Establishment and refinement of segmental pattern in the *Drosophila* embryo: spatial control of *engrailed* expression by pair–rule genes

Stephen DiNardo and Patrick H. O'Farrell

Department of Biochemistry and Biophysics, University of California at San Francisco, San Francisco, California 94143 USA

We are examining the development of the segmental body pattern of *Drosophila* by immunolocalization of *engrailed*, a developmental regulatory protein that maintains segmental subdivisions in the embryo, and is expressed in a spatially restricted (striped) manner that persists while the body pattern is being established and refined. A regulatory network among pair–rule segmentation genes establishes the striped pattern of *engrailed* expression. In general, mutations in particular pair–rule genes affect either even- or odd-numbered *engrailed* stripes. For example, *fushi tarazu* or *odd-paired* mutations delete even-numbered stripes, whereas *paired* mutations delete odd-numbered stripes. An analysis of *engrailed* expression in other mutants, including *even-skipped odd-skipped* double mutants, indicates that some pair–rule genes play a rule in establishing the correct width and position of *engrailed* stripes. Overall, the changes in *engrailed* pattern have consequences for final embryonic body pattern. Thus, the pair–rule loci, acting through *engrailed*, establish an early, general outline of body pattern. However, in several pair–rule mutants, *engrailed* patterns are dynamic, suggesting that as later events build upon this general rule to form the final body pattern, adjustments are made in response to the earlier pair–rule defect—that is, the pattern regulates.

The basic segemental body pattern of the fruit fly *Drosophila melanogaster* is established early in embryogenesis. After fertilization, a series of rapid nuclear division cycles converts the embryo into a shell of about 6000 nuclei at the periphery of a syncytial cytoplasm. The cellular blastoderm is formed by cellularization of each of these peripheral nuclei [2.5 hr after egg laying (AEL)]. By this stage the basic outlines of the segmental pattern have been established, as determined by cell marking and cell transplantation experiments (Wieschaus and Gehring 1976; Simcox and Sang 1983), although no morphological consequences of the pattern formation process are yet visible. We are interested in the molecular mechanisms that establish the early segmental body pattern and that build upon this early outline to establish the final form of the embryo.

Developmental regulatory genes that are thought to control the process of pattern formation have been identified (Nüsslein-Volhard and Wieschaus 1980; Jurgens et al. 1984; Nüsslein-Volhard et al. 1984; Wieschaus et al. 1984). Twenty or so zygotically expressed genes have been grouped into "segmentation gene" classes according to the positions and extent of cuticle pattern defects caused by mutations at these loci. The "gap" loci, such as *Krüppel* (Wieschaus et al. 1984b), are required for the establishment of relatively large contiguous do-mains (about seven segments). The pair–rule loci, such as *fushi tarazu* (Wakimoto et al. 1981), are required for the patterning of domains spaced roughly at two-segment intervals. A combined molecular and genetic analysis indicates that the gap and pair–rule loci function by the cellular blastoderm stage. Therefore, these genes are thought to be important in the establishment of early segmental body pattern (Nüsslein-Volhard and Wieschaus 1980; Hafen et al. 1984; Wieschaus et al. 1984b; Gergen et al. 1986).

A third class of segmentation gene—the "segment polarity" loci, such as *wingless*—is required for the development and polarity of domains within each segment. This class acts over a broad developmental period, beginning at the cellular blastoderm. These genes are probably involved in building upon the early segmental body pattern, establishing more detail in the pattern of cell fates. This period of embryogenesis covers the morphogenic movements of gastrulation (3.5 hr AEL), germband extension (3.5–5.5 hr AEL) and retraction (7–9 hr AEL), and two postblastoderm cell divisions. Although little is known of the mechanisms used to create more detail in pattern during these later stages, the suggestion that the *wingless* product may function in morphogenesis as a signal in cell communication is intriguing (Rijsewik et al. 1987).

Molecular probes for segmentation genes allow us to visualize the pattern formation process with single-cell precision, long before the morphological consequences are apparent. Perhaps the clearest manifestation of the early segmental pattern is the exquisite expression pattern of *engrailed*, a gene required for the maintenance of segmental pattern (Nüsslein-Volhard and Wieschaus 1980; Kornberg 1981a). In the adult, *engrailed* is required for the development of a portion of each segment (the posterior compartment; Garcia-Bellido 1975; Lawrence and Morata 1976; Kornberg 1981b). During embryonic development, the *engrailed* locus has attributes of both the pair–rule and segment polarity gene classes. [*engrailed* also has a role in precellular blastoderm development (Karr et al. 1986). It is unclear as yet whether this role is distinct from its role in segmentation.] The molecular analyses show that *engrailed* is expressed during embryogenesis in a position-specific manner such that 14 stripes are seen transecting the anteroposterior axis at intervals that correspond to the posterior portions of each developing segment (Fjose et al. 1985; Kornberg et al. 1985). Immunocytochemical localization of the *engrailed* protein in fixed whole-mount embryos shows that the antigen accumulates in stripes at the cellular blastoderm (3 hr AEL) and that this pattern persists for an extended period (DiNardo et al. 1985). It is this period during which patterns of cell fates are further specified. Therefore, by monitoring the *engrailed* pattern, we can follow both the establishment of the early segmental pattern outline and its refinement into a more detailed pattern (DiNardo et al. 1985).

To define the processes that are used during the establishment and refinement of pattern, we have determined the consequences that mutations in each of the pair–rule genes have on the *engrailed* pattern. The early *engrailed* pattern shows that correct width and placement of each *engrailed* stripe requires the combined activity of several pair–rule genes. Even-numbered and odd-numbered stripes require distinct, but partly overlapping, subsets of pair–rule activities. When a given *engrailed* stripe is altered, so is the consequent development of posterior compartment structures in that region of the embryo. These results indicate that the pair–rule genes, acting through *engrailed*, are indeed primary regulatory loci that control early segmental patterning.

The results also reveal aspects of the process that build upon the early segmental pattern. In effect, each pair–rule mutation introduces a characteristic pattern defect at cellular blastoderm, as determined by alterations in the early *engrailed* pattern. During the course of continued development, we find that the system responds to this early pattern defect in ways suggesting that cell–cell interactions are important in the establishment of final cell fates.

Results

The control of early patterning events by pair–rule segmentation loci

The pattern of *engrailed* expression at cellular blasto-derm is altered in each pair–rule mutant. Although we have examined the strongest alleles available (unless specifically noted), in no case is *engrailed* expression abolished or globally induced throughout the embryo. Rather, particular stripes of expression seen in wild-type embryos are deleted, broadened, or repositioned, producing a distinct pattern in each mutant.

To describe the *engrailed* pattern, we number stripes in an anteroposterior sequence, 1–14, and indicate their segmental fate parenthetically (Mn, Mx, La; T1–T3; A1–A8) (Figs. 1a; 2d). The segments are defined morphologically. Along the ventral surface of the larval cuticle, repeating groups of hairs, the denticle belts, mark thoracic (T1, T2, T3) and abdominal (A1–A8) segments. The oral segments, mandibular (Mn), maxillar (Mx), and labial (La), largely involute and do not contribute to the external markers considered here. In Figure 1, the stippled area within each segment approximates the region where *engrailed* gene activity is required earlier in development (Nüsslein-Volhard and Wieschaus 1980; Kornberg 1981a). These *engrailed*-requiring regions cover some naked cuticle in each segment and the most anterior row of denticles in A2–A8 (Gergen et al. 1986; S. DiNardo and P. O'Farrell, unpubl.). By convention we refer to segments, their primordia, and component pattern elements (e.g., denticle belts) as odd numbered (T2, A1, A3, A5, and A7) or even numbered (T1, T3, A2, A4, A6, and A8).

Expression of odd- and even-numbered engrailed *stripes is controlled by different combinations of pair–rule genes*   Whether a pair–rule mutation deletes, broadens, or repositions *engrailed* stripes, each mutation has distinct effects on even- versus odd-numbered stripes (Fig. 6). Furthermore, these genes act in combinations. For example, the combined activity of six pair–rule genes is required for the production of even-numbered stripes of correct width and position (*ftz, opa, eve, runt, hairy,* and *odd*). In this section we focus on mutations that cause the deletion of specific stripes; *fushi tarazu (ftz)* and *odd-paired (opa)* mutants lack even-numbered stripes, whereas *paired (prd)* and *unpaired (upd)* mutants lack odd-numbered stripes.

Figure 2 shows the pattern of *engrailed* expression in wild-type, *ftz,* and *prd* mutant embryos at two stages of development [3.5 hr AEL (a, b, and c) and ~ 7 hr AEL (d, e, and f)]. In the mutants only 7, rather than 14, *engrailed* stripes are formed. The intensity of the developing stripes and their position indicate that *ftz* mutants lack even-numbered stripes (Figs. 1b and 2b,e), whereas *prd* mutants lack odd-numbered stripes (Figs. 1c and 2c,f). As in wild type, the *engrailed* stripes that form in *ftz* and *prd* mutants are one single cell wide. However, these stripes are now separated by six to eight cells rather than two or three cells. If *engrailed* expression reflects the specification of cell fates, a compound segment having an enlarged anterior compartment and a normal posterior compartment would be anticipated. Indeed, the effects on *engrailed* expression anticipate the morphological defects. First, cuticular pattern elements
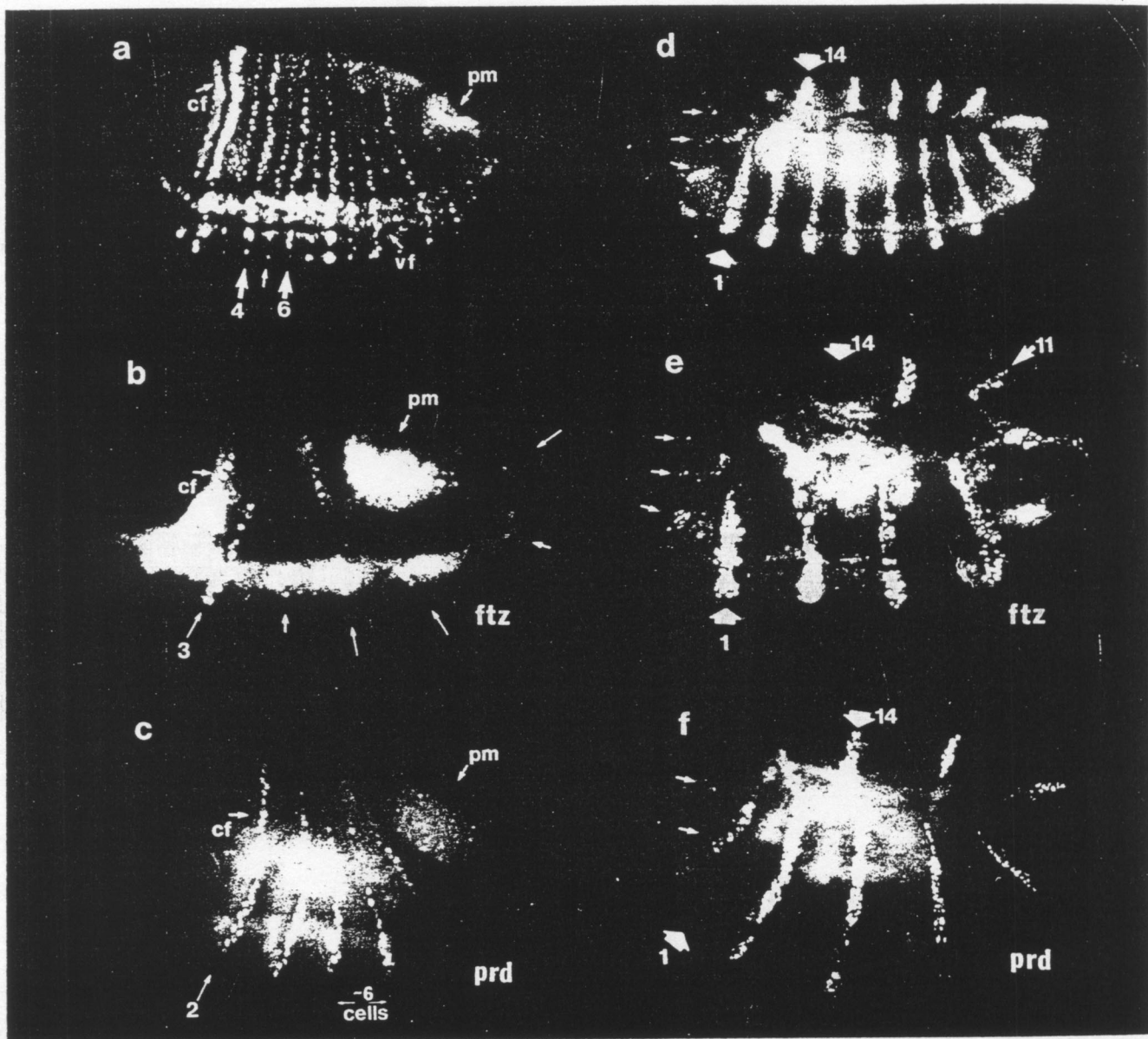
Figure 2. *ftz* is necessary for even-numbered stripes, and *prd* is necessary for odd-numbered stripes. Embryos are oriented anterior left and ventral down (roughly) in all images, unless otherwise noted. (*a*) Wild-type, early gastrula. The stripes are roughly one cell (nucleus) wide, with alternating (even-numbered) stripes higher in signal intensity. The stripes are about 2.5 cells apart (S. DiNardo, unpubl.). (*b*) *ftz*[W20] mutant, beginning germ-band elongation. Fewer stripes are visible, and those that are present are particularly light. These are odd-numbered stripes, which may take longer to appear than in wild-type (cf. *a*). The 1(Mn) stripe is within the cephalic furrow (cf) and not in this focal plane. The 3(La) stripe and other stripes more easily visible in the original preparations [7(A1), 9(A3), and 13(A7); larger arrows] mark a four-segment periodicity. The stripes are one cell wide. This is seen clearly for 3(La). *ftz*[E66-1] exhibits the same pattern as *ftz*[W20] at all stages. (*c prd*[2.45] mutant, early gastrula. Five even-numbered stripes are visible here, with a hint of the sixth (the seventh will become apparent as development progresses). These are one cell wide, as in wild type, and there are about six to seven cells between stripes. (*d*) Wild-type embryo at ~7 hr AEL. Fourteen stripes representing the posterior compartment primordia of the oral, thoracic, and eight abdominal segments are indicated. Small arrows point out other pockets of *engrailed* expression, but these are not of primary concern here. The 1(Mn) and the 14 (A8) stripes (broad arrows) are good reference points in comparisons between this and the mutant embryos in panel *e* and *f*. (*e*) *ftz*[W20] mutant at 7 hr AEL. Seven odd-numbered stripes are present. Stripe 11(A5) is sometimes missing. In agreement with this, we find that a fraction of *ftz* embryos have a compound fusion of A4 with A6. Small arrows point to preoral pockets of *engrailed* expression, which are identical to wild type (*d*). (*f*) *prd*[2.45] mutant at 7 hr AEL. There are seven even-numbered stripes, starting at 2 (Mx), and some preoral expression (small arrows). Segment primordia and *engrailed* stripe designations are described in the legend to Fig. 1 and the text. (vf) Ventral furrow; (cf) cephalic furrow; (pm) posterior midgut invagination.

early *engrailed* expression appears normal, but defects develop after the germ band is fully extended. In these cases, stripe 10(4) is partially or totally deleted (Fig. 3d). When visible, the remnant of the 10A(4) stripe is shifted posteriorly (Fig. 3d). These late alterations in *engrailed* pattern are compatible with the most frequent cuticular defect, which is a deletion of the A5 denticle belt and the posterior portion of A4 (Gergen and Wieschaus

1986). These investigators also describe a more severe and complex cuticular phenotype in a fraction of *upd* mutant embryos. This class of embryos probably derives from the mutants that show a severe and early effect on *engrailed* pattern. It is not clear why such disparate phenotypes arise.

We conclude that even- and odd-numbered *engrailed* stripes are controlled by distinct genes: *ftz* and *opa* ac-
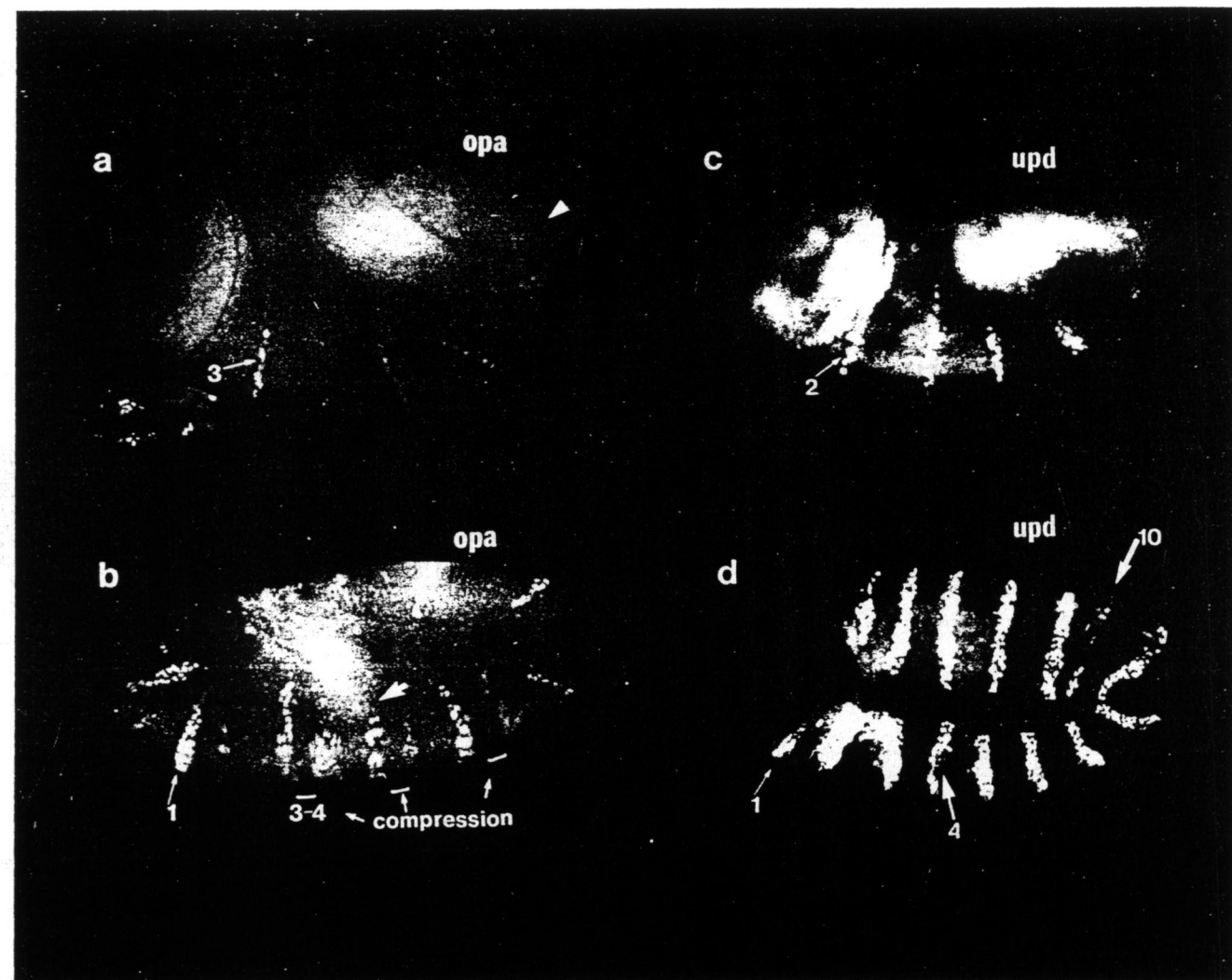

Figure 3. *opa* and *upd* are also required for even-numbered and odd-numbered stripes, respectively. (*a*) Early *opa*[IIC41], beginning germ band extension. *engrailed* antigen accumulation is similar to that in *ftz* embryos, with only odd-numbered stripes developing. The 3(La) stripe is most evident. The 1(Mn) stripe is within the cephalic furrow (cf). There is a hint of one even-numbered stripe, 12(A6), at this early stage (arrowhead). The developing stripes are one cell wide. *opa*[II32] exhibits a similar pattern. (*b*) *opa*[IIC41] embryo at 7 hr AEL. The seven odd-numbered stripes are most evident beginning with 1(Mn); however, there is expression to varying degrees between odd-numbered stripes. The expression here could be even-numbered stripes, but they are displaced anteriorly (brackets reflect this compression). The cuticular pattern deletions in *opa* embryos are centered within the denticle bands of even-numbered segments. The compression of segment primordia between *engrailed* stripes at the expense of anterior (denticle) portions of even-numbered segments anticipates these cuticle defects. The even-numbered stripes are not complete in the dorsoventral direction. Some odd-numbered stripes are incomplete at this stage also [see, e.g., 5(T2), bold arrow]. (*c*) *upd*[YC43] embryo beginning germ band extension. Six or seven stripes are developing. Analysis of later embryos with similar staining patterns (not shown) indicates that these are probably even-numbered stripes, as in *prd* mutants. Only one quarter of mutant embryos show this early and clearly pair–rule effect on the *engrailed* pattern. The rest are normal at this stage. (*d*) Late *upd*[YC43] embryo (~6 hr AEL), showing the most penetrant effect on *engrailed* pattern. Defects are apparent in the 10(A4) stripe (arrow) and frequently in the 12(A6) (not seen in this embryo) and 4(T1) stripes. These defects develop over time. They are not apparent in early embryos.

tivity are required for even-numbered stripes, and *prd* and *upd* (to a variable extent) are required for odd-numbered stripes. In addition, we show below that other pair–rule genes are required for the appropriate placement and width of either even- or odd-numbered stripes (see Fig. 6).

Regulatory interactions among genes that control engrailed expression and cell fate Although it is possible that some pair–rule gene products directly regulate *engrailed* expression, the existence of regulatory interactions among particular pair–rule genes (Carroll and Scott 1986; Harding et al. 1986; Howard and Ingham 1986) makes the assignment of a direct regulatory role to these genes problematic. Our analysis of a loss-of-function mutation of *even-skipped* (an *eve* null allele) illustrates the consequences that such regulatory interactions have for *engrailed* expression.

The *eve* null mutation leads to the absence of 14 *engrailed* stripes (Fig. 4a; Harding et al. 1986; MacDonald et al. 1986) and produces an unsegmented embryo (Nüsslein-Volhard et al. 1985). These results would appear to indicate an essential role for *eve* in establishing *engrailed* expression in stripes and for segmentation. However, Gergen et al. (1986) showed that the double mutant combination of *eve* and *odd* has appreciable segmentation (but not resembling wild type). Figure 4b shows that a striped pattern of *engrailed* expression returns in this double mutant. Thus, striped *engrailed* expression per se does not require *eve* function, nor does (partial) segmentation of the embryo. Rather, *eve* function is coupled to the activity of the *odd* locus; and therefore, we cannot determine whether either gene has a direct or indirect role in regulating *engrailed*. However, although striped *engrailed* expression is produced in the *eve odd* double mutants, the pattern is neither wild type nor is it similar to the pattern observed in *odd* single mutants (described below). In the double mutant, even-numbered stripes are two cells wide (Fig. 4b), and odd-numbered stripes are present in only 50% of mutant embryos. Therefore, it would be inappropriate to conclude that *eve* and *odd* are dispensable with respect to *engrailed* expression. Rather, the observations here and below show that the full complement of pair–rule gene activities, along with their attendant interactions, is required for the proper spatial programming of this striped expression.

Pair–rule mutations can produce spatially complex engrailed patterns The simple deletion of alternate *engrailed* stripes is unique to *ftz* and *prd* mutations. In other pair–rule mutants the width, spacing, or integrity of the stripes is affected upon their establishment (Fig. 6), and these characteristics change progressively during development. Such changes are found in embryos having strong or null mutations in genes such as *hairy*, *runt*, and *odd-skipped*. Rather than describe each case in detail, we describe the effects of a particular mutation selected because it illustrates all of these types of changes.

This allele *eve^{3.77}*, is not null for function (Nüsslein-Volhard et al. 1985). The spatial changes are considered here, whereas the temporal changes are reserved for the next section where the primary focus will be temporal aspects of patterning.

Although all *engrailed* stripes are missing in *eve* null mutants (above), the *eve^{3.77}* mutant shows complex alterations in the pattern of *engrailed* expression. First, the relative spacing between *engrailed* stripes is altered, for example, brackets point out the apposition of the 5(T2) and 6(73) stripes in Figure 4c and d. This spacing change is observed for each odd/even pair of stripes. Because *engrailed* marks the prospective posterior compartment of segmental primordia, the reduced spacing between certain pairs of *engrailed* stripes in *eve^{3.77}* indicates that the anterior compartment primordia are smaller. This could account, at least in part, for the ultimate cuticular pattern deletions, which are centered in the anterior region (denticle belt) of each even-numbered segment.

The repositioning of a stripe is a complex change in regulation. A row of cells that would normally express *engrailed* no longer does so in the mutant, but rather an adjacent row expresses. To effect such a change in pattern, the distribution of several developmental regulatory proteins, such as other pair–rule gene products (see Discussion; Harding et al. 1986), is probably altered by this allele. It is possible that other pair–rule mutations that affect the positioning of *en* stripes (e.g., *runt*, Fig. 6) do so by affecting the expression of several pair–rule genes.

Second, although all odd-numbered stripes are affected, the particular effect of this *eve* allele varies, depending on the particular odd-numbered *engrailed* stripe considered. Stripes 7(A1), 9(A3), and 11(A5) are incomplete ventrally, stripe 3(La) is incomplete laterally, and stripe 1(Mn) is absent (Fig. 4d). Stripe 5(T2), the only odd stripe intact along its dorsoventral axis, shows a singular alteration, being broader than normal (Fig. 4d), although this increase in breadth develops over time (see below). Thus, although *eve^{3.77}* has a pair–rule effect on *engrailed*—each odd *engrailed* stripe is altered—the nature of the alteration in each stripe varies. The *en* pattern in *runt* and *hairy* mutants is not altered in a precisely periodic fashion, with particular stripes being wider than others or spaced more closely to others (Fig. 6). Such observations suggest that the requirement for the activity of particular pair–rule genes varies along the anteroposterior axis.

The dynamic nature of the engrailed *pattern in pair–rule mutants reveals progressive aspects of the pattern formation process*

Late changes are evident in the *engrailed* pattern observed in the *eve* hypomorph already discussed. For example, there is a striking increase in the width of stripe 5(T2) (cf. Fig. 4c and d). In addition, odd stripes such as

7(A1) and 9(A3) are faint in the early gastrula, only becoming prominent later. We also observe complex temporal alterations in *engrailed* pattern in *hairy*, *runt*, and *sloppy-paired* mutant embryos, but we will reserve their full description for elsewhere (for *hairy*, see also Howard and Ingham 1986). We have already alluded to late changes that occur in *opa* and in some *upd* mutants. We note only that, as in the case of the *eve* hypomorph, each

of these mutations causes early and reproducible *engrailed* pattern defects (Fig. 6) and that in each case these early defects are further altered during the course of development. Some insight into the process(es) that are driving these further patterning changes comes from an analysis of the dynamic nature of the *engrailed* pattern in *odd-skipped* embryos.

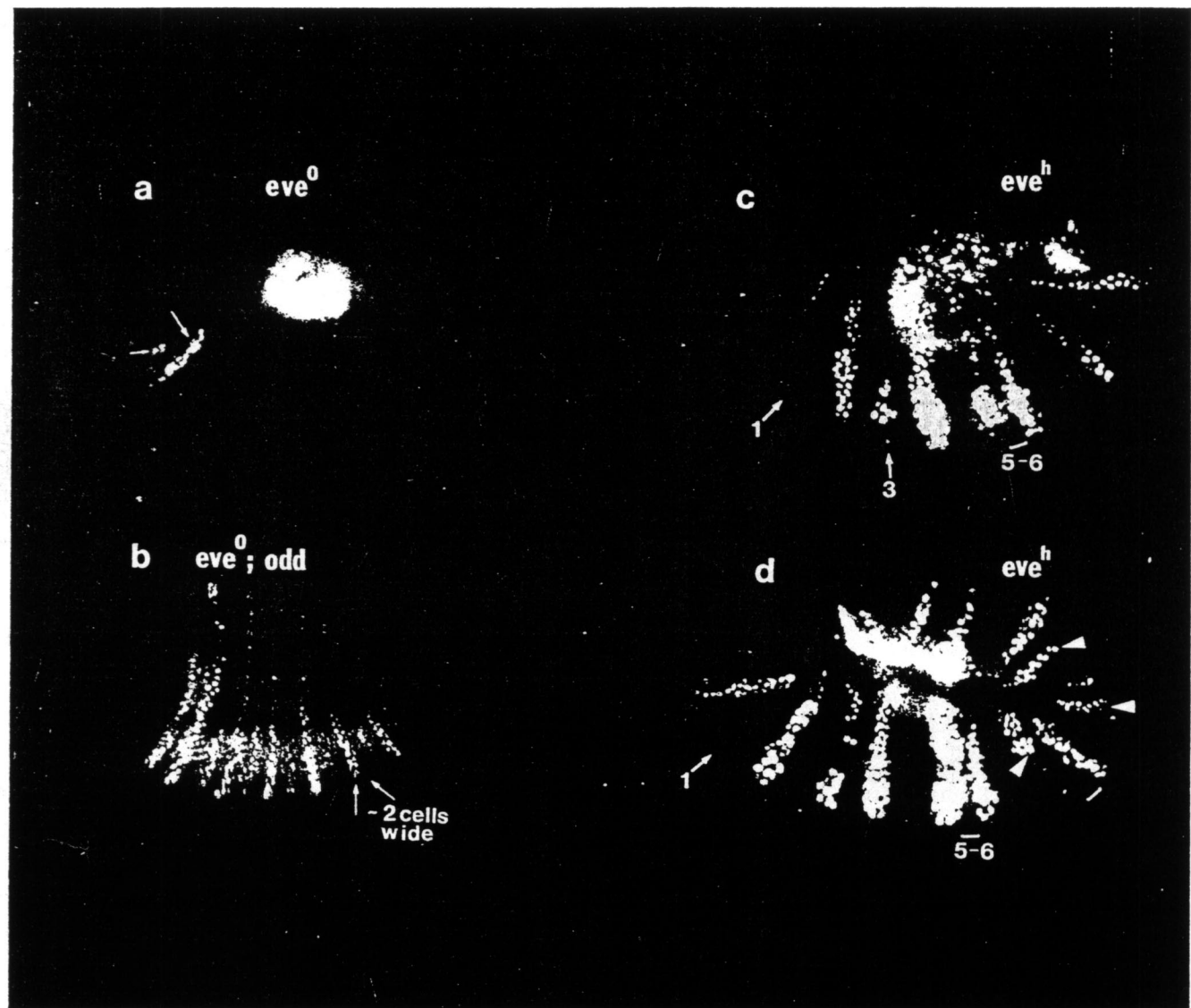Early in the development of *odd* mutants, even-num-

Figure 4. Pair–rule genes interact to establish cell fate. (*a*) Early $eve^{1.27}$ (null) embryo. Only preoral expression is present (arrows). No striped expression is apparent along the oral, thoracic, and abdominal region. eve^0 indicates a null allele. (*b*) $eve^{1.27}$ (null), odd^{IIID36} double mutant, early gastrula. A striped pattern of *engrailed* expression is restored. The pattern is not wild type, with particular stripes being two cells wide instead of one. The effect of this double-mutant combination on the *engrailed* pattern will be analyzed in detail elsewhere. (*c*) $eve^{3.77}$ (hypomorph), beginning germ band extension. There are several alterations in the *engrailed* pattern. Odd-numbered stripes are defective. For example, the 1(Mn) stripe is not present; the 3(La) stripe is confined to a few ventral cells. Second, odd-numbered stripes are shifted, appearing closer to the next most posterior stripe. The 5(T2)–6(T3) pair is bracketed to show this shift. The 4(T1) stripe is broader than usual. eve^h indicates a hypomorphic allele. (*d*) $eve^{3.77}$ (hypomorph) at 6 hr AEL. The shifted position of odd-numbered stripes at the expense of anterior portions of the next, even-numbered segment is indicated for the pairs 5(T2)—6(T3) and 7(A1)–8(A2) and is true for other pairs as well. The 5(T2) stripe has broadened dramatically relative to other stripes, [cf. 5(T2) in *c*]. Several stripes are present dorsolaterally [7(A1), 9(A3), and 11(A5) arrowheads], with only a minority of the ventral cells expressing (not in this plane of focus).

bered *engrailed* stripes are two cells wide instead of one, whereas the odd-numbered stripes are normal (Fig. 5a). The distance between the broadened even stripes and the next most posterior stripe is two cells rather than the normal three (Fig. 6). Thus, the expansion of the even-numbered stripe is at the expense of the primordia for anterior parts (denticle belts) of odd-numbered segments.

After the germ band is fully elongated (~6 hr AEL), the aberrant *engrailed* pattern changes. Each broad *engrailed* stripe splits almost completely into two stripes. The embryos in Figure 5 (b,c,d) show this progression. It appears that cells toward the middle of the broad stripe lose *engrailed* signal. The position and extent of this split is variable both in different *odd* embryos and in comparing stripes within the same embryo (Fig. 5d). Cells that have lost signal are grouped and not intermingled among expressing cells.

The initial *engrailed* pattern and the later transition can be correlated with the cuticular phenotype of *odd* mutant embryos (first described by D. Coulter and E. Wieschaus, pers. comm.). The region of cuticle deleted is shown schematically in Figure 5e. The odd-numbered denticle belts are almost completely missing. In place of these, there is a mirror-image duplication of the derivatives of even-numbered *engrailed* stripes (Fig. 5f). Up to this point in the description, part of the pattern deletion and duplication can be accounted for by the misspecification of cell fate at blastoderm as seen by changes in the early *engrailed* pattern. The even-numbered *engrailed* domains have been "duplicated" and this duplication is at the expense of a row of cells normally specified with anterior (denticle) cell fates. Therefore, the outlines of the final cuticular pattern are established early in *odd* mutants. This holds for mutations at other pair–rule loci also and confirms the suggestion that pair–rule genes are among the primary loci to consider in contemplating the establishment of segmental pattern (Gergen et al. 1986). However, a close look at the denticle remnant in *odd* mutants suggests that not all pattern elements within the duplication are accounted for by derivatives of *engrailed*-expressing cells (Fig. 5f) and, therefore, that some further alteration of this early "framework" of pattern must occur. This further alteration seems to be reflected in the splitting of the broad *engrailed* stripes.

The denticle remnant is composed of an anterior row of small denticles, each pointing anteriorly and resembling those normally secreted by *engrailed*-expressing cells. Behind this row are one or two rows of large denticles ordinarily produced by non-*engrailed*-expressing cells (cells in the anterior region of a segment). The polarity of denticles in these two rows is somewhat variable, possibly reflecting the fact that the plane of mirror-image symmetry lies here. At the posterior edge of this remnant is another row of small denticles of the type normally secreted by *engrailed*-expressing cells but with opposite polarity (pointing posteriorly). It is the "intercalated" anterior pattern elements that are not accounted for by the blastoderm *engrailed* pattern. It is

possible that some of the cells that lose *engrailed* signal within the broad stripes give rise to the intercalated row of large denticle hairs, that is, the final fate of these cells is being respecified. If so, these transitions in *engrailed* staining reflect a process that intercalates new cell fates during the postblastoderm stage of pattern refinement (see Discussion).

Discussion

The results reveal two aspects of the pattern formation process during *Drosophila* embryogenesis. The first concerns the establishment of early segmental pattern through the regulatory activities of the pair–rule segmentation genes. We show that the precise spatial pattern of *engrailed* expression, which is a marker for the establishment of segmental pattern, depends upon combinatorial interactions among these segmentation genes. The correct width and spacing of even- and odd-numbered stripes requires two distinct subsets of pair–rule gene activity. Importantly, if the *engrailed* pattern at cellular blastoderm is altered in a pair–rule mutant, say, by a broadening or repositioning of particular *engrailed* stripes, then so is the consequent development of posterior compartment pattern elements in the corresponding region of the embryo. This demonstrates that pair–rule gene activity is a primary determinant in establishing an outline for the final body pattern and, furthermore, that this role is mediated, in part, through the spatial regulation of *engrailed*.

The second aspect of our analysis is that the final body pattern is not necessarily a simple projection forward from the pattern at blastoderm. We find changes in the pattern of *engrailed* expression over time in several of the pair–rule mutants. This appears to be the result of embryonic pattern regulation, visualized at the level of gene expression.

Establishment of blastoderm pattern

Pattern formation relies on the expression of developmental regulatory genes in precise spatially restricted domains Developmental regulatory gene products are expressed in very precise patterns in the early embryo (e.g., Hafen et al. 1984; Harding et al. 1986; Kilcherr et al. 1986; MacDonald et al. 1986; for a review, see Scott and O'Farrell 1986). There is a correlation between the expression pattern for a given gene and the placement of pattern defects due to mutations in that gene. Such a correlation has suggested that ectopic expression of these genes would redirect the fates of expressing cells. Evidence supporting this is accumulating. For example, presumed unregulated (Gergen and Wieschaus 1986a) or global (Struhl 1985) expression of particular pair–rule genes causes pattern defects. Indeed, we find that alterations in *engrailed* gene expression that are induced by pair–rule mutations are associated with corresponding alterations in cell fate. Perhaps most instructive, the duplication of even-numbered *engrailed* stripes seen in *odd-skipped* mutants gives rise to correspondingly du-

plicated posterior compartment structures and segment borders. A similar correlation between ectopic *en* expression and changes in body pattern is observed in the segment polarity mutant *patched* (in prep.). In these cases, inappropriate *engrailed* expression correlates with the production of new posterior pattern elements. Therefore, the establishment of precise, spatial patterns of segmentation gene expression is a crucial step in the development of embryonic pattern.

Combinatorial signals determine patterns of cell fate

A knowledge of the effects of pair–rule mutations on *engrailed* pattern cannot define in mechanistic detail the complex interactions involved in the establishment of *engrailed* expression and, therefore, patterns of cell fate at blastoderm. However, the overall features that govern these processes are revealed (see also, Howard and Ingham 1986). First, the pair–rule gene products required for odd-numbered stripes differ in part from those required for even-numbered stripes (Fig. 6). For example, *ftz* and *opa* activity are necessary for the establishment of expression in even-numbered stripes but not odd-numbered stripes. Reciprocally, *prd* activity is required only for the odd-numbered stripes. Thus, *engrailed* responds to two distinct sets of regulatory factors, which in turn suggests that there may be distinct *cis*-acting control regions, one each for even- and odd-numbered *en* stripes. In addition, we expect that some of the pair–rule gene products are direct *trans*-acting regulators of the *engrailed* gene. Three of the four pair–rule gene products characterized to date contain homeo domains
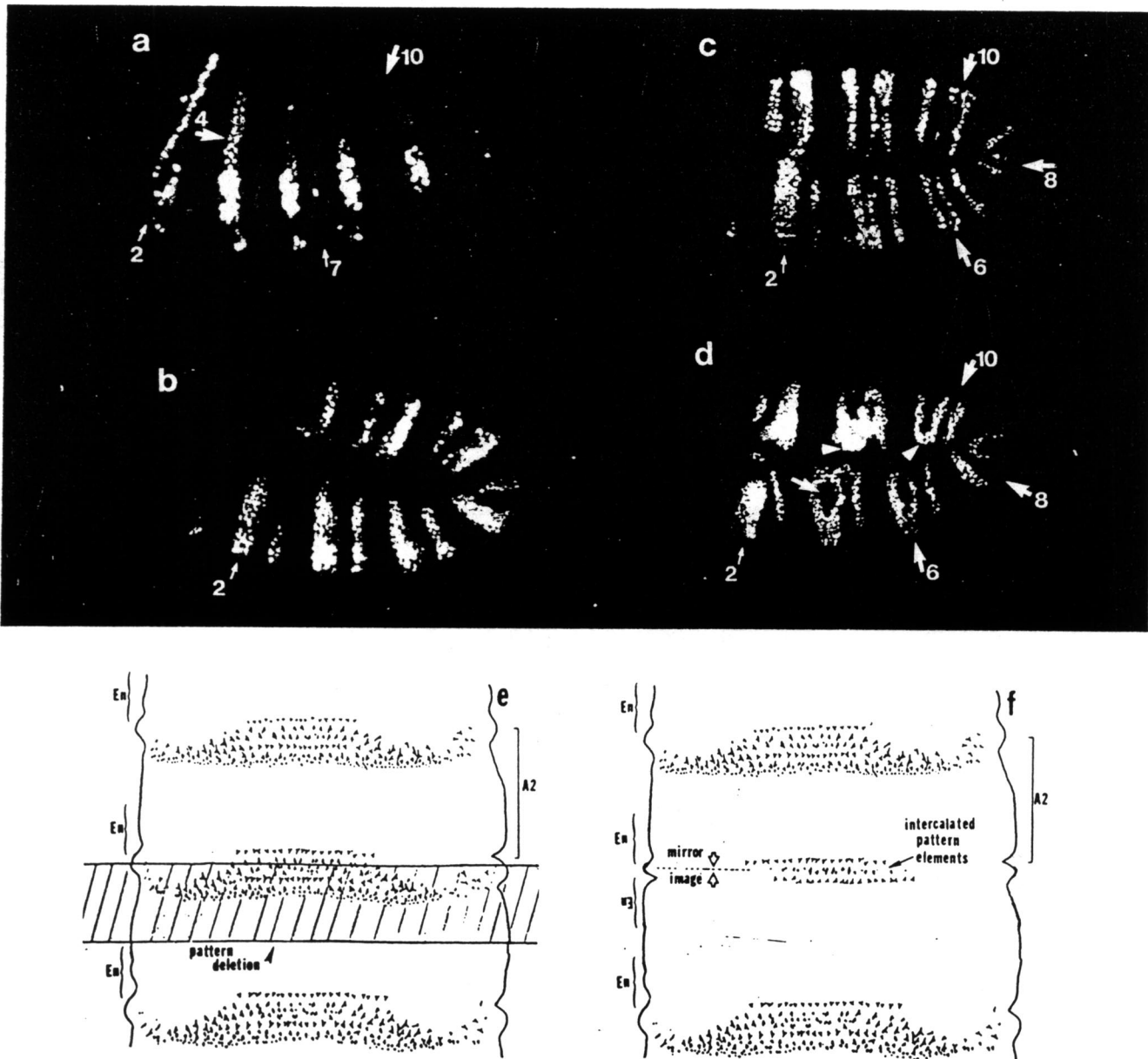

Figure 5. (*See facing page for legend.*)

[*ftz, prd*, and *eve* (Laughon and Scott 1984; Frigerio et al. 1986; MacDonald et al. 1986; Frasch et al. 1987)]. Furthermore, this domain has sequence-specific DNA-binding activity (Desplan et al. 1985) and binds to several sites in the *engrailed* upstream regulatory region. The results reported here cannot distinguish between the role of an activator or a repressor of *engrailed* expression.

A second overall feature revealed by these analyses is that although a particular pair–rule product is necessary for expression in even-numbered stripes, it is not sufficient. As shown schematically in Figure 7, pair–rule products are distributed in relatively broad stripes at the onset of gastrulation. If, for example, the presence of *ftz* were sufficient for *engrailed* induction, the *engrailed* stripes would be three cells wide rather than one cell wide. Because *ftz* is not sufficient to specify *engrailed* expression, *engrailed* must respond to a combination of input signals (perhaps other pair–rule products). And, in fact, there must be two "combinatorial codes," one for even- and one for odd-numbered stripes. Combinatorial control could imply that several gene products act together to affect *engrailed* or, alternatively, imply an indirect pathway where one gene product affects the expression or activity of another, which in turn affects *engrailed*.

Such combinatorial control of cell fate reflects the refinement of a relatively coarse pattern (broad pair–rule stripes) into a more detailed pattern (single-cell-wide segmental stripes). Stripes of expression for different pair–rule genes are offset from one another by increments of one or two cell diameters (Fig. 7 and references therein). Such overlap gives rise to particular combinations of regulatory products in each row of cells. This would be sufficient to distinguish the fate of one row from the next (also see Gergen et al. 1986). One outcome of this is the production of stripes of *engrailed* expres-sion that are one cell wide. Therefore, by responding to a specific combinatorial code, *engrailed* marks one row of cells out of every three or four as distinct. We would predict that other segmentation genes (some of those among the segment polarity class) would respond to a different combinatorial code and, therefore, mark each of the remaining rows of cells in the segment primordium. In this manner, the combinatorial control of gene expression by developmental regulatory products has refined patterning domains from three or four cell widths (the domain of any one pair–rule product) to a single cell width.

Regulatory interactions connect the segmentation genes in a network

Recent work has shown that pair–rule genes themselves interact in the establishment of positional identity. For example, the *ftz* pattern of expression is dependent on *eve, hairy,* and *runt* (Carroll and Scott 1986; Howard and Ingham 1986). *eve, odd,* and *runt* mutations have consequences on the *engrailed* pattern that appear to involve such interactions.

First, even though the 14 *engrailed* stripes are absent in an *eve* null, striped expression returns in *eve, odd* double mutants. The molecular nature of this interaction between *eve* and *odd* is not clear. Because stripes of *eve* expression overlap odd-numbered *en* stripes, *eve* could play a direct role in establishing these *en* stripes (Lawrence et al. 1987), although we would argue that *eve* would not be sufficient in this role for the same reasons argued above for *ftz*. In contrast, *eve* stripes do not overlap developing even-numbered *en* stripes, suggesting that *eve* may only affect these stripes indirectly. In particular, *eve* may affect these stripes through some regulatory consequences on the *odd* gene. This possibility arises because in an *odd* mutant, even-numbered

Figure 5. The late changes in *engrailed* pattern in *odd* mutants may reflect the intercalation of new cell identities. (*a*) *odd*[IID36] early gastrula. The even-numbered stripes are two cells wide, rather than one [see stripes 4(T1) and 10(A4)]. The position of one developing odd-numbered stripe is indicated [7(A1)]. The spacing between the odd-numbered stripes and each previous broad stripe is less than that in wild type, indicating that the expansion of even-numbered *engrailed* primordia is at the expense of the anterior domain of odd-numbered segments. The low intensity of the odd-numbered stripes is probably normal as this is also a feature of odd-numbered stripes in early wild-type embryos (Fig. 2a). The strength of the ventral-most signal is an optical effect due to the contribution of expressing cells in the invaginated mesoderm. (*b*) Embryo at 5 hr AEL. The pattern described in *a* is quite apparent. Note the broad even-numbered *engrailed* stripes and the reduced primordia of the next most posterior (odd) segment. This broadening is at the expense of anterior (denticle) regions. (*c*) Embryo at 6.5 hr AEL. The pattern is beginning to change. Cells within most broad stripes seem to be losing signal [6(T3), 8(A2), 10(A4) shown by arrows]. (*d*) Embryo at ~7–7.5 hr AEL. The broad stripes have split almost completely in two. The same three primordia are shown by arrows, as in *c*. In cases where stripes have not completely split, contiguous groups of cells have changed [arrow, 4(T1)]. In some cases, odd-numbered stripes and part of the split even-numbered stripes have reorganized dorsally so that they are joined [10(A4)–11(A5) and 12(A6)–13(A7), arrowheads]. (*e,f*) Schematic representation of the pattern changes in A2 and A3 and part of A4 ventral cuticle in *odd* embryos. The regions of naked cuticle and anterior-row denticle hairs that require *engrailed* function are indicated by brackets in the left margin. (*e*) The region deleted in *odd* embryos includes most of the A3 denticle belt, as indicated by hatching. The regions requiring *engrailed* function are not within the domain deleted in *odd* mutants. Therefore, we expect and observe the correct number of *engrailed* stripes in the early embryo. (*f*) In *odd* embryos the deleted region is replaced by a duplication of naked cuticle and anterior-row denticle hairs. The orientation of denticle hairs indicates that the duplication has a reversed polarity. The resulting mirror image is indicated by head-to-head arrows. The duplicated pattern elements that require *engrailed* function are indicated by brackets in the margin. The blastoderm *engrailed* pattern anticipates both this pattern duplication and at least part of the loss of anterior (denticle) region in odd-numbered segments. Recall that single-cell-wide, even-numbered *engrailed* stripes have been "duplicated" and are thus two cells wide in early *odd* embryos (*a*). Intercalated, large denticle hairs are pointed out, nestled between the anterior-row hairs. These (anterior) pattern elements are not accounted for in the blastoderm pattern. They may be derived as a consequence of the postblastoderm splitting of the broad *engrailed* stripes.

is consistent with the production of new cell identities during regulation. Second, the production of a mirror-image duplication is characteristic of many patterns produced during a regulative response. Clearly, the process of pattern regulation suggests the involvement of cell communication during this period of development.

The extent of the pattern adjustment accomplished by this regulative response is variable, perhaps because the decision to respecify is probabilistic when the starting

overlap among pair-rule gene products may specify blastoderm cell fates
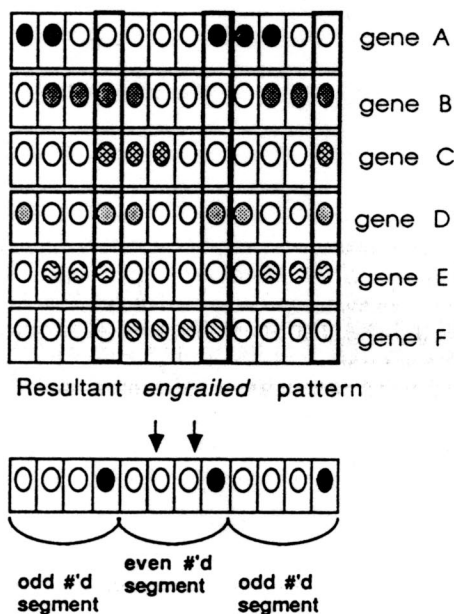

Resultant *engrailed* pattern

Figure 7. A scheme depicting one model for the combinatorial code of pair–rule gene products at cellular blastoderm and the resultant *engrailed* pattern. The pattern of expression of each gene, *A–F*, is shown for 12 cells (corresponding to three segment primordia) at an arbitrary position along the A–P axis of the embryo. The patterns for genes *E* and *F* are hypothetical but that for *A–D* are compiled from the literature. The accumulated products are shown as nuclear proteins for convenience. Gene *A* represents *ftz*; the overlap between *ftz* and *engrailed* (nuclear) protein stripes has been directly determined (S. DiNardo and S.B. Carroll, unpubl.). (Gene *B*) *hairy* (Ingham et al. 1985); (gene *C*) *eve* (Harding et al. 1986; MacDonald et al. 1986); (gene *D*) *prd* (Kilcherr et al. 1986). Note that each pair-rule gene is expressed in relatively broad stripes, which overlap to varying extents. As a result, any vertical row of blastoderm cells has a unique combination of pair–rule products. Combinations that allow *engrailed* induction in this scheme are highlighted. Note that the particular combinations alternate for odd- and even-numbered stripes. It is likely that some other segment polarity genes would respond to other combinations and, therefore, come to be expressed in single-cell-wide stripes, marking other cells within a segment primordium. We emphasize that this is a static picture of a very dynamic process, where the expression patterns are developing and decaying quickly. The precise kinetics of accumulation and decay may be of paramount importance (see MacDonald et al. 1986).
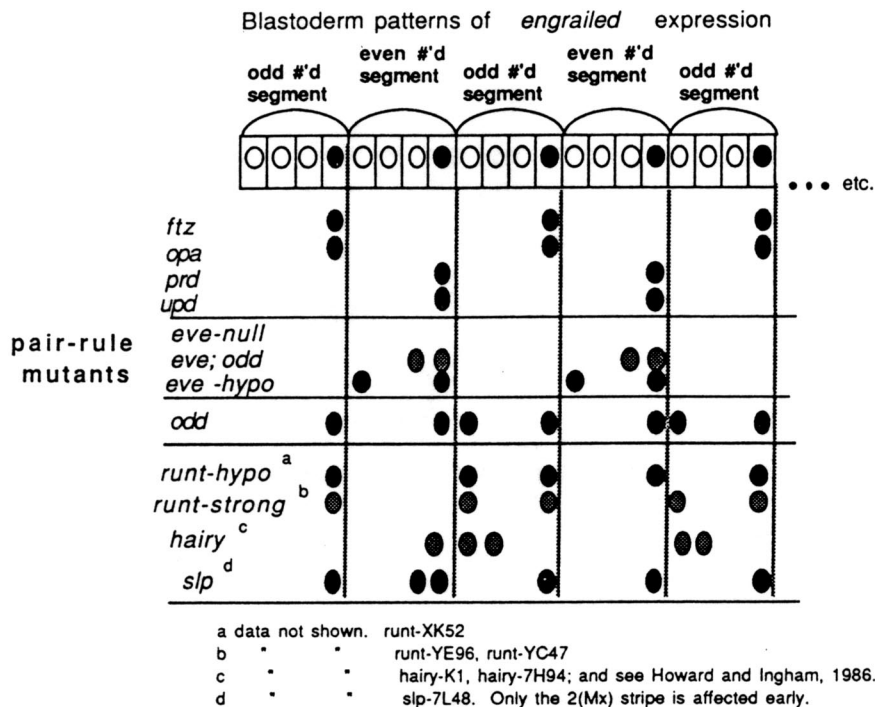
pattern is highly abnormal and the developmental time during which adjustments can be made is short. In their analysis of *runt*, Gergen and Weischaus (1985) have also suggested that cell–cell interactions may be responsible for intercalating new cell identity at the borders of pattern defects.

In summary, by the cellular blastoderm stage, one accomplishment of the interactive set of regulatory products produced by the pair–rule genes is the establishment of the precise metameric nature of the embryo. This is elegantly manifested by the single-cell-wide stripes of expression of *engrailed*. Moreover, at least some of these same pair–rule gene products are involved in the establishment of the identity of these segment primordia, by virtue of their proposed control over homeotic gene expression (Duncan 1986; Ingham and Martinez-Arias 1986). The further elaboration of pattern within the segmental primordia relies on a (continued) gene regulatory hierarchy, involving *engrailed*, homeotic genes (Struhl 1982), and segment polarity genes, some of which may play a role in cell–cell interactions (Rijsewijk et al. 1987).

Materials and methods

Culture conditions

Flies were kept as balanced stocks and reared at 25°C.

Genotypes used

Pair–rule mutants were generously provided by the laboratories of C. Nüsslein-Volhard and E. Wieschaus. The strongest alleles available were used (genetic nulls or molecular nulls, if known), unless indicated. The specific alleles are designated in the appropriate figure legend. Similar staining patterns have been observed using different alleles for *ftz* (*ftz^{W20}* and *ftz^{E66-1}*), *opa* (*opa^{IIC-2}* and *opa^{II32}*), and *h* (*h^{K1}* and *h^{7H94}*). With the exception of stocks or crosses involving the FM7 balancer chromosome, we find no complications due to the specific balancer chromosome used (most often CyO and TM3, Sb, or Ser). The segregation of the FM7 chromosome gives rise to defective blastoderm embryos at a frequency of about 5%, as determined by outcrosses. Therefore, this minor class of embryos, which die shortly after gastrulation, has been taken into account in our analysis of *runt* and *upd*. We have also checked the *engrailed* patterns in *runt* mutant embryos derived from crosses in which no balancer chromosomes were segregating. Mutant patterns observed in (hemi- or homozygous) *runt^{YE96}*, *runt^{YC43}*, and heteroallelic *runt^{YC43}/runt^{YE96}* embryos are similar.

Cuticle preparations

In most cases, collections from the same parents as those used for analysis of *engrailed* patterns were aged until all viable animals had hatched. Mutant embryos were then mounted and cleared for cuticular analysis (Van der Meer 1977; Gergen and Weischaus 1985).

Immunocytochemistry

Fixation and indirect immunofluorescent staining was performed as in DiNardo et al. (1985) with minor modifications. Detailed protocols are available upon request. Primary anti-

Figure 6. Summary of the changes in *engrailed* expression at cellular blastoderm in pair–rule mutant embryos. Roughly five segments worth of blastoderm cells (rectangular boxes containing oval nuclei) are shown, where every fourth cell expresses *engrailed*. This is the wild-type configuration. For convenience, a segment primordium is assigned four cells. Actual cell counts give an average of 3.4 cell diameters in ventrolateral regions (S. DiNardo, unpubl.). Odd-numbered segments alternate with even-numbered segments. The pair–rule mutants considered in this work are listed along the left-hand margin, roughly in the order considered in the Results. Registration lines are placed at the presumptive segment boundaries. The shaded ovals represent nuclei where *engrailed* expression is found in each of the mutants examined. Black ovals have been as precisely aligned as possible (not more than one cell diameter off). Thus, in *odd* mutants, the expansion in *engrailed* domain is into the posteriorly adjacent row of cells. Those nuclei shaded with hatching are only approximately positioned and could be off a few cell diameters. Because the *runt* hypomorphic and *hairy* mutants do not exhibit precisely periodic patterns, successive double segment intervals show alternative patterns. Strong *runt* alleles exhibit a more complicated *engrailed* pattern at some positions along the anteroposterior axis (S. DiNardo, unpubl.). Also, in early *slp* mutants, only one *engrailed* stripe is altered; the 2(Mx) stripe is broadened. Note that with the exception of *ftz* and *prd*, the mutant patterns are altered dramatically by postblastoderm events, and these changes are not illustrated here.

en stripes are expanded to two cells, as if *odd* activity normally serves to repress *en*. In an *eve* mutant, the domain of *odd* expression or activity may be expanded and effectively repress the even-numbered *en* stripes.

Second, we are intrigued by those pair–rule mutations that cause a shift in position, rather than a deletion, of particular *engrailed* stripes (e.g., *runt*). The simplest explanation for such a shift is that the particular mutation causes alterations in the expression patterns of several of the other pair–rule genes as a consequence of the regulatory interactions among these genes. This suggests that a gene such as *runt* may only exert an effect on *en* indirectly, possibly through direct control of other pair–rule genes. This supports the notion that a functional subdivision(s) exists within the pair–rule gene class (Carroll and Scott 1986; Howard and Ingham 1986).

Elaborating upon the blastoderm framework of pattern as development continues

We have cataloged the stereotyped alterations in *en* blastoderm pattern due to pair–rule mutations (Fig. 6). However, in most mutants we find that the *en* pattern

changes, often dramatically, after the germ band is fully extended, and that these late changes have consequences for the final body pattern. Therefore, an understanding of pattern formation not only requires a determination of the processes that establish spatially patterned gene expression at blastoderm but also an identification of the processes that cause the later changes that are superimposed on the early pattern. Because these changes occur after the major time period of pair–rule activity (in epidermal precursors), it is likely that these late changes are a consequence of the earlier pattern defects. The changes could result from cell death or cell rearrangements at the later embryonic stages. To some extent, both of these probably contribute to the final patterns we observe. In addition, some of the changes may be due to changes in the state of gene expression, reflecting a respecification of cell fate.

Two features of the development of *odd* mutants suggest that pattern regulation (French et al. 1976; Wright and Lawrence 1981) is responsible for the temporal alteration of *engrailed* signal described in *odd* mutants. First, the production of non-*engrailed*-staining cells with anterior fates in the middle of an *engrailed*-expressing region

bodies used were the previously described affinity-purified rabbit polyclonal sera directed against the *engrailed* protein and monoclonal antibody FP.3.38 directed against Ubx, a kind gift of R. White and M. Wilcox (1984). Secondary antibodies in more recent work are biotinylated (Vector Labs) and used in conjunction with streptavidin horseradish peroxidase (BRL). In this case, the cytological stain is developed with diaminobenzidine and hydrogen peroxide (Sigma) according to protocols from manufacturers. Embryos are dehydrated, cleared in xylene or methylsalicylate, and mounted for viewing with Nomarski optics.

Identity of engrailed stripes

The segmental identity of a particular *engrailed* stripe in the mutants is determined by its correlation in position relative to morphological markers in wild type. In addition, these have been verified (especially in the complex cases, such as *runt*) by double labeling with anti-Ubx, because the pair–rule mutations generally do not alter segmental identity. Mutant embryos are identified by characteristic and reproducible changes in the pattern of *engrailed* expression. The fraction of such embryos approximated 25%, except where noted in the text. For each mutant, roughly 10–50 mutant embryos at each developmental stage have been examined.

References

Cabrera, C., J. Botas, and A. Garcia-Bellido. 1985. Distribution of *Ultrabithorax* proteins in mutants of *Drosophila* bithorax complex and its transregulatory genes. *Nature* **318**: 569–571.

Carroll, S.B. and M.P. Scott. 1985. Localization of the *fushi tarazu* protein during *Drosophila* embryogenesis. *Cell* **43**: 47–57.

———. 1986. Zygotically active genes that affect the spatial expression of the *fushi tarazu* segmentation gene during early *Drosophila* embryogenesis. *Cell* **45**: 113–126.

Desplan, C., J. Theis, and P.H. O'Farrell. 1985. The *Drosophila* developmental gene, *engrailed*, encodes a sequence specific DNA binding activity. *Nature* **318**: 630–635.

DiNardo, S., J.M. Kuner, J. Theis, and P.H. O'Farrell. 1985. Development of embryonic pattern in *D. melanogaster* as revealed by accumulation of the nuclear *engrailed* protein. *Cell* **43**: 59–69.

Doe, C.Q. and C.S. Goodman. 1985. Early events in insect neurogenesis. II. The role of cell interactions and cell lineage in the determination of neuronal precursor cells. *Dev. Biol.* **111**: 206–219.

Duncan, I.M. 1986. Control of bithorax complex functions by the segmentation gene *fushi tarazu* of *Drosophila melanogaster*. *Cell* **47**: 297–309.

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

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

French, V., P.J. Bryant, and S. Bryant. 1976. Pattern regulation in epimorphic fields. *Science* **193**: 969–981.

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

Garcia-Bellido, A. 1975. Genetic control of wing disc development in *Drosophila*. *CIBA Found. Symp.* **29**: 161–182.

Gehring, W. 1967. Clonal analysis of determination dynamics in cultures of imaginal disks in *Drosophila melanogaster*. *Dev. Biol.* **16**: 438–456.

Gergen, J.P. and E.F. Wieschaus. 1985. The localized requirements for a gene affecting segmentation in *Drosophila*: Analysis of larvae mosaic for *runt*. *Dev. Biol.* **109**: 321–335.

———. 1986a. Dosage requirements for *runt* in the segmentation of *Drosophila* embryos. *Cell* **45**: 289–299.

———. 1986b. Localized requirements for gene activity in segmentation of *Drosophila* embryos: Analysis of *armadillo*, *fused*, *Giant*, and *unpaired* mutations in genetic mosaics. *Wilhelm Roux's Arch. Dev. Biol.* **195**: 49–62.

Gergen, J.P., D. Coulter, and E. Wieschaus. 1986. Segmental pattern and blastoderm identities. *Symp. Soc. Dev. Biol.* **43**: 195–220.

Hafen, E., A. Kuroiwa, and W.G. Gehring. 1984. Spatial distribution of transcripts from the segmentation gene *fushi tarazu* during *Drosophila* embryonic development. *Cell* **37**: 439–445.

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

Howard, K. and P. Ingham. 1986. Regulatory interactions between the segmentation genes *fushi tarazu*, *hairy*, and *engrailed* in the *Drosophila* blastoderm. *Cell* **44**: 949–957.

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

Ingham, P.W., K.R. Howard, and D. Ish-Horowicz. 1985. Transcription pattern of the *Drosophila* segmentation gene *hairy*. *Nature* **318**: 439–445.

Jurgens, G., E. Wieschaus, C. Nüsslein-Volhard, and M. Kluding. 1984. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. II. Zygotic loci on the third chromosome. *Wilhelm Roux's Arch. Dev. Biol.* **193**: 283–295.

Karr, T.L., Z. Ali, B. Drees, and T. Kornberg. 1985. The *engrailed* locus of *D. melanogaster* provides an essential zygotic function in precellular embryos. *Cell* **43**: 591–601.

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

Kornberg, T. 1981a. *engrailed*: A gene controlling compartment and segment formation in *Drosophila*. *Proc. Natl. Acad. Sci.* **78**: 1095–1099.

————. 1981b. Compartments in the abdomen of *Drosophila* and the role of the *engrailed* locus. *Dev. Biol.* **86**: 363–372.

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

Laughon, A. and M.P. Scott. 1984. Sequence of a *Drosophila* segmentation gene: Protein structure homology with DNA binding proteins. *Nature* **310**: 25–31.

Lawrence, P.A. and G. Morata. 1976. Compartments in the wing of *Drosophila*: A study of the *engrailed* gene. *Dev. Biol.* **50**: 321–337.

MacDonald, P.M. and G. Struhl. 1986. A molecular gradient in early *Drosophila* embryos and its role in specifying the body pattern. *Nature* **324**: 537–545.

MacDonald, P.M., P. Ingham, and G. Struhl. 1986. Isolation, structure, and expression of *even-skipped*: A second pair-rule gene of Drosophila containing a homeobox. *Cell* **47**: 721–734.

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

Nüsslein-Volhard, C., E. Wieschaus, and H. Kluding. 1984. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. I. Zygotic loci on the second chromosome. *Wilhelm Roux's Arch Dev. Biol.* **193**: 267–282.

Nüsslein-Volhard, C., H. Kluding, and G. Jurgens. 1985. Genes affecting the segmental subdivision of the *Drosophila* embryo. *Cold Spring Harbor Symp. Quant. Biol.* **50**: 145–154.

Ready, D.F., T.E. Hanson, and S. Benzer. 1976. Development of the *Drosophila* retina, a neurocrystalline lattice. *Dev. Biol.* **53**: 217–240.

Rijsewijk, F., M. Schuermann, E. Wagenaar, P. Parren, D. Weigel, and R. Nusse. 1987. The *Drosophila* homology of the mouse mammary oncogene *int-1* is identical to the segment polarity gene *wingless*. *Cell* **50**: 649–657.

Scott, M.P. and P.H. O'Farrell. 1986. Spatial programming of gene expression in early *Drosophila* embryogenesis. *Annu. Rev. Cell Biol.* **2**: 49–80.

Simcox, A.A. and J.H. Sang. 1983. When does determination occur in *Drosophila* embryos? *Dev. Biol.* **97**: 212–221.

Struhl, G. 1982. Genes controlling segmental specification in the *Drosophila* thorax. *Proc. Natl. Acad. Sci.* **79**: 7380–7384.

————. 1985. Near-reciprocal phenotypes caused by inactivating or indiscriminately expressing the *Drosophila* segmentation gene *ftz*. *Nature* **318**: 677–680.

Szabad, J., T. Schupbach, and E. Wieschaus. 1979. Cell lineage and development in the larval epidermis of *Drosophila melanogaster*. *Dev. Biol.* **73**: 256–271.

Van der Meer, S. 1977. Optical clean and permanent whole mount preparation for phase contrast microscopy of cuticular structures of insect larvae. *Dros. Inf. Serv.* **52**: 160.

Wakimoto, B.T. and T.C. Kaufman. 1981. Analysis of larval segmentation in lethal genotypes associated with the *Antennapedia* gene complex in *Drosophila melanogaster*. *Dev. Biol.* **81**: 51–64.

Weischaus, E. and W. Gehring. 1976. Clonal analysis of primordial disc cells in the early embryo of *Drosophila melanogaster*. *Dev. Biol.* **50**: 249–263.

Weischaus, E., C. Nüsslein-Volhard, C. and G. Jurgens. 1984a. 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.

————. 1984b. *Kruppel*, a gene whose activity is required early

in the zygotic genome for normal embryonic segmentation. *Dev. Biol.* **104**: 172–186.

White, R.A.H. and M. Wilcox. 1984. Protein products of the Bithorax Complex in *Drosophila*. *Cell* **39**: 163–171.

Wright, D.A. and P.A. Lawrence. 1981. Regeneration of the segment boundary in *Oncopeltus*. *Dev. Biol.* **85**: 317–327.