripe 6 which precedes stripe 5, and stripe 4 precedes stripe 3. As prd protein levels rise on the ventral side, prd expression expands laterally to finally form five circumferential transverse stripes (Fig. 1E-H) exhibiting a periodicity of two segment anlagen (pair-rule pattern). The areas between the stripes (‘interstripes’) either stop expressing prd protein or even reduce its levels (Fig. 1D-G).

On the ventral side, prd protein thus first accumulates in stripes 7 and 4, which are followed by stripes 3 and 6 and finally by stripe 5. Astonishingly, the order of prd protein accumulation on the dorsal side is different from that on the ventral side, mainly because stripe 3 accumulates higher prd protein levels prior to stripe 4 (Fig. 1F,G). Concomitantly with the division of the two broad domains into stripes 3 to 7, stripe 1-2 begins to split. In stripe 1-2, this process is restricted to the lateral and dorsal sides of the embryo and is somewhat asymmetric since the resulting stripe 1 is much narrower than stripe 2.

As prd protein levels continue to increase, both within the stripes and dorsally along the circumference of the embryo, the anterior and posterior boundaries of the stripes become gradually more distinct (end of phase A1.2/beginning of phase A1.3; Fig. 1G). Furthermore, the bell-shaped distribution within individual stripes of prd protein changes to a gradient with its maximum at the posterior margin (Fig. 1G,H). Again, stripe 1 behaves differently. Its width is reduced due to loss of prd protein in its anterior portion (Fig. 1G-I), and its anterior ventral margin remains diffuse (not shown).

At mid-cellularization, prd protein begins to be expressed in an anterior dorsal spot and in a posterior eighth stripe (phase A1.3, Fig. 1G-I). In contrast to all other stripes, stripe 8 is first expressed on the lateral sides of the embryo (Fig. 1H,I) and remains largely excluded from the ventral side (not shown). Furthermore, stripe 8 is several nuclei wider than the other stripes and is expressed in a wedge-shaped gradient with high protein levels at the anterior margin (Fig. 1K).
Towards the end of cellularization (phase A1.3), the levels of prd protein continue to rise in the posteriormost cells of stripes 3 to 7 (beginning of phase A2). As a result, the protein distribution changes from a continuous to a step-like gradient (Fig. 1G-I). Furthermore, prd protein has reached similar levels in all seven stripes. Subsequently, with some delay in time, prd protein accumulates to higher levels in the anterior one or two cells of all stripes, again with the exception of stripe 1, which further narrows to two or three cells (Fig. 1I,K). However, prd protein concentra-

Fig. 1. Expression of prd protein in wild-type embryos during embryogenesis. Whole-mount preparations of fixed wild-type embryos were stained with purified anti-prd antiserum. The panels show successive stages of embryos, prior to the 13th nuclear division (A), just after the 13th nuclear division (B), at the onset of cellularization (C), at progressive stages of cellularization (D-I), at early gastrulation (K), at mid-gastrulation (L), towards the end of gastrulation (M), at successive stages of germ band elongation (N-P), at the fully extended germ band stage (Q), at late germ band retraction (R), at the end of germ band retraction (S), undergoing head involution (T), and at the onset of nerve cord retraction (U). Numbers in panels A-I refer to time (minutes) of development at 25°C before and after the 13th nuclear division, calibrated according to Foe and Alberts (1983). The inserts on the right of panels A-I are optical mid-plane sections at high magnification of the corresponding embryos to reveal the degree of cellularization (arrowheads indicate the position of the leading edge
tions in anterior cells never quite attain the same levels as in posterior cells of stripes 2 to 7 (Fig. 1K-P). In contrast to the cells of the stripe margins, expression of prd protein in the centrally located cells is gradually lost (‘late interstripes’). These processes seem to occur in an anterior to posterior direction, resulting in a periodic pattern of 14 stripes (Fig. 1K,L) with a single-segment repeat (segment-polarity pattern). During this process, both anterior and posterior margins of all 14 stripes become quite distinct, with the exception of the anterior ventral portion of stripe 1 (not of the progressing cellular membranes). All embryos are shown with their anterior poles to the left. Lateral views are shown in panels B-L, P, R and U, ventrolateral views in panels A, Q, S and T, and dorsal or dorsolateral views in panels M-O. Panel A is a bright-field image while the remaining panels were photographed by partial Nomarski optics. Note that the single stained cells revealed by the plane of focus in panel S are neurons. In panel T, the single, most laterally staining cells (in focus only in the right abdominal half of the embryo) are epidermal while the remaining single stained cells, located symmetrically with respect to the ventral midline in the trunk, are neurons. The anterior spots, which are out of focus in panels R and S, are due to the strong prd expression in the right-side maxillary lobe below the plane of focus. Abbreviations: LB, labial segment; MX, maxillary segment; MD, mandibular segment; HY, hypopharyngeal segment; CL, clypeolabral segment.
shown). However, prd protein is still detectable at very low levels in cells of the late interstripes.

With the onset of germ band extension, prd protein levels begin to drop in stripe 1 and in stripes 2 to 7, which have split by this stage (Fig. 1M-Q). During germ band elongation, stripe 8 appears to be split in its ventral portion into an anterior and posterior stripe. This splitting of stripe 8 (Fig. 1M,N) is obscured by posterior folds, yet is more easily detected in the splitting of prd-transcripts that precedes it (cf. Fig. 1h of Baumgartner and Noll, 1990). As evident from a comparison of the dorsal views in Fig. 1M and N, stripe 8 is better described at this stage as an outer ring enclosing the posterior ‘ventral’ stripe 8 which is eventually engulfed by the posterior midgut invagination (Fig. 1O). The anterior ‘ventral’ stripe 8 moves anteriorly and approaches what seems to be the original dorsal portion of stripe 8 (Fig. 1N,O). Levels of prd protein decrease in the anterior ‘ventral’ stripe 8 as in all other stripes, except in the originally dorsal portion of stripe 8 and in the dorsal spot of the head where they remain high until the end of germ band extension (Fig. 1O,P). By the end of germ band extension, when the gnathal segments appear, prd protein is detectable in a few patches of cells at the bases of the labial, maxillary and mandibular lobes (Fig. 1R). The most prominent expression of prd protein is observed in the maxillary lobe (Fig. 1Q,R). At the onset of germ band retraction, the number of cells as well as the levels at which prd protein is expressed begin to increase mainly in the maxillary segment (Fig. 1Q,R). In the clypeolabrum, four cells begin to accumulate prd protein to high levels (one of which can be seen in Fig. 1R,S). Beginning with the end of germ band retraction, the number of cells in the labial and mandibular lobes expressing prd protein continuously decreases (Fig. 1S,T). In the maxillary lobe (Fig. 1T) as well as in the clypeolabrum, prd protein remains expressed at high levels until the beginning of nerve cord retraction (Fig. 1U). At about the same time, prd protein can be detected at very low levels in a few lat-

Relative positions of paired to engrailed expression

Since the en gene is expressed in the posterior compartment of each segment (Kornberg, 1981; Kornberg et al., 1985; Fjose et al., 1985; Lawrence et al., 1987) and since en expression depends on the prd product in odd-numbered stripes (DiNardo and O’Farrell, 1987), we wished to determine which of the prd- and en-expressing cells overlap. Therefore, prd and en proteins were visualized in the same embryos by immunofluorescence double-labeling. As shown in Fig. 2, prd and en proteins are coexpressed in the posterior row of cells of the prd stripes, yet there seems to be no consistent correlation between the levels of prd and en protein in these cells. The few cells that seem to express en protein exclusively and which are located just posterior to cells expressing both prd and en might be cells in the process of switching off en (Vincent and O’Farrell, 1992). Although Fig. 2 might suggest that these cells are mainly located in even-numbered en stripes, analysis of several other embryos indicates no such preference.

Late, region-specific expression of paired protein in the head

The region-specific phase of prd protein expression in the head (phase B) briefly overlaps with the end of the striped phase A (Fig. 1Q). The beginning of this phase is marked by the decrease of prd protein levels in the dorsal eighth stripe as well as in the anterior dorsal spot. By the time the gnathal segments appear, prd protein is detectable in a few patches of cells at the bases of the labial, maxillary and mandibular lobes (Fig. 1R). The most prominent expression of prd protein is observed in the maxillary lobe (Fig. 1Q,R). At the onset of germ band retraction, the number of cells as well as the levels at which prd protein is expressed begin to increase mainly in the maxillary segment (Fig. 1Q,R). In the clypeolabrum, four cells begin to accumulate prd protein to high levels (one of which can be seen in Fig. 1R,S). Beginning with the end of germ band retraction, the number of cells in the labial and mandibular lobes expressing prd protein continuously decreases (Fig. 1S,T). In the maxillary lobe (Fig. 1T) as well as in the clypeolabrum, prd protein remains expressed at high levels until the beginning of nerve cord retraction (Fig. 1U). At about the same time, prd protein can be detected at very low levels in a few lat-

Fig. 2. Expression of prd relative to en at gastrulation. Embryos were stained with rabbit anti-prd (revealed by TRITC) and mouse anti-en antiserum (FITC) as described in Materials and methods. Black and white photographs were taken from the same embryo. The photographs were aligned and prd and en coexpressing cells were determined graphically. The upper panel shows the result of such a graphical superimposition of prd and en proteins in nuclei of an embryo at gastrulation shown in the two lower panels when fluorescence of either the prd (left) or en (right) conjugate is excited. Note that the prd stripes seem to merge on the dorsal side, an effect due to the onset of morphogenetic movements which lead to the formation of deep folds. The arrowheads mark en stripe 3 and the posterior stripe of the split prd stripe 2. Symbols in the upper panel denote cells expressing both prd and en (●), cells expressing only prd (+), and cells expressing only en (○).
eral cells in the epidermal region (Fig. 1T). Since these cells express prd protein at very low levels and since their number and locations seem to be erratic, we have not attempted to further identify them.

**Specific cells of the CNS express the paired protein**

At late germ band retraction and until head involution, prd protein is detectable at low levels in single cells of the CNS which appear to be segmentally repeated but to vary considerably in staining intensity (Figs. 1S,T; 3; CNS-staining is already present at stage shown in Fig. 1R where it is out of plane of focus). The two to three neurons in each hemisegment expressing prd protein are located laterally of the three longitudinal axon bundles on the dorsal side of the CNS (Fig. 3). The prd-expressing neurons do not seem to overlap with any previously identified neurons, as inferred from double-labeling experiments using mAb 22C10 (Zipursky et al., 1984) - which stains the same specific subset of neurons in the CNS as mAb SOX2 (Goodman et al., 1984; N. Patel and C.S. Goodman, personal communication) - and anti-prd antiserum (Fig. 3).

The low level and short duration of prd expression, as well as the small number of cells expressing prd protein, suggest that prd could specify the fate of a very small number of unique and highly specific neurons.

**Expression of paired in primary pair-rule mutants**

Since the primary pair-rule genes run, h and eve have been shown to regulate prd expression (Baumgartner and Noll, 1990) and are thought to mediate the transition from the non-periodic expression pattern of the gap genes to the periodic expression pattern of the secondary and tertiary pair-rule genes (Ingham, 1988; Ingham and Gergen, 1988), we reexamined prd expression in these mutant backgrounds. In particular, in light of our observations of early prd expression in broad domains, we wished to determine the earliest stage at which an impact on the prd pattern could be observed. To exclude residual activities of the mutated genes, prd expression was analyzed in deletion mutants of these genes.

In eve^- embryos (Fig. 4A-D), the early prd pattern differs from that in wild-type embryos. The anterior portion of stripe 1-2 (future stripe 1) fails to accumulate high levels of prd protein by the time stripes 3 to 7 begin to appear (compare Fig. 4A with Fig. 1D). Accumulation of prd protein in the region of stripes 3 to 7 is also different from wild-type as the interstripes develop only partially. Furthermore, the order of prd accumulation in the stripes is different from that observed in wild-type embryos. In particular, stripe 5 is expressed early in comparison to stripes 3, 4, 6 and 7. At gastrulation, relatively broad interstripes begin to form (Fig. 4C), resulting in a regular pattern of seven equally wide and spaced stripes with a double-segment repeat (Fig. 4D).

In h^- embryos (Fig. 4E-H), the initial prd pattern appears relatively normal (Fig. 4E). The first deviation from the wild-type prd pattern is observed in the emerging stripe 6, which is more strongly expressed and at similar levels on the dorsal and ventral sides (Fig. 4F). In addition, prd protein levels are slightly enhanced in interstripes as compared to the wild-type pattern (cf. Fig. 4F with Fig. 1E,F). At the onset of gastrulation, prd is expressed in a continuous but periodically modulated pattern with strongly elevated levels at the anterior borders of stripes 3 to 8 (Fig. 4G). Subsequently, prd protein concentrations decrease anterior to these most prominent regions of prd expression, thus producing three- to four-cell-wide gaps and generating a regular pattern of eight stripes (Fig. 4H). Stripes 3 to 8 continue to express high levels of prd protein at their anterior margins and appear to be shifted with respect to the positions of the corresponding wild-type stripes as judged by the position of stripe 2 with respect to the cephalic furrow and by the enlarged gap between stripes 2 and 3 (cf. Fig. 4G,H with Fig. 1L,K). The observed late pattern (Fig. 4H) is consistent with a model of prd regulation by h, which predicts that prd expression in specific cells of the CNS. Dorsal view of a portion of a dissected CNS of an early stage 14 embryo (Campos-Ortega and Hartenstein, 1985). Embryos were stained with anti-prd (black) and mAb 22C10 (brown). The latter was used to reveal aCC, RP1 and RP2 cells (Goodman et al., 1984) and to indicate the positions of the anterior (RP1, RP2) and posterior commissures (aCC) and of the intersegmental nerve (aCC, RP2). A schematic view of the photograph on the left is shown on the right. Filled cells express prd more strongly in the photograph, stippled cells more weakly. Other cells and axons indicated all stained with mAb 22C10. The open triangles representing 22C10-positive cells might correspond to VUM cells (Goodman et al., 1984). Abbreviations: AC, anterior commissure; PC, posterior commissure; IS, intersegmental nerve.
is ectopically expressed in the interstripes but fails to remain expressed in the posterior regions of the stripes during their splitting (Baumgartner and Noll, 1990). During gastrulation, prd levels decrease prematurely in stripes 1 and 6 (cf. Fig. 4G,H with Fig. 1K,L). In stripe 6, this process begins on the ventral side and proceeds dorsally (Fig. 4H).

In run embryos (Fig. 4I-M), stripe 1-2 accumulates high levels of prd protein but fails to develop an interstripe at mid-cellularization (Fig. 4I,K). In the region corresponding to stripes 3 to 7, prd protein begins to accumulate in stripe 5 prior to stripes 3, 4, 6 and 7, which appear with a significant delay as compared to the wild-type situation (Fig. 4K; a delay of about 5-10 minutes at 25°C may be estimated from the progression of the cleavage furrows formed by the inward growing plasma membrane). In addition, stripes 5 to 7 appear unequally spaced, probably due to shifts by one nucleus of stripe 5 posteriorly and of stripe 6 anteriorly, resulting in a fused stripe 5-6 and in enlarged interstripes between stripes 4, 5-6 and 7 (Fig. 4K-M). Moreover, stripes 3, 4 and 7 appear narrower by one to two cells than their wild-type counterparts (cf. Fig. 4L with Fig. 1I). The distribution of prd protein within the stripes is fairly homogeneous, and both the anterior and the posterior margins appear to be relatively sharp (Fig. 4L).

The anterior dorsal spot appears unaffected in all three mutants (Fig. 4D,H,M). Stripe 8 seems to be normal in h- and eve- embryos, although initially it is not separated from stripe 7 by an interstripe in either mutant (Fig. 4C,G). In run embryos, stripe 8 fails to accumulate high levels of prd protein and remains much narrower than in wild-type embryos (cf. Fig. 4L,M with Fig. 1K).

In summary, the primary pair-rule gene h shows no clear effects on the initial activation of prd. In contrast, eve is required early to activate stripe 1 and to repress prd in the early interstripes while run represses prd to resolve stripes 1 and 2 and strongly enhances activation of stripe 8 and somewhat of stripes 3, 4, 6 and 7. Nevertheless, in primary pair-rule mutants, the initial prd expression patterns exhibit the normal number of stripes with only minor shifts with respect to their normal positions rather than a complete loss of stripe activation, with the exception of eve-dependent stripe 1.

Expression of paired in gap mutant embryos
Since the initial activation of prd is largely independent of primary pair-rule gene products, we examined whether it might depend on the preceding activity of gap genes. Therefore, we analyzed the expression of prd in gap mutant

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Fig. 4. Expression of prd protein in primary pair-rule mutants. Whole-mount preparations of fixed eve- (A-D), h- (E-H), and run- (I-M) embryos of various stages were stained with anti-prd antiserum as in Fig. 1. Ventrolateral (A-C, G-I, L,M) or lateral views (D-F,K) of embryos are shown at early (A,E,I) and late stages of cellularization (B,F,K), and during the first (C,G,L) and second half of gastrulation (D,H,M). Numbers in panels A-C, E-G, and I-L refer to time (minutes) of development at 25°C after the 13th nuclear division as in Fig. 1.
embryos (Fig. 5). While no effect on prd expression was detectable in embryos mutant for orthodenticle (otd), empty spiracles (ems), buttonhead (btd) (Cohen and Jürgens, 1990) and huckebein (hkb) (Weigel et al., 1990), the initial prd pattern was drastically altered in the gap mutants hb, Kr, kni (Nüsslein-Volhard and Wieschaus, 1980), gt (Wieschaus et al., 1984) and tailless (tl) (Strecker et al., 1986).

Expression of prd in homozygous hb embryos is altered in two regions corresponding to the anterior and posterior hb expression domains in wild-type embryos. In the anterior domain, prd stripes 2 to 4 fail to form properly whereas, in the posterior domain, stripe 8 is never activated (Fig. 5A-C). In homozygous tl embryos, stripes 1 to 5 form normally, stripes 6 and 7 are broader and shifted posteriorly, while stripe 8 never appears (Fig. 5D-F). In kni− embryos, stripe 4 fails to separate from 5, and 6 from 7 (Fig. 5G-I).

Most severely affected is the expression of prd in homozygous Kr embryos (Fig. 5K-M). Initially, stripes 2 and 3 are replaced by a broader stripe, posterior to the position of wild-type stripe 2 and not completely separated from stripes 1 and 4. Stripes 5 and 6 are replaced by a stripe that is located posterior to the position of wild-type stripe 5 and fails to separate from stripe 4. Finally, in gr− embryos, prd stripes 6 and 7 do not resolve while stripes 1, 2 and 8 are not properly activated (Fig. 5N-P).

DISCUSSION

An affinity-purified antibody was used to monitor the dynamic and rapidly evolving expression of prd protein during embryogenesis in wild-type and mutant embryos. At all stages, prd protein is localized to the nuclei, a finding that is consistent with the suggested role for the prd protein as a gene regulatory factor (Bopp et al., 1986, 1989; DiNardo and O’Farrell, 1987; Treisman et al., 1991). Comparison of prd protein with prd RNA expression patterns (Kilcherr et al., 1986) reveals no obvious incongruities in either wild-type or mutant embryos (Baumgartner and Noll, 1990). However, novel expression patterns, including the cap at the anterior pole and two broad domains corresponding to the prospective stripes 3 to 7 during the initial stages of expression, segmentally repeated expression in the CNS and a region-specific expression in head segments were discovered.

The striped expression patterns evolve in several phases

For the sake of clarity, we have divided the complex and dynamic development of the prd expression pattern, which includes multiple phases of activation, repression, refinement and modulation, into two main phases: an early striped phase (phase A) and a late region- and tissue-specific phase (phase B). Phase A is characterized by a pair-rule phase, A1, and a segment-polarity phase, A2. The two phases overlap in time to some extent.

The pair-rule phase A1 was further subdivided into three subphases: expression of prd at the anterior pole prior to the 13th nuclear division (phase A1.1), initial expression of prd in the region of prospective stripes 3 to 7, which starts at the onset of cellularization (phase A1.2), and late initial expression of prd in the anterior dorsal spot and in stripe 8, which begins at mid-cellularization (phase A1.3). All three subphases are characterized by their clear separation with respect to time and prd expression along the antero-posterior axis. In addition, they exhibit obvious differences in the temporal course of prd activation along the dorsoventral axis. In phase A1.1, prd is expressed simultaneously along the dorsoventral axis, during phase A1.2, it is first expressed ventrally and, in phase A1.3, it is initially expressed either laterally (stripe 8) or exclusively on the dorsal side (anterior dorsal spot).

Below we discuss evidence suggesting that the initial pair-rule pattern of prd, which evolves during phase A1, largely depends on the activation by gap rather than primary pair-rule genes. The primary pair-rule genes eve, h and run are only required for the proper establishment of the most anterior and posterior prd stripes, 1-2 and 8, and have an early effect on the precise positioning of stripes 3 to 7. The secondary pair-rule genes ftz, odd, oga and slp have no effect on prd expression during this phase (Baumgartner and Noll, 1990). In contrast, the segment-polarity pattern, initiated during phase A1.3 and established during phase A2, depends on the activity of all pair-rule genes except of prd itself (Baumgartner and Noll, 1990). Similar biphasic modes of expression have been reported for eve (Macdonald et al., 1986; Frasch and Levine, 1987; Frasch et al., 1987), run (Kania et al., 1990) and odd (Coulter et al., 1990), but not for h or ftz.

**Paired determines the posterior boundary of odd-numbered engrailed stripes**

A major function of pair-rule proteins is to establish the proper expression of segment-polarity genes. It has been shown previously that odd-numbered en stripes are expressed in the most anterior row of cells of each eve stripe (Lawrence et al., 1987) and that en expression posterior to stripe 1 depends on eve (Harding et al., 1986; Macdonald et al., 1986). Therefore, eve delineates the anterior boundary of odd-numbered en stripes (Lawrence et al., 1987). The question then arises which pair-rule gene(s) determine(s) the posterior boundary of these en stripes. In prd− embryos, odd-numbered en stripes fail to be activated (DiNardo and O’Farrell, 1987). Here we show that the posterior cells of the prd stripes coincide with the en stripes (Fig. 2). Hence we conclude that it is the prd protein that determines the posterior boundary of the odd-numbered en stripes. Few cells posterior and adjacent to the prd stripes have been observed to express en (Fig. 2). However, this observation is not in conflict with our conclusion because no cells have been found at the posterior boundary of en stripes that express prd but not en. We think that the few cells that exclusively express en protein are cells in the process of switching off en (Vincent and O’Farrell, 1992) due to the preceding disappearance of prd protein (Fig. 1).

Further evidence that prd might specify the posterior boundary of odd-numbered en stripes has been provided recently by the ubiquitous expression of prd which results in a posterior expansion of the odd-numbered en stripes corresponding to the expression of eve (Morrissey et al., 1991). Although prd is expressed at the same relative position and...
precision with respect to odd- and even-numbered en stripes, \(en\) expression does not depend on \(prd\) in even-numbered stripes (DiNardo and O’Farrell, 1987). The role of \(prd\) in these regions remains unclear.

**Differential regulation of paired along the dorsoventral axis**

As discussed above, \(prd\) expression during each subphase of A1 not only occurs in distinct regions along the antero-posterior axis but exhibits a characteristic temporal course of activation along the dorsoventral axis as well. While \(prd\) accumulates at similar levels around the periphery of the embryo in stripe 1-2, there is a marked difference in protein levels on the ventral and dorsal sides in the early stripes 3 to 7. Since, at the onset of gastrulation, the gap genes are rather uniformly expressed along the dorsoventral axis, other factor(s) that are differentially distributed along this axis, like the activated dorsal protein (Steward, 1989; Roth et al., 1989; Rushlow et al., 1989), must be involved in the initial activation of \(prd\). These factors, in combination with gap proteins, activate \(prd\) differentially along the antero-posterior axis, such that stripes 3 to 7 accumulate in different orders on the ventral and dorsal sides. The complex interactions of these dorsoventral activating factors and of gap or pair-rule proteins with cis-regulatory regions of \(prd\) might also explain the lack of dorsoventral polarity in the activation of stripe 6 and its premature disappearance from ventrolateral regions in \(h^+\) embryos (Fig. 4F, H) as well as the changes observed in \(prd\) expression along the dorsoventral axis in gap mutants (Fig. 5).

**Primary pair-rule genes activate stripes 1 and 8 and modulate paired expression in stripes 2 to 7**

It has been proposed that the primary pair-rule genes \(h, run\) (Ingham, 1988) and \(eve\) (Ingham and Gergen, 1988) are responsible for the generation of the periodic pattern of the other pair-rule genes by responding to nonperiodic cues provided by the gap gene products. Here, we concentrated on the various early effects on \(prd\) expression in primary pair-rule mutants during phase A1 since the later effects have been previously shown to result from complex regulatory interactions among primary and secondary pair-rule genes (Baumgartner and Noll, 1990). Although absence of any primary pair-rule gene has distinct and stripe-specific
Fig. 5. Expression of prd protein in gap mutants. Whole-mount preparations of fixed hbr- (A-C), tll- (D-F), kni- (G-I), Kr- (K-M), and gtr- (N-P) embryos during cellularization or early gastrulation (F) were stained with anti-prd antigen as in Fig. 1. Embryos are shown with their anterior pole to the left and dorsal side up. Numbers in panels refer to time (minutes) of development at 25°C after the 13th nuclear division as in Fig. 1.

In hbr- embryos, the anterior boundary of Kr expression expands anteriorly by 10% EL (Jäckle et al., 1986; Hülskamp et al., 1990), kni expression is also extended anteriorly by 5% EL (Hülskamp et al., 1990) while the posterior limit of the anterior domain of gt expression is shifted slightly anteriorly and its posterior domain expands posteriorly (Eldon and Pirrotta, 1991; Kraut and Levine, 1991a). The observed alterations of prd and gap gene expression in hbr- embryos (A-C) are consistent with the postulated effect of gap proteins on prd expression (Table 1): (i) below a threshold concentration of hbr protein, Kr protein activates prd, generating the broad prd band with its maximum coinciding with that of Kr protein in hbr- embryos; (ii) low kni protein represses prd at high concentrations of Kr protein, which accounts for the enlarged gap posterior to the broad prd band; (iii) gt protein activates prd above a threshold concentration in stripes 2 and 7 in wild-type embryos; in hbr- embryos, reduction of gt protein in the posterior part of its anterior domain results in its failure to activate stripe 2 whereas ectopic expression of gt posterior to its posterior domain activates prd ectopically a few cells posterior to stripe 7 at the onset of gastrulation (not shown); (iv) the activation of stripe 8 depends completely on hbr protein. Although stripe 8 appears relatively late and also requires run protein for full activation, the effect cannot be mediated entirely by run or other pair-rule proteins as its dependence on run is incomplete and other pair-rule products have failed to exhibit an effect on stripe 8 expression (Baumgartner and Noll, 1990). The observation that prd remains largely inactive in the dorsal region of band 2 in hbr- embryos indicates an interaction of gap proteins with gene products activating prd along the dorsoventral axis.

In tll- embryos, the altered prd expression consists of a posteriorly extended stripe 6, a much broader, posteriorly shifted, stripe 7 with its maximum at 13% EL and a missing stripe 8 (D-F). In these mutant embryos, hbr is not activated in its posterior domain (Casanova, 1990; Brönner and Jäckle, 1991), kni expression expands posteriorly at relatively low levels to about 20% EL (Pankratz et al., 1989), the posterior domain of gt expression is shifted posteriorly by about 5% EL to extend between about 10% and 32% EL (Eldon and Pirrotta, 1991; Kraut and Levine, 1991a), while Kr expression remains unaffected (Hülskamp et al., 1990). Since the expression of no other gap gene is affected, the changed expression of stripes 6 and 7 can only be generated by the altered expressions of kni and gt in tll- embryos. We interpret these results as follows. In wild-type embryos, stripe 6 is activated by low concentrations of kni and gt proteins and limited by high kni protein anteriorly and high gt protein posteriorly (Table 1). In agreement with this hypothesis, the posterior shifts of kni and gt expression account for the observed posterior extension of stripe 6 in tll- embryos. In addition, stripe 7 is activated by gt only when kni protein has dropped below a very low threshold concentration. The observed posterior shift of the posterior limit of kni expression from 29% to 20% agrees well with the shift in position of the anterior boundary of prd stripe 7. Finally, consistent with the prd pattern in hbr- embryos, stripe 8 fails to appear since it depends on hbr which is not activated in tll- embryos.

In kni- embryos, Kr expression expands posteriorly by about 10% EL (Jäckle et al., 1986) and gt protein is reduced in the posterior gt domain (Eldon and Pirrotta, 1991; Kraut and Levine, 1991a) while the expression of other gap genes remains unchanged. In such embryos, prd fails to be repressed between stripes 4 and 5, between stripes 6 and 7, and to a lesser extent also between stripes 5 and 6 (G-I). Hence, the fused prd stripes 4 and 5 are activated in kni- as stripe 4 in wild-type embryos, by high concentrations of Kr protein (Table 1). Their anterior and posterior limits are determined by increasing hb protein above and decreasing Kr protein below certain threshold concentrations. Similar to stripe 7 in wild-type embryos, the fused prd stripes 6 and 7 are activated by gt protein in the absence of kni. The premature reduction of prd protein, first in the region of the fused stripes 4 and 5 and subsequently more posteriorly, is a late effect and might occur in response to altered expression patterns of pair-rule genes.

In Kr- embryos, prd stripes 2 and 3 are replaced by a single stripe with its peak slightly posterior to wild-type stripe 2 and not completely separated from its neighboring stripes 1 and 4. Stripe 1 is slightly reduced in intensity while stripes 5 and 6 appear with a delay and are later replaced by a broad stripe fused to stripe 4 but separated from stripe 7 (K-M). The observed alterations of gap gene expression in these mutant embryos consist of a considerable anterior expansion of the posterior gt domain to 50% EL and of a minor posterior shift by about 2% EL of the anterior gt domain (Eldon and Pirrotta, 1991; Kraut and Levine, 1991a). In addition, kni expression is reduced and its anterior limit shifted posteriorly by about 5% EL (Pankratz et al., 1989; Capovilla et al., 1992). The slight posterior shift of prd stripe 2 correlates well with that of the anterior gt domain and hence is consistent with its activation by low concentrations of gt protein (Table 1). Low activation or incomplete repression of prd posterior to stripe 2 is explained by the presence of hb and lack of Kr protein (Table 1). The formation of the central prd stripe in homozygous Kr embryos is induced by gt protein which activates prd below a certain low level of kni protein, similar to stripe 7 in wild-type embryos (Table 1). Activation of prd posterior to this central stripe and anterior to what appears a normal stripe 7 is first delayed due to relatively high kni to gt protein levels, and derepression occurs only after kni protein has disappeared from the most anterior portion of this region due to its repression by gt (Capovilla et al., 1992). In the middle of this region, kni (and gt) protein concentrations remain sufficiently high to activate prd while in the posterior portion, kni protein levels drop to low levels that repress prd in combination with high concentrations of gt protein (Table 1).

In homozygous gt embryos, kni expression expands posteriorly by about 4% EL (Eldon and Pirrotta, 1991; Kraut and Levine, 1991a) while expression of Kr (Gaul and Jäckle, 1987; Eldon and Pirrotta, 1991; Kraut and Levine, 1991b) and hb (Eldon and Pirrotta, 1991) remain unchanged. Since no effect on posterior hb expression, which depends on tll (Casanova, 1990; Brönner and Jäckle, 1991), is observed, we assume that the posterior tll domain is not affected either. Since prd stripes 1 and 2 are considerably reduced (N), they require gt protein for full activation (Table 1). Stripe 1 is later activated to high levels by low concentrations of eve protein, on which it depends at this time (see above), while the slight anterior extension of stripe 2 (by one nucleus) is probably due to its activation by hb in the absence of gt (O). The posterior extension of the kni domain results in a slightly enlarged gap posterior to stripe 5 due to high kni protein concentrations repressing prd while, more posteriorly, lower kni protein concentrations activate the fused stripes 6 and 7 in the absence of gt (Table 1). Finally, activation of stripe 8 is delayed in the absence of gt protein (P), indicating that its initial activation depends on both gt and hb protein (Table 1). Since, in this case, the dependence on gt is incomplete, gt might activate stripe 8 indirectly via run (see above).
and Levine, 1991a), have been omitted. Similarly, the initial patterns of prd protein during phase A1.1 (corresponding to Fig. 1A-C) and the beginning of phase A1.2 (Fig. 1D,E), leading to stripes 1 to 7 of the pair-rule pattern, are illustrated below in panels A-E. In addition, the early position of prd stripe 8 (Fig. 1I), which is activated only during phase A1.3, is inserted as dotted line in panel E. For prd protein patterns, times of development at 25°C since the onset of cellularization have been estimated by comparison with studies by Foe and Alberts (1983) and are indicated (in minutes) in parentheses. The locations of the mandibular (MD), maxillary (MX), labial (LB), thoracic (T1-T3), and first seven abdominal segment anlagen (A1-A7) are shown at the bottom.

effects on early prd expression, activation of prd in stripes 2 to 7 remains largely unaffected in all three primary pair-rule mutants. Therefore, primary pair-rule proteins are not involved in the initial activation of stripes 2 to 7.

In two instances, however, primary pair-rule mutants show clear effects on the initial activation of prd. In eve− embryos, the anteriormost portion of stripe 1-2, which would later resolve as stripe 1 in wild-type embryos, fails to accumulate high levels of prd protein, suggesting that eve protein is required for high levels of prd expression in stripe 1. Since this effect is apparent only a few minutes after the onset of cellularization (at a stage shown in Fig. 1C), at which eve is expressed at low levels in a broad anterior domain (Frasch and Levine, 1987; Yu and Pick, personal communication; our own unpublished observations), we conclude that relatively low concentrations of eve protein influence prd expression dramatically. The second effect of a primary pair-rule gene on the initial activation of a prd stripe is observed in run− embryos in which stripe 8 is only weakly activated. Hence, activation of stripe 8 depends strongly, though not completely, on run protein.

The principal effects of primary pair-rule mutants on prd expression occur after the initial activation of stripes and concern the modulation of the initial pair-rule stripes as well as their conversion to the segment-polarity stripes (Baumgartner and Noll, 1990). For example, in eve− embryos prd protein disappears prematurely from the posterior portions of stripes 2 to 7. This effect may be explained by a mechanism similar to that of stripe 1 activation, namely an activation of prd by eve. The two effects are separated in time because eve is initially expressed in an anterior broad band in a region roughly corresponding to that of prd stripe 1-2 and only later appears in more posterior stripes (Macdonald et al., 1986; Frasch and Levine, 1987; Yu and Pick, personal communication; our own unpublished results).

Another effect in eve− embryos consists in the continued expression of prd in the early interstripes. This effect, combined with the apparently normal repression of prd in the late interstripes (splitting of early stripes 2 to 7) and the premature disappearance of prd in the posterior portions of stripes 2 to 7, generates a late prd pattern of equally wide and spaced stripes with a double-segment periodicity (Baumgartner and Noll, 1990). Thus, eve protein influences the early prd pattern in two ways: it is required (i) to activate stripe 1 and (ii) to repress prd in the early interstripes. These opposing roles in prd regulation of eve protein probably depend on its interaction with gap and/or other pair-rule gene products.

Also in the absence of run product, most effects on prd expression consist of modulations of the stripe pattern, such as the failure to repress prd between stripes 1 and 2. Later, at cellular blastoderm and subsequent stages, no late interstripes appear (see also Baumgartner and Noll, 1990). The delayed and altered order of appearance of stripes 3 to 7 and the irregular spacing of these stripes in run− embryos suggest that run protein interacts with prd-activating factors, such as gap gene products, to modify their action in defining the timing and precise position at which prd is expressed. Similar effects have been observed on ftz (Carroll and Scott, 1986) and eve expression in run− embryos (Frasch and Levine, 1987).

The absence of h product does not have a strong impact on early prd expression. However, during late cellularization, gastrulation and early germ band extension, absence
of h protein exhibits a similar, but delayed effect on prd expression as observed in eve− embryos. This is apparent from the delayed decay of stripe 1 and the delayed reduction in width of the posterior stripes in h− embryos. This delay is easily understood by assuming that h acts via eve on prd expression, which is strongly supported by the observation that h is required for continued rather than initial eve expression (Frasch and Levine, 1987). Hence, the effect on prd expression of missing eve product is delayed in h− as compared to eve− embryos.

A model of initial activation of paired by gap proteins

As the initial activation of prd during phase A1 remains largely unaffected by primary pair-rule gene products, it seems probable that it occurs through the action of gap genes. Fig. 6 depicts schematically how the initial pattern of prd expression evolves in stripes 1 to 7 during phase A1.I and the beginning of phase A1.2 as shown in Fig. 1A-E. In addition, the patterns of the gap proteins hb, Kr, kni, gt and tll, and of the maternal bicoid (bcd) protein are shown at the onset of cellularization. We propose that prd is activated by different combinations of gap proteins active above certain threshold concentrations as indicated in Table 1. Such an activation by gap and maternal genes has been demonstrated previously for individual stripes of the primary pair-rule genes h and eve. A model emerged in which different combinations and threshold concentrations of gap proteins and of the maternal bcd protein interact with multiple copies of specific cis-regulatory sequences of h and eve to determine their activation or repression (Stanojevic et al., 1989; Pankratz et al., 1990; Howard and Struhl, 1990; Small et al., 1991; Riddihough and Ish-Horowicz, 1991).

If prd is activated by a similar mechanism, prd expression must be consistent with the known preceding expression of gap genes and possibly bcd. In agreement with the expression patterns shown in Fig. 6 and documented in the literature and in Fig. 1, Table 1 demonstrates that it is indeed possible to explain the initial prd activation by the preceding activities of gap genes. For example, the sequence of appearance of stripes 3 to 7 parallels the rising concentrations of gt, Kr and kni proteins: while gt protein activates prd in the regions of stripe 1-2 and 6-7 and Kr protein activates prd stripes 3-4, low concentrations of kni protein repress prd at relatively high levels of Kr protein (Fig. 6D). Subsequently, increasing kni protein levels begin to activate stripe 5 at relatively low concentrations of Kr protein whereas no activation occurs in the absence of Kr product between stripes 5 and 6 (Fig. 6E). Similarly, prd is repressed at relatively high gt protein concentrations between stripes 6 and 7 by low kni protein levels extending posteriorly (Fig. 6E).

In contrast, it is not yet clear how prd is initially activated at very low levels in the anterior ‘cap’ (Fig. 1A), which appears unrelated to the following patterns of prd activation. A possible explanation might be its low activation by maternal hb protein - as it seems to occur more posteriorly at subsequent stages in Kr− embryos as argued below - and its subsequent repression by rising bicoid protein levels.

Model of paired activation is consistent with altered expression patterns in gap mutants

If the model explaining the initial activation of prd by gap gene products (Table 1, Fig. 6) is correct, then in a particular gap mutant it has to be consistent with the changed initial expression of prd, taking into account both the absence of that gap protein and the documented altered early expression patterns of all other gap genes in that gap mutant (Jäckle et al., 1986; Pankratz et al., 1989; Casanova, 1990; Hälskamp et al., 1990; Brönner and Jäckle, 1991; Eldon and Pirrotta, 1991; Kraut and Levine, 1991a; Capovilla et al., 1992). An analysis of these altered initial expression patterns of prd in gap mutants shows that the model indeed fulfills this criterion, as explained in detail in the legend to Fig. 5. Moreover, the changes also correlate with respect to timing and hence are consistent with a direct activation of prd by gap gene products. In contrast, the reported early expression patterns of primary pair-rule genes in gap mutants show no correlation with the initial expression of prd in these mutants.

While we find an excellent correlation between gap gene activities and the initial activation of prd in stripes 2 to 8 of wild-type and gap mutant embryos, no evidence was

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<th>Table 1. Model of paired activation in pair-rule stripes by different combinations and threshold concentrations of gap gene products</th>
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The proposed activation of prd stripes 1 to 8 by different combinations and relative concentrations of gap proteins is consistent with the observed levels of gap proteins in the stripe and early interstripe regions of wild-type embryos as illustrated in Fig. 6. As explained in the legend to Fig. 5, the postulated effects of these combinations and concentrations on prd activity are also consistent with the changes observed in gap mutants during early expression of prd under the influence of altered distributions of gap proteins. Only the requirements for activation by gap proteins are listed, the early dependence on eve and run protein to activate stripes 1 and 8, respectively, and on run to resolve stripe 1 from 2 by repression are not included. Moreover, we assume that the requirement for the tll protein to activate stripe 8 is indirect, reflecting the requirement for activation by hb in this region (Casanova, 1990; Brönner and Jäckle, 1991).
obtained for a direct participation of gap proteins in the activation of stripe 1 and its subsequent separation from stripe 2. As discussed above, high-level activation of stripe 1 depends on low concentrations of eve protein whereas repression between stripes 1 and 2, which occurs relatively late (Fig. 1D-G), depends on run protein. High concentrations of eve protein are further required to repress prd between stripes (Fig. 4B,C; Baumgartner and Noll, 1990). We also find a good correlation of low levels of prd with high concentrations of eve protein in gap mutants at cellular blastoderm (Fig. 5; Frasch and Levine, 1987), supporting the notion that eve protein acts as a repressor of prd during late cellularization. However, with the exception of stripe 1, eve is clearly not required for the initial activation of the prd stripes (Fig. 4A,B).

Are all pair-rule genes initially activated by gap genes?

The observation that the initial activation of the tertiary pair-rule gene prd is not influenced by the products of pair-rule genes but rather by those of gap genes suggests that not only primary but also secondary and tertiary pair-rule genes are initially activated by gap genes. Hence, we envision that all initial pair-rule expression patterns are set up by gap and maternal genes and are only modulated by primary pair-rule gene activities. This subsequent modulation is more complex for tertiary pair-rule genes like prd (Baumgartner and Noll, 1990) than for primary and secondary pair-rule genes. The distinction between primary, secondary and tertiary pair-rule genes consists thus in the degree of modulation by other pair-rule genes of their later expression patterns rather than in a direct or indirect regulation by gap genes. For example, the expression of the primary pair-rule gene eve is modulated only by the primary pair-rule genes h and run, yet remains unaffected by the remaining, secondary and tertiary, pair-rule genes (Frasch and Levine, 1987). By this definition of pair-rule gene categories, based on the hierarchical interaction among pair-rule genes, we do not wish to exclude the possibility that pair-rule genes of a lower category affect the expression patterns of primary or secondary pair-rule genes. However, if such effects exist, we would predict them to be minor and to occur relatively late (germ band extension).

Support for the proposal that all pair-rule genes are initially activated by gap rather than primary pair-rule proteins comes from experiments which demonstrate that the initial activation of the pair-rule gene ftz, previously considered as secondary pair-rule gene regulated by primary pair-rule genes, in seven stripes is independent of primary pair-rule genes (Yu and Pick, personal communication).

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REFERENCES


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