Development 117, 609-623 (1993)
Printed in Great Britain © The Company of Biologists Limited 1993

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

Thomas Gutjahr*, Erich Frei and Markus Noll

Institute for Molecular Biology II, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

*Present address: Laboratory of Biochemistry and Molecular Biology, the Rockefeller University, New York, NY 10021, USA

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 metamerization, 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 metamerization, 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 *ftz* gene lacking the CNS-specific regulatory element in a *ftz*$^-$ 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 *paired* (*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, *prd* is expressed in an unusually dynamic manner, showing both pair-rule and segment-polarity transcript patterns (Kilchherr et al., 1986). Finally, *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 antiserum was used in a detailed study of prd protein expression throughout embryogenesis. To clarify whether the pair-rule pattern of prd results from activation by primary pair-rule proteins or gap gene products, *prd* expression was examined in all gap mutants and in embryos deficient for any one of the primary pair-rule genes *eve*, *hairy* (*h*) or *runt* (*run*). Our results suggest that the initial activation of *prd* in a pair-rule pattern of eight stripes occurs through the products of the gap genes *hunchback* (*hb*), *Krüppel* (*Kr*), *knirps* (*kni*) and *giant* (*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 *prd*- and *engrailed* (*en*)-expressing cells was determined with single cell resolution. Finally, we have shown that 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 prd protein was constructed by cloning a 1.95 kb *Bam*HI fragment, generated by partial digestion of a c7340.4 *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 *Hin*dIII fragment from a c7340.4 *prd* cDNA subclone in pGEM-2 into pTRB0. A plasmid expressing the 'box-less' prd protein (C-terminal half of prd protein without N-terminal paired- and homeodomain) was constructed by ligating a 1.3 kb *Pvu*II-*Sma*I fragment, obtained from a c7340.6 *prd* cDNA subclone in pGEM-2, into the blunt-ended *Bam*HI site of the vector pAR3039. Expression of the plasmids in *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-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-HCl, pH 8.0) containing 1 M NaCl; 3M NaCl; 50 mM NaCl, 1% Triton X-100; and finally in H_2O. 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 antiserum

White New Zealand rabbits were immunized with the full-length prd protein (150 µg per injection) dialyzed against buffer A containing 2 M urea. The crude anti-prd antiserum 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' 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-HCl, pH 8.5 to neutralize the eluate. The eluate was dialyzed against PBS. Antibodies from the 50 ml dialyzate were concentrated by ammonium sulfate precipitation (44% saturated at 4°C), dissolved in 5 ml of PBS, dialyzed overnight twice against 2 liters of 0.2× 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 antiserum, 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_2HPO_4/NaH_2PO_4, pH 7.8, 100 mM NaCl, 5mM $MgCl_2$, 0.05% Tween-20). The affinity-purified antiserum 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 antiserum 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-HCl, pH 7.1, 0.2% Ni_2Cl, 0.003% H_2O_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 Axiophot. Kodak black and white T-MAX 100 film was developed for lowest contrast using Microdol-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 ascites 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^{2}*/SM1;* kni^{IID}*/TM3, Sb;* tll^{L10}*/TM3, Sb; st* hb^{14F} *e/TM3, Sb;* gt^{YA} *w/FM7;* otd^{YH}*/FM7; st* ems^{10A37} *e/TM3, Sb; y* btd^{IIIA}*/FM7;* $Df(2R)eve^{1.27}$*, cn sca bw sp/CyO;* $Df(1)run^{III2}$*, y* f^{36a}*/FM7/Y* mal^{+} y^{+} (all kindly provided by the Tübingen stock center), $Df(3L)h^{i22}$*, Ki roe* p^{p}*/TM3* (kindly provided by W. Gehring), and *st* hkb^{2} p^{p} *ca/TM3*, Sb (a generous gift of H. Jäckle). The allele used to test the specificity of the prd antiserum was $Df(2L)prd^{1.7}$*/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 gooseberry 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 subphases (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 circumference (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-

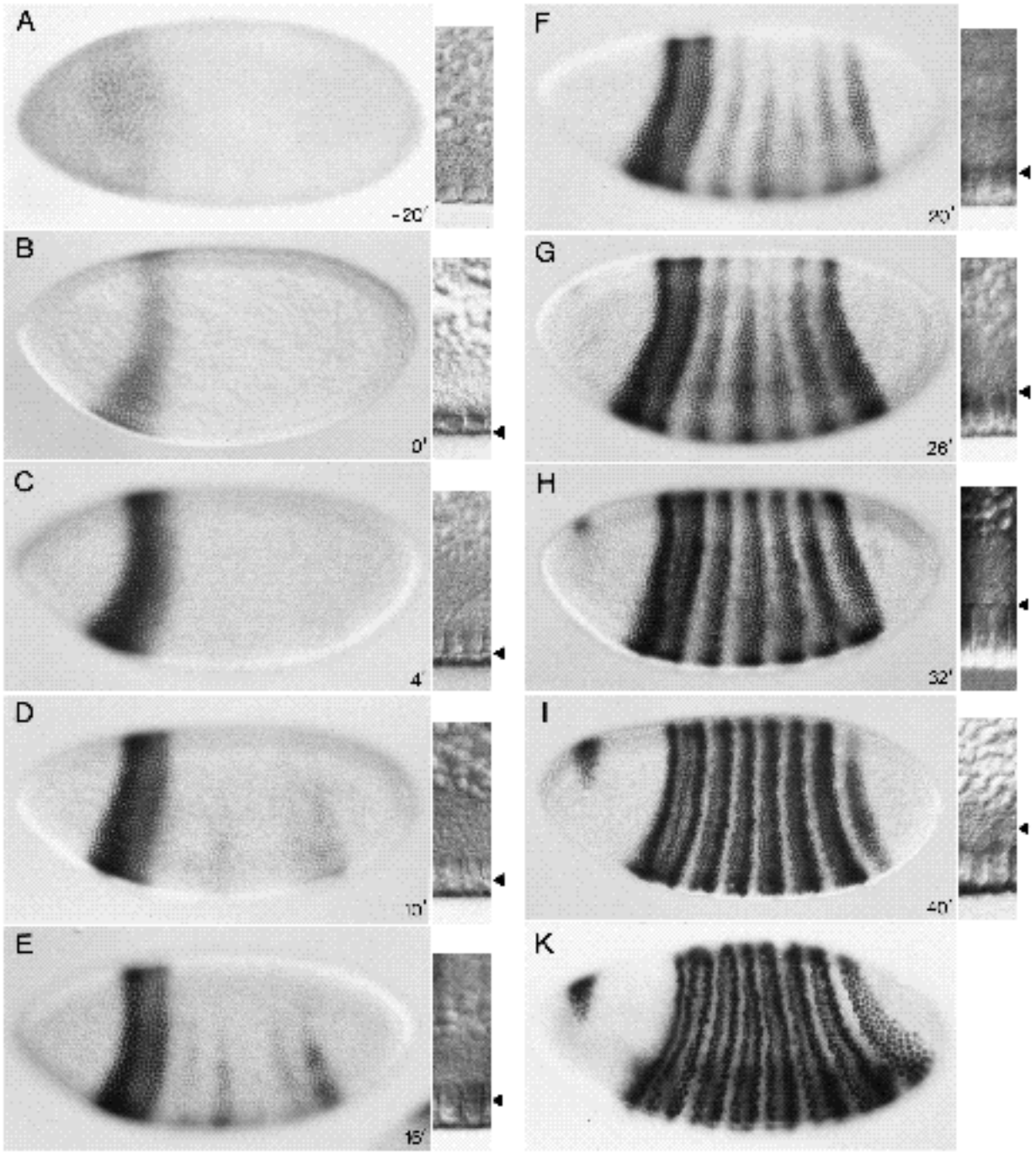
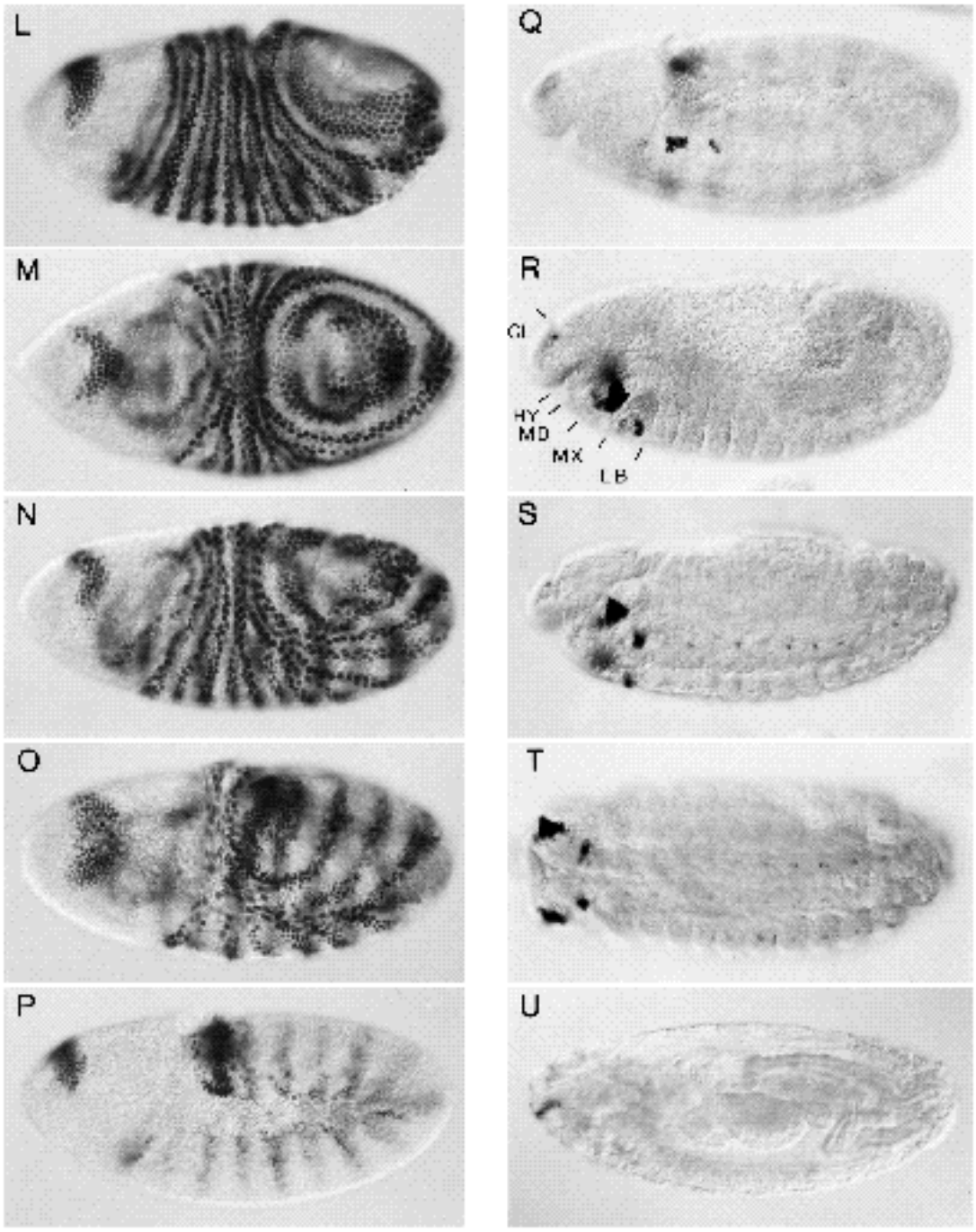


Fig. 1. Expression of prd protein in wild-type embryos during embryogenesis. Whole-mount preparations of fixed wild-type embryos were stained with purified anti-prd antiserum. The panels show successive stages of embryos, prior to the 13th nuclear division (A), just after the 13th nuclear division (B), 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 begin to differentiate, prd stripes have almost completely disappeared except for the dorsal stripe 8 and the anterior dorsal spot which are still detectable (Fig. 1Q).

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-

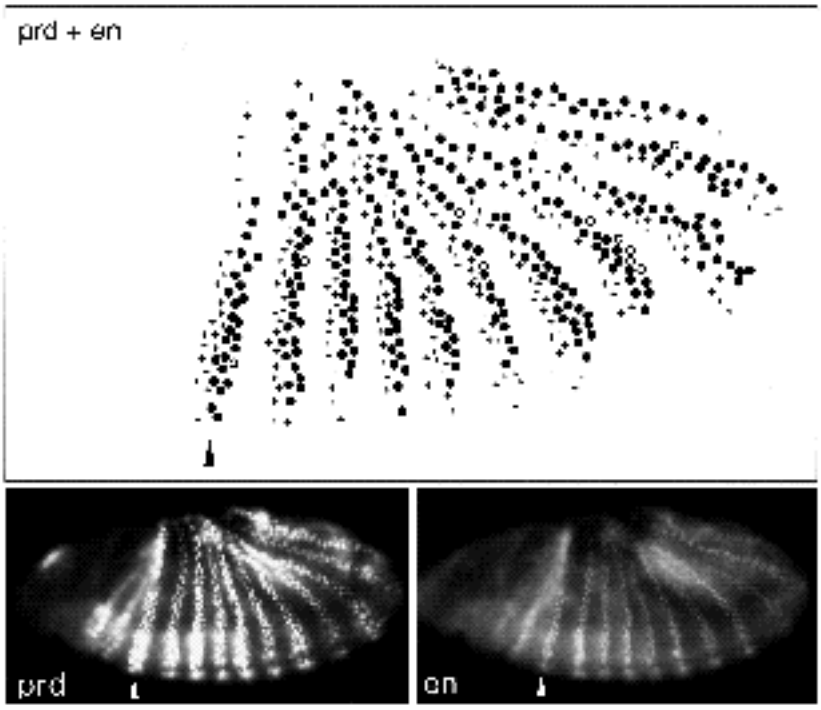


Fig. 2. Expression of *prd* relative to *en* at gastrulation. Embryos were stained with rabbit anti-prd (revealed by TRITC) and mouse anti-en antiserum (FITC) as described in Materials and methods. Black and white photographs were taken from the same embryo. The photographs were aligned and *prd* and *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. 1I,K). The observed late pattern (Fig. 4H) is consistent with a model of *prd* regulation by *h*, which predicts that *prd*

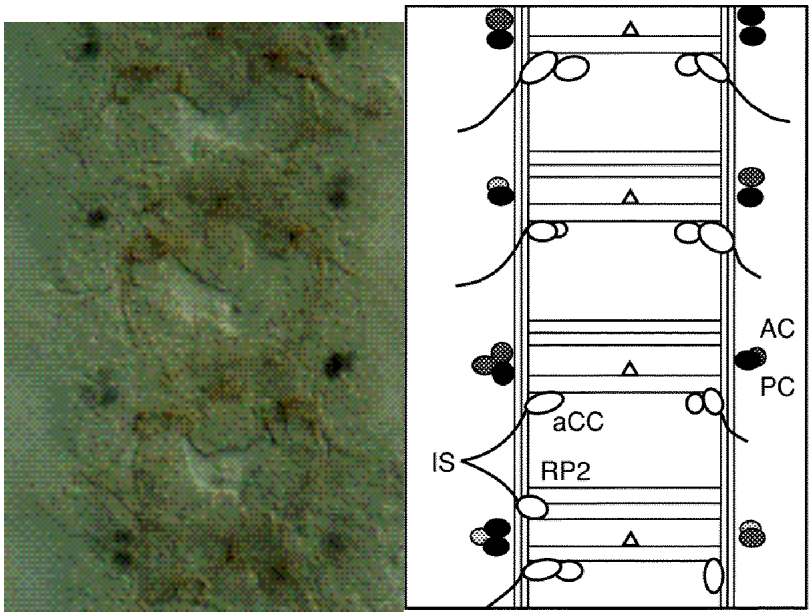


Fig. 3. Expression of *prd* in specific cells of the CNS. Dorsal view of a portion of a dissected CNS of an early stage 14 embryo (Campos-Ortega and Hartenstein, 1985). Embryos were stained with anti-prd (black) and mAb 22C10 (brown). The latter was used to reveal aCC, RP1 and RP2 cells (Goodman et al., 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 triangels 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

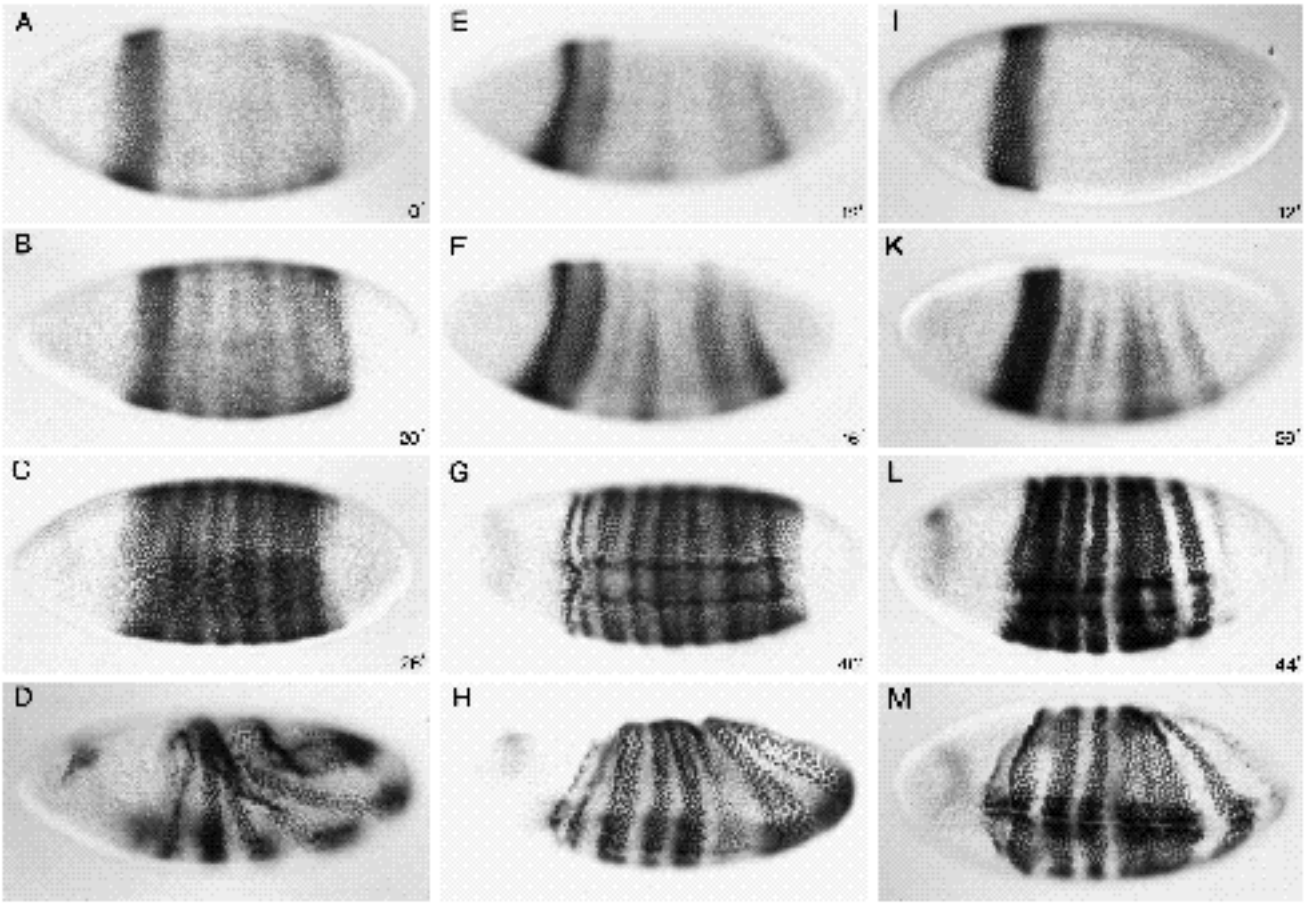


Fig. 4. Expression of prd protein in primary pair-rule mutants. Whole-mount preparations of fixed *eve*$^-$ (A-D), *h*$^-$ (E-H), and *run*$^-$ (I-M) embryos of various stages were stained with anti-prd antiserum as in Fig. 1. Ventrolateral (A-C, G-I, L,M) or lateral views (D-F,K) of embryos are shown 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* (*tll*) (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 *tll* 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 *gt*$^-$ 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 (Kilchherr 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 anteroposterior 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*, *opa* 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 anteroposterior 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 anteroposterior 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

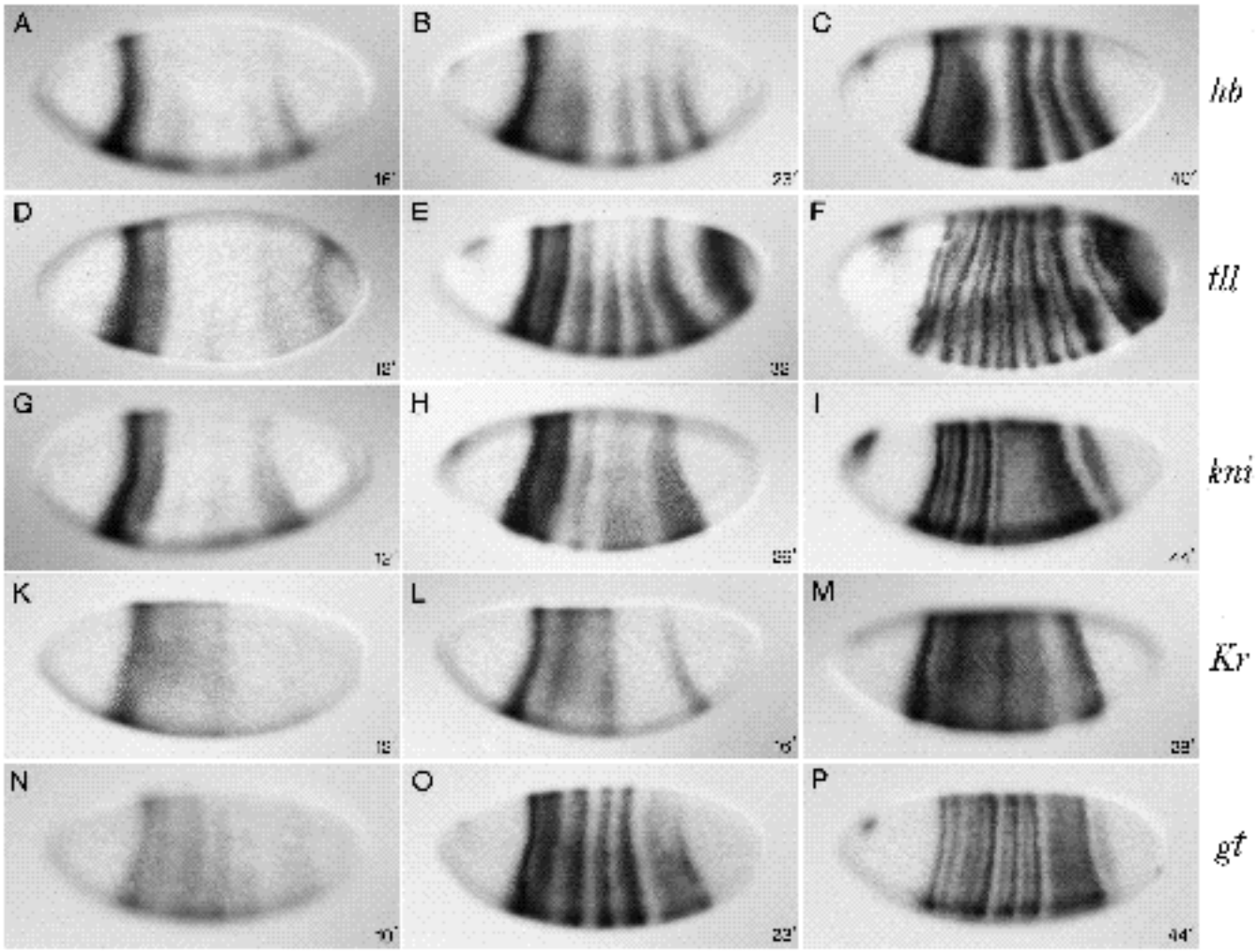


Fig. 5

Fig. 5. Expression of prd protein in gap mutants. Whole-mount preparations of fixed *hb*⁻ (A-C), *tll*⁻ (D-F), *kni*⁻ (G-I), *Kr*⁻ (K-M), and *gt*⁻ (N-P) embryos during cellularizition or early gastrulation (F) were stained with anti-prd antiserum 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 *hb*⁻ 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 *hb*⁻ embryos (**A-C**) are consistent with the postulated effect of gap proteins on *prd* expression (Table 1): (i) below a threshold concentration of hb protein, Kr protein activates *prd*, generating the broad prd band with its maximum coinciding with that of Kr protein in *hb*⁻ 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 *hb*⁻ 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 hb 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 *hb*⁻ 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, *hb* 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 *hb*⁻ embryos, stripe 8 fails to appear since it depends on *hb* 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 is 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 expansion 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).

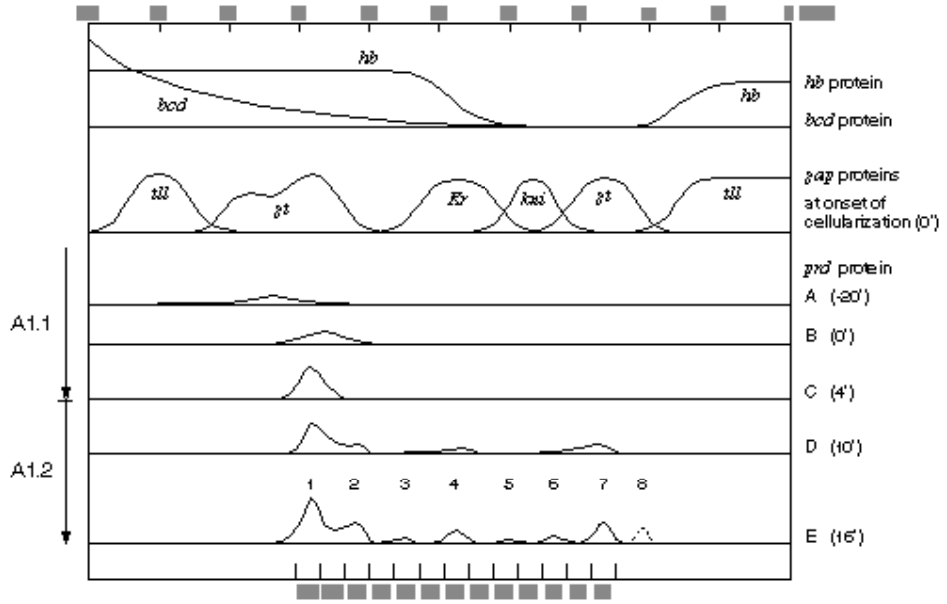


Fig. 6. Schematic illustration of the initial *prd* expression patterns and their relation to those of gap genes and *bcd*. The two top panels illustrate the amount of gene products (in arbitrary units on the ordinate) of the maternal gene *bcd* (Driever and Nüsslein-Volhard, 1988) and the gap genes *hb* (Tautz, 1988; Gaul and Jäckle, 1989; Kraut and Levine, 1991a), *Kr* (Gaul and Jäckle, 1989), *kni* (Pankratz et al., 1989; Kraut and Levine, 1991a), *gt* (Mohler et al., 1989; Eldon and Pirrotta, 1991; Kraut and Levine, 1991a) and *tll* (Pignoni et al., 1990) along the anteroposterior axis (in % EL on the abscissa) at the onset of cellularization. For simplicity, the most anterior *gt* domain (Eldon and Pirrotta, 1991) and the anterior *kni* domain, which extends between the two peaks of the anterior *gt* domain (Eldon and Pirrotta, 1991; Kraut 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 A1.1 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; Hülskamp et al., 1990; Brönner and Jäckle, 1991; Eldon and Pirrota, 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

Table 1. Model of *paired* activation in pair-rule stripes by different combinations and threshold concentrations of gap gene products

	gap protein concentrations along A-P axis (paired stripes 1-8)															
	1		2		3		4		5		6		7		8	
giant	high	moderate	low	–	–	–	–	–	–	–	low	high	high	low	low	–
hunchback	high	high	high	high	high	high	low	–	–	–	–	–	–	–	low	low
Krüppel	–	–	–	low	moderate	high	high	high	low	–	–	–	–	–	–	–
knirps	–	–	–	–	–	–	–	low	high	high	low	low	–	–	–	–
paired activation	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–
position along A-P axis (% EL)	70		60			50			40			30			20	

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).

We thank Fritz Ochsenbein for his generous help in preparing the figures and Patrick Spielmann for expert technical assistance, Theo Staehelin and Tom Rolink for their advice in antibody techniques, R. Smith, M. Wilcox, K. Coleman, and T. Kornberg for generously providing en antibody, N. Patel for providing 22C10 mAb and helping with the 22C10/prd double-labeling as well as teaching T. G. the technique of CNS dissection. The 22C10/prd double-labeling experiment was performed in Corey Goodman's lab, to whom we are very grateful for his generous support. We thank the Tübingen stock center, W. Gehring and H. Jäckle for gap and pair-rule mutant stocks. We would also like to thank Xuelin Li for discussions and Leslie Pick for comments on the manuscript. We are deeply indebted to Leslie Pick for her continued interest in this project, intellectual stimulation and support. This work has been supported by the Swiss National Science Foundation grant 31-26652.89 and by the Kanton Zürich.

REFERENCES

Akam, M. (1987). The molecular basis for metameric pattern in the *Drosophila* embryo. *Development* **101**, 1-22.

Baumgartner, S. and Noll, M. (1990). Network of interactions among pair-rule genes regulating *paired* expression during primordial segmentation of *Drosophila*. *Mech. Dev.* **33**, 1-18.

Bopp, D., Burri, M., Baumgartner, S., Frigerio, G. and Noll, M. (1986). Conservation of a large protein domain in the segmentation gene *paired* and in functionally related genes of *Drosophila*. *Cell* **47**, 1033-1040.

Bopp, D., Jamet, E., Baumgartner, S., Burri, M. and Noll, M. (1989). Isolation of two tissue-specific *Drosophila* paired box genes, *pox meso* and *pox neuro. EMBO J.* **8**, 3447-3457.

Brönner, G. and Jäckle, H. (1991). Control and function of terminal gap gene activity in the posterior pole region of the *Drosophila* embryo. *Mech. Dev.* **35**, 205-211.

Bürglin, T. R. and De Robertis, E. M. (1987). The nuclear migration signal of *Xenopus leaevis* nucleoplasmin. *EMBO J.* **6**, 2617-2625.

Burri, M., Tromvoukis, Y., Bopp, D., Frigerio, G. and Noll, M. (1989). Conservation of the paired domain in metazoans and its structure in three isolated human genes. *EMBO J.* **8**, 1183-1190.

Campos-Ortega, J. A. and Hartenstein, V. (1985). *The Embryonic Development of* Drosophila melanogaster. Berlin/Heidelberg/New York/Toronto: Springer-Verlag.

Capovilla, M., Eldon, E. D. and Pirrotta, V. (1992). The *giant* gene of *Drosophila* encodes a b-ZIP DNA-binding protein that regulates the expression of other segmentation gap genes. *Development* **114**, 99-112.

Carroll, S. B. and Scott, M. P. (1986). Zygotically active genes that affect the spatial expression of the *fushi tarazu* segmentation gene during early *Drosophila* embryogenesis. *Cell* **45**, 113-126.

Casanova, J. (1990). Pattern formation under the control of the terminal system in the *Drosophila* embryo. *Development* **110**, 621-628.

Cohen, S. M. and Jürgens, G. (1990). Mediation of *Drosophila* head development by gap-like segmentation genes. *Nature* **346**, 482-485.

Coulter, D. and Wieschaus, E. (1986). Segmentation genes and the distributions of transcripts. *Nature* **321**, 472-474.

Coulter, D. E., Swaykus, E. A., Beran-Koehn, M. A., Goldberg, D., Wieschaus, E. and Schedl, P. (1990). Molecular analysis of *odd-skipped*, a zinc finger encoding segmentation gene with a novel pair-rule expression pattern. *EMBO J.* **9**, 3795-3804.

DiNardo, S. and O'Farrell, P. H. (1987). Establishment and refinement of segmental pattern in the *Drosophila* embryo: spatial control of *engrailed* expression by pair-rule genes. *Genes Dev.* **1**, 1212-1225.

Doe, C. Q., Hiromi, Y., Gehring, W. J. and Goodman, C. S. (1988a). Expression and function of the segmentation gene *fushi tarazu* during *Drosophila* neurogenesis. *Science* **239**, 170-175.

Doe, C. Q., Smouse, D. and Goodman, C. S. (1988b). Control of neuronal fate by the *Drosophila* segmentation gene *even-skipped*. *Nature* **333**, 376-378.

Dressler, G. R., Deutsch, U., Balling, R., Simon, D., Guenet, J.-L. and Gruss, P. (1988). Murine genes with homology to *Drosophila* segmentation genes. *Development* **104 Supplement**, 181-186.

Driever, W. and Nüsslein-Volhard, C. (1988). A gradient of *bicoid* protein in *Drosophila* embryos. *Cell* **54**, 83-93.

Eldon, E. D. and Pirrotta, V. (1991). Interactions of the *Drosophila* gap gene *giant* with maternal and zygotic pattern-forming genes. *Development* **111**, 367-378.

Fjose, A., McGinnis, W. J. and Gehring, W. J. (1985). Isolation of a homeo box-containing gene from the *engrailed* region of *Drosophila* and the spatial distribution of its transcripts. *Nature* **313**, 284-289.

Foe, V. and Alberts, B. M. (1983). Studies of nuclear and cytoplasmic behaviour during the five mitotic cycles that precede gastrulation in *Drosophila* embryogenesis. *J. Cell Sci.* **61**, 31-70.

Frasch, M., Hoey, T., Rushlow, C., Doyle, H. and Levine, M. (1987). Characterization and localization of the *even-skipped* protein of *Drosophila*. *EMBO J.* **6**, 749-759.

Frasch, M. and Levine, M. (1987). Complementary patterns of *even-skipped* and *fushi tarazu* expression involve their differential regulation by a common set of segmentation genes in *Drosophila*. *Genes Dev.* **1**, 981-995.

Frigerio, G., Burri, M., Bopp, D., Baumgartner, S. and Noll, M. (1986). Structure of the segmentation gene *paired* and the *Drosophila* PRD gene set as part of a gene network. *Cell* **47**, 735-746.

Gaul, U. and Jäckle, H. (1987). Pole region-dependent repression of the *Drosophila* gap gene *Krüppel* by maternal gene products. *Cell* **51**, 549-555.

Gaul, U. and Jäckle, H. (1989). Analysis of maternal effect mutant combinations elucidates regulation and function of the overlap of *hunchback* and *Krüppel* gene expression in the *Drosophila* blastoderm embryo. *Development* **107**, 651-662.

Goodman, C. S., Bastiani, M. J., Doe, C. Q., du Lac, S., Helfand, S. L., Kuwada, J. Y. and Thomas, J. B. (1984). Cell recognition during neuronal development. *Science* **225**, 1271-1279.

Harding, K., Rushlow, C., Doyle, H. J., Hoey, T. and Levine, M. (1986). Cross-regulatory interactions among pair-rule genes in *Drosophila. Science* **233**, 953-959.

Howard, K. R. and Struhl, G. (1990). Decoding positional information: regulation of the pair-rule gene *hairy*. *Development* **110**, 1223-1231.

Hülskamp, M., Pfeifle, C. and Tautz, D. (1990). A morphogenetic gradient of *hunchback* protein organizes the expression of the gap genes *Krüppel* and *knirps* in the early *Drosophila* embryo. *Nature* **346**, 577-580.

Ingham, P. W. (1988). The molecular genetics of embryonic pattern formation in *Drosophila*. *Nature* **335**, 25-34.

Ingham, P. and Gergen, P. (1988). Interactions between the pair-rule genes *runt*, *hairy*, *even-skipped* and *fushi tarazu* and the establishment of periodic pattern in the *Drosophila* embryo. *Development* **104 Supplement**, 51-60.

Ingham, P. W. and Martinez-Arias, A. (1986). The correct activation of *Antennapedia* and bithorax complex genes requires the *fushi tarazu* gene. *Nature* **324**, 592-597.

Ingham, P. W. and Martinez-Arias, A. (1992). Boundaries and fields in early embryos. *Cell* **68**, 221-235.

Jäckle, H., Tautz, D., Schuh, R., Seifert, E. and Lehmann, R. (1986). Cross-regulatory interactions among the gap genes of *Drosophila. Nature* **324**, 668-670.

Kania, M. A., Bonner, A. S., Duffy, J. B. and Gergen, J. P. (1990). The *Drosophila* segmentation gene *runt* encodes a novel nuclear regulatory protein that is also expressed in the developing nervous system. *Genes Dev.* **4**, 1701-1713.

Kilchherr, F., Baumgartner, S., Bopp, D., Frei, E. and Noll, M. (1986). Isolation of the *paired* gene of *Drosophila* and its spatial expression during early embryogenesis. *Nature* **321**, 493-499.

Kornberg, T. (1981). Compartments in the abdomen of *Drosophila* and the role of the *engrailed* locus. *Dev. Biol.* **86**, 363-372.

Kornberg, T., Siden, I., O'Farrell, P. and Simon, M. (1985). The *engrailed* locus of *Drosophila*: *in situ* localization of transcripts reveals compartment-specific expression. *Cell* **40**, 45-53.

Kraut, R. and Levine, M. (1991a). Spatial regulation of the gap gene *giant* during *Drosophila* development. *Development* **111**, 601-609.

Kraut, R. and Levine, M. (1991b). Mutually repressive interactions between the gap genes *giant* and *Krüppel* define middle body regions of the *Drosophila* embryo. *Development* **111**, 611-621.

Lawrence, P. A., Johnston, P., Macdonald, P. and Struhl, G. (1987). Borders of parasegments in *Drosophila* embryos are delimited by the *fushi tarazu* and *even-skipped* genes. *Nature* **328**, 440-442.

Macdonald, P. M., Ingham, P. and Struhl, G. (1986). Isolation, structure, and expression of *even-skipped*: a second pair-rule gene of *Drosophila* containing a homeo box. *Cell* **47**, 721-734.

Mohler, J., Eldon, E. D. and Pirrotta, V. (1989). A novel spatial transcription pattern associated with the segmentation gene, *giant*, of *Drosophila*. *EMBO J.* **8**, 1539-1548.

Morrissey, D., Askew, D., Raj, L. and Weir, M. (1991). Functional dissection of the *paired* segmentation gene in *Drosophila* embryos. *Genes Dev.* **5**, 1684-1696.

Nüsslein-Volhard, C. and Wieschaus, E. (1980). Mutations affecting segment number and polarity in *Drosophila*. *Nature* **287**, 795-801.

Pankratz, M.J. and Jäckle, H. (1990). Making stripes in the *Drosophila* embryo. *Trends Gen.* **6**, 287-292.

Pankratz, M. J., Hoch, M., Seifert, E. and Jäckle, H. (1989). *Krüppel* requirement for *knirps* enhancement reflects overlapping gap gene activities in the *Drosophila* embryo. *Nature* **341**, 337-340.

Pankratz, M. J., Seifert, E., Gerwin, N., Billi, B., Nauber, U. and Jäckle, H. (1990). Gradients of *even-skipped* and *knirps* gene products direct pair-rule gene stripe patterning in the posterior region of the *Drosophila* embryo. *Cell* **61**, 309-317.

Patel, N. H., Schafer, B., Goodman, C.S. and Holmgren, R. (1989). The role of segment-polarity genes during *Drosophila* neurogenesis. *Genes Dev.* **3**, 890-904.

Pignoni, F., Baldarelli, R. M., Steingrimsson, E., Diaz, R. J., Patapoutian, A., Merriam, J. R. and Lengyel, J. A. The *Drosophila* gene *tailless* is expressed at the embryonic termini and is a member of the steroid receptor superfamily. *Cell* **62**, 151-163.

Ridddihough, G. and Ish-Horowicz, D. (1991). Individual stripe regulatory elements in the *Drosophila hairy* promoter respond to maternal, gap, and pair-rule genes. *Genes Dev.* **5**, 840-854.

Roth, S., Stein, D. and Nüsslein-Volhard, C. (1989). A gradient of nuclear localization of the *dorsal* protein determines dorsoventral pattern in the *Drosophila* embryo. *Cell* **59**, 1189-1202.

Rushlow, C. A., Han, K., Manley, J. L. and Levine, M. (1989). The graded distribution of the *dorsal* morphogen is initiated by selective nuclear transport in *Drosophila. Cell* **59**, 1165-1177.

Small, S., Kraut, R., Hoey, T., Warrior, R. and Levine, M. (1991). Transcriptional regulation of a pair-rule stripe in *Drosophila*. *Genes Dev.* **5**, 827-839.

Stanojevic, D., Hoey, T. and Levine, M. (1989). Sequence-specific DNA-binding activities of the gap proteins encoded by *hunchback* and *Krüppel* in *Drosophila*. *Nature* **341**, 331-335.

Steward, R. (1989). Relocalization of the *dorsal* protein from the cytoplasm to the nucleus correlates with its function. *Cell* **59**, 1179-1188.

St Johnston, D. and Nüsslein-Volhard, C. (1992). The origin of pattern and polarity in the *Drosophila* embryo. *Cell* **68**, 201-219.

Strecker, T. R., Kongsuwan, K., Lengyel, J. A. and Merriam, J. R. (1986). The zygotic mutant *tailless* affects the anterior and posterior ectodermal regions of the *Drosophila* embryo. *Dev. Biol.* **113**, 64-76.

Studier, F. W. and Moffat, B. A. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **198**, 113-130.

Tautz, D. (1988). Regulation of the *Drosophila* segmentation gene *hunchback* by two maternal morphogenetic centres. *Nature* **332**, 281-284.

Treisman, J., Harris, E. and Desplan, C. (1991). The paired box encodes a second DNA-binding domain in the *paired* homeo domain protein. *Genes Dev.* **5**, 594-604.

Vincent, J.-P. and O'Farrell, P. H. (1992). The state of *engrailed* expression is not clonally transmitted during early *Drosophila* development. *Cell* **68**, 923-931.

Weigel, D., Jürgens, G., Klingler, M. and Jäckle, H. (1990). Two gap genes mediate maternal terminal pattern information in *Drosophila*. *Science* **248**, 495-498.

Wieschaus, E., Nüsslein-Volhard, C. and Jürgens, G. (1984). Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster.* III. Zygotic loci on the X-chromosome and fourth chromosome. *Wilhelm Roux's Arch. Dev. Biol.* **193**, 296-307.

Zipursky, S. L., Venkatesh, T. R., Teplow, D. B. and Benzer, S. (1984). Neuronal development in the *Drosophila* retina: monoclonal antibodies as molecular probes. *Cell* **36**, 15-26.

(Accepted October 28 1992)