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Odd-paired: a zinc finger pair-rule protein required for the timely activation of engrailed and wingless in Drosophila embryos

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odd-paired: a zinc finger pair-rule protein required for the timely activation of *engrailed* and *wingless* in *Drosophila* embryos

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Doctor of Philosophy

This thesis is dedicated to my parents.

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I also thank the rest of the DiNardo lab for their encouragement and friendship. Along with members of the Desplan lab, they made the fifth floor of Bronk a fun place to do science and throw Christmas parties. I would especially like to thank Janet Mullen, the first postdoc to come out of the DiNardo lab. Much of this work arose through a recurring collaboration between us. In particular, her thorough genetic characterization of *odd-skipped* and her construction of the hs-*opa* transgenic strains were enormously important in helping place my own molecular characterization of *odd-paired* in a broader biological context.

"Development proceeds from a few prearranged conditions, that are given in the structure of the egg, and these conditions, by reacting on each other, produce new conditions, and these may in turn react on the first ones, etc. With every effect there is at the same time a new cause, and the possibility of a new specific action, i.e., the development of a specific receiving station for stimuli. In this way there develops from the simple conditions existing in the egg the complicated form of the embryo."

Thomas Hunt Morgan on embryonic development in Regeneration (1901)

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ABSTRACT

Here I report a molecular and genetic analysis of the pair-rule gene *odd-paired* (*opa*). Genetically, *opa* is essential for the appropriate level and timing of *en* and *wg* expression in all parasegments, but *opa* activity alone does not determine the restricted spatial domains of these genes. I summarize some of the pair-rule circuitry through which *opa* acts to regulate *en* and *wg* expression in parasegment border cells. In addition, I describe how the *opa* cuticle phenotype is generated by cell signalling interactions between *en*- and *wg*- expressing cells late in development. I also describe molecular characterization of the *opa* gene and embryonic expression of *opa* mRNA and protein. I present models describing how Opa might interact with other pair-rule proteins to regulate target gene expression in the context of the genetic and molecular data described here. Finally, I propose further experiments to address: (1) how *opa* expression might be controlled, (2) the possible function(s) of Opa expression during different stages of embryogenesis, and (3) specific molecular mechanisms by which Opa might interact with other proteins to directly regulate target gene expression.

Introduction

A basic problem in developmental biology is understanding the conversion of the apparently uniform egg into a more patterned adult. This patterning arises from the establishment and subdivision of the anteroposterior (AP) and dorsoventral (DV) axes of the embryo body. Throughout embryogenesis, this patterning occurs as cells within the embryo adopt discrete fates. Thus, somehow the uniform cells of the early embryo (or nuclei of a syncytial embryo) must distinguish themselves from their neighbors along these axes as development proceeds.

In insect embryos, several classical embryological experiments revealed that AP axis specification is completed early in development. Ligation experiments demonstrated that communication between morphogenetic signalling centers at the egg termini generate patterning. If this communication is blocked, then normal patterning is disrupted. For example, if developing embryos are ligated prior to cellularisation, neither embryo half develops body segments normally present in the embryo midsection (Schubiger et al., 1977a), and embryos ligated earlier lack more parts than those ligated later (Vogel, 1977; Schubiger et al., 1977a). Ligation causes a membrane to form which physically separates the two halves of the treated embryo. If this membrane is punctured soon after ligation this restores continuity of the egg and a complete embryo can then form (Schubiger et al., 1977b).

Other experiments implied that these signals are carried by localized cytoplasmic components at the ends of the egg. For example, *Drosophila* embryos from *Bicaudal* (*Bic*) mutant females have a mirror image abdomen in the place of anterior structures (Bull, 1966). The *Bic* mutation could be phenocopied in other insect species by either centrifuging embryos or irradiating anterior egg cytoplasm (Kalthoff, 1971; Kalthoff, 1977; Yajima, 1983). Conversely, irradiation of posterior egg cytoplasm creates double-headed embryos in many insects (Kalthoff, 1978; Yajima, 1983). Together, these data demonstrate that progressive interactions between cytoplasmic signals located at the embryo poles

generate AP polarity. Formal models designed in the context of these experiments proposed that this patterning might arise through the formation of one or more morphogen gradients peaking at the ends of the egg (Wolpert, 1968; Crick, 1970; Gierer et al., 1972).

After the AP axis is set up in the insect embryo, the body plan is subdivided into cephalic, thoracic, and abdominal segments. Early experiments demonstrated that this segmentation is completely determined by formation of the cellular blastoderm. Embryos ligated after cellularisation form complete pattern of segments, but prior to cellularization ligation results in loss of segments from the middle of the embryo (Schubiger, 1976). Consistent with this, marked cells from halves of blastoderm stage embryos develop into distinct adult structures with positional character corresponding to that of specific segment primordia along the blastoderm AP axis (Chan et al., 1971; Schubiger, 1976). How might this segmentation be generated? Numerical and computer graphic models proposed interactions that might form and stabilize axial subdivisions to generate a periodic, segmented pattern from an intially aperiodic one (Turing, 1952; Cooke, 1976).

Although these approaches described how different egg components might interact to generate body pattern, they did not address what these components were or the specific biochemical mechanisms behind their interactions. Initial direct biochemical experiments attempted to answer these questions, but this strategy proved somewhat limiting. For instance, the *Bic* mutation mentioned above could be phenocopied in the midge by injection of RNase into the anterior of wildtype embryos (Kandler-Singer et al., 1976). This pointed to a localized RNA being important for anterior patterning, but such an approach did not identify the source of this RNA, how it was anteriorly localized, or its mechanism of action. In contrast, a concerted molecular and genetic approach to studying pattern formation has identified specific genes and biochemical pathways crucial for patterning in a number of organisms (Redei, 1970; Nusslein-Volhard et al., 1987; Kimmel, 1989; Meyerowitz et al., 1989; Jurgens et al., 1991). In particular, the early genetic experiments of Ed Lewis (Lewis, 1978) and Christiane Nusslein Vollhard

(Nusslein-Volhard et al., 1987) identified specific maternal and zygotic genes which interact to generate pattern in *Drosophila* embryos. Further molecular characterisation of these genes revealed the biochemical mechanisms underlying these interactions (for recent reviews see [St. Johnston et al., 1992; Hoch et al., 1993; Tabata et al., 1993]).

Below I summarize how proteins encoded by the *bicoid* and *hunchback* genes set up the AP axis in *Drosophila*. Coarse division of this axis arises via expression of discrete domains of gap gene expression. The first evidence of metameric subdivision of the fly embryo then occurs when the pair-rule genes are expressed in periodic patterns of stripes. I describe mechanisms by which this periodic expression is established and refined through a network of pair-rule genetic interactions. These interactions, in turn, serve to precisely activate expression of the segment polarity genes *wg* (Baker, 1987) and *en* (Kornberg et al., 1985) in discrete, periodic striped domains across the AP axis. These domains subdivide the embryo into intervals roughly four cells in width called parasegments (PS) (Martinez-Arias et al., 1985).

Here I report that activity of the pair-rule gene *odd-paired* (*opa*) is essential for the appropriate level and timing of *en* and *wg* expression in all parasegments, but *opa* activity alone does not determine the restricted spatial domains of these genes. I report Janet Mullen's work in which she describes how *opa* acts through the pair-rule genes *odd* and *ftz* to regulate even PS domains of *en* expression. In addition, I describe how post-blastoderm cell signalling interactions between *en*- and *wg*- expressing cells generate the *opa* cuticle phenotype. I also describe molecular characterization of the *opa* gene and embryonic expression of *opa* mRNA and protein. I present models describing how Opa might interact with other proteins to regulate target gene expression in the context of Janet Mullen's genetic data. Finally, I propose further experiments to address: (1) how *opa* expression might be controlled, (2) the possible function(s) of Opa expression during different stages of embryogenesis, and (3) specific molecular mechanisms by which Opa might interact with other proteins to directly regulate target genes.

The Bicoid morphogen gradient establishes AP polarity in the *Drosophila* embryo

In *Drosophila*, establishment of the anteroposterior (AP) axis begins with deposition of maternal gene products in gradients across the embryo body. Subdivision of the axis proceeds via a regulatory hierarchy of gene expression up to the time of cellular blastoderm formation (Nusslein-Volhard et al., 1980) (for reviews, see [Nusslein-Volhard et al., 1985; Carroll et al., 1986; Ingham, 1988a]). First, a morphogen gradient forms through diffusion and uniform degradation of the maternally encoded Bicoid (Bcd) homeoprotein (Driever et al., 1988a; Driever et al., 1988b; Struhl et al., 1989b). This gradient peaks at the embryo anterior, and altering Bcd distribution along this gradient directly alters embryonic patterning. For instance, embryos from *bcd* mothers lack anterior head and thoracic structures, as do wildtype embryos which have lost anterior cytoplasm (Frohnhofer et al., 1986). Such mutant embryos can be rescued by anterior injection of wildtype anterior cytoplasm or *bcd* mRNA, and injection elsewhere in the embryo induces ectopic head structures at the site of injection (Driever et al., 1988a; Driever, 1990). Altering the absolute amount of Bicoid in the gradient also affects patterning. Injection of large amounts of wildtype cytoplasm into the anterior of bcd mutant embryos leads to formation of head structures while injection of lower amounts generates more posterior pattern elements (Frohnhofer et al., 1986). Changing *bcd* dosage genetically can also affect anterior patterning, as embryos with half the normal amount of Bcd develop reduced head and thoracic structures (Frohnhofer , 1986; Berleth et al., 1988). Conversely, embryos from female flies containing 4 copies of *bicoid* have expanded anterior structures extending into the embryo posterior (Driever et al., 1988a).

The *hunchback* (hb) gene is also maternally transcribed, but the *hb* transcript is uniformly distributed throughout the embryo body (Tautz et al., 1987). Translation of this message is repressed by Nanos in the embryo posterior (Hulskamp et al., 1989; Irish et al., 1989). Zygotic transcription of *hb* is activated in the embryo anterior by high concentrations of Bicoid (Tautz et al., 1987; Schroder et al., 1988; Tautz, 1988; Driever

et al., 1989a; Struhl et al., 1989b). As a result of these interactions, the Hb morphogen is translated in a gradient peaking at the embryo anterior and extending to the midpoint of the embryo. In contrast to *bicoid*, altering this gradient alters posterior patterning in the embryo. For example, in *nanos* (*nos*) mutant embryos, Hb accumulates in the embryo posterior, giving rise to embryos lacking posterior segments (Tautz et al., 1987; Struhl, 1992). Together, both the Bcd and Hb gradients help to set up the *Drosophila* AP axis prior to its segmentation later in embryogenesis.

Gap genes roughly subdivide the AP axis

The gap genes *tailless* (*tll*), *hunchback* (*hb*), *Krüppel* (*Kr*), *knirps* (*kni*) and *giant* (*gt*) are transcribed in restricted domains in response to different threshold concentrations of Hb and Bcd across the AP axis (Knipple et al., 1985; Strecker et al., 1986; Gaul and Jackle, 1987; Lehmann et al., 1987; Nauber et al., 1988; Kraut et al., 1991a; Kraut et al., 1991b). This transcription is thought to be due to direct interactions of Hb and Bcd on a given gap gene promoter. For example, *Kr* expression is activated by different concentrations of both Bcd and Hb binding to multiple sites within a 730 bp element to its promoter (Hoch et al., 1990). Mutations in either the Bicoid homeodomain or this element result in aberrant *Kr* expression (Hoch et al., 1991; Hoch et al., 1992). Refinement of this initial domain is thought to occur via antagonistic binding of other activators or repressors to these sites (Gaul et al., 1987; Eldon et al., 1991; Steingrimsson et al., 1991; Kraut et al., 1991a; Kraut et al., 1991b; Capovilla et al., 1992; Hoch et al., 1992). The gap gene *kni*, on the other hand is initially activated throughout the entire embryo at low levels (Nauber et al., 1988; Pankratz et al., 1992). Later, Hb and Tll repress *kni* expression in the embryo anterior and posterior, respectively, to generate the single central striped domain of *kni* occurring just posterior to the Kr domain (Pankratz et al., 1989; Hulskamp et al., 1990; Pankratz et al., 1992; Struhl, 1992). This has been shown by examining *kni* expression in either *hb* or *tll* mutant embryos. In *hb* mutant

embryos the *kni* domain expands anteriorly (Nauber et al., 1988; Hulskamp et al., 1990). Loss of Hb binding sites in the *kni* promoter also results in anterior expansion of *kni* expression (Pankratz et al., 1989; Pankratz et al., 1992). Conversely, *kni* expression is expanded posteriorly in *tll* mutant embryos (Jackle et al., 1986). Mutually repressive interactions such as these refine initial patterns of expression of the other gap genes as well (Jackle et al., 1986; Bronner et al., 1991).

Gap genes regulate primary pair-rule stripe expression

In addition to mutually refining their own expression, domains of gap gene expression serve as short range gradients which activate transcription of the primary pair-rule genes, *hairy* (*h*), *even-skipped* (*eve*), and *runt* (*run*), each of which is expressed in a striped pattern (Ingham et al., 1985; MacDonald et al., 1986a; Frasch et al., 1987; Carroll et al., 1988; Gergen et al., 1988; Goto et al., 1989; Stanojevic et al., 1989; Pankratz et al., 1990). Consistent with this, *eve* expression is altered relative to wildtype in *hb*, *Kr*, or *gt* mutant embryos (Frasch et al., 1987). All of the pair-rule genes are expressed in dynamic, striped patterns with each stripe encompassing two to four cells (Hafen et al., 1984a; Ingham et al., 1985; Kilchherr et al., 1986; MacDonald et al., 1986a; Gergen et al., 1988; Coulter et al., 1990; Grossniklaus et al., 1992). This restricted, periodic expression is necessary for proper segmental subdivision of the embryo, as embryos mutant for *h*, *eve*, or *run* have severe segmentation defects (Nusslein-Volhard et al., 1980).

What are some of the molecular mechanisms which generate pair-rule expression in discrete, confined domains? Restricted primary pair-rule gene expression arises in response to different concentrations of various gap proteins in nuclei across the AP axis of the syncytial embryo (Howard et al., 1988; Warrior et al., 1990; Riddihough et al., 1991; Small et al., 1991; Small, 1992). At least two of the primary pair-rule genes, *h* and *eve* contain stripe specific promoter elements which direct expression of a given stripe (Howard et al., 1988; Goto et al., 1989; Harding et al., 1989; Lardelli et al., 1993). For

instance, the *h* promoter contains elements which activate transcription in response to different concentrations of maternal, gap, and pair-rule proteins across the AP axis. Each of these elements can direct expression of *lacZ* in a domain closely approximating a given *h* stripe (Riddihough et al., 1991). Expression of individual stripes is abolished when these elements are mutated (Howard et al., 1988). The primary pair-rule gene *eve* is also regulated in a stripe specific fashion at blastoderm (Goto et al., 1989; Harding et al., 1989). Small, et al. (1992) showed that expression of the *eve* stripe 2 is controlled by a 480 bp promoter element. This element can direct expression of *lacZ* in a domain corresponding to that of *eve* stripe 2, and this expression depends upon cooperative interactions between Bcd, Hb, Kr,and Gt binding at this element (Goto et al., 1989; Stanojevic et al., 1989; Small et al., 1991; Stanojevic, 1991; Small, 1992). Presumably, Bcd and Hb initially activate transcription of this stripe. Gt and Kr repress expression at the anterior and posterior of this domain, respectively, to generate the restricted stripe (Harding et al., 1989; Small et al., 1991; Stanojevic, 1991). *eve* stripe 3 expression is also discretely activated by a specific promoter element (Goto et al., 1989; Harding et al., 1989). This enhancer is located on a 500 bp element 1.7 kb upstream of the stripe 2 element. Proper spacing between these enhancers is crucial for wild type expression of *eve*, as when the enhancers are brought together in tandem, stripe 2 is more intense in expression than wild type and is expanded posteriorly (Small et al., 1993).

Primary pair-rule proteins regulate secondary pair-rule gene expression

The primary pair-rule proteins, probably with assistance from the gap proteins, activate the secondary pair-rule genes, *fushi tarazu* (*ftz*), *paired* (*prd*), *oddskipped* (*odd*) and *sloppy-paired* (*slp*) (Carroll et al., 1986; Howard et al., 1986; Frasch et al., 1987; Baumgartner et al., 1991; Grossniklaus et al., 1992). The stripe-specific control of primary pair-rule gene expression is in marked contrast to that of the secondary pair-rule genes. For instance, *ftz* is expressed in a broad domain across all segment primordia prior

to cellularisation (Edgar et al., 1986b; Karr et al., 1989). At this stage, many other *Drosophila* genes are generally transcribed throughout the embryo (Edgar et al., 1989). Perhaps the activators responsible for this general activation drive initial *ftz* expression. At cellularisation, initial broad expression of *ftz* evolves into a pattern of seven discrete stripes of *ftz* expression with double segment periodicity (Hafen et al., 1984a; Carroll et al., 1985). Presumably these stripes form via repression of interstripe regions by a repressor translated prior to cellularisation, as *ftz* remains broadly expressed in precellular embryos injected with cycloheximide (Edgar et al., 1986b). The *ftz* promoter contains three primary *cis*-regulatory elements (Hiromi et al., 1985). When fused to a *lacZ* reporter gene and transformed into *Drosophila* embryos, each drives expression of *lacZ* in a distinct pattern. The neurogenic element drives *lacZ* expression in specific neuronal cells in the developing central nervous system (Carroll et al., 1985; Hiromi et al., 1985). The zebra element activates striped *lacZ* expression in primordial mesodermal tissue (Hiromi et al., 1985; Hiromi et al., 1987). The remaining element, the upstream element (Hiromi et al., 1985; Hiromi et al., 1987) contains two distinct enhancers. The distal enhancer, directs *lacZ* expression in 7 mesodermally restricted stripes, while the proximal enhancer drives *lacZ* expression in both ectodermal and mesodermal tissues (Pick et al., 1990). Expression of upstream element-*lacZ* constructs is reduced in *ftz* mutant embryos (Hiromi et al., 1987). In addition, each enhancer within the upstream element is independently capable of being positively autoregulated by Ftz (Pick et al., 1990).

Once the 7-stripe pattern is initiated, stripe expression is maintained by at least two different mechanisms. The *ftz* mRNA is short-lived, and continuous transcriptional activation is required to maintain the steady state levels of expression seen in wildtype embryos (Edgar et al., 1987). The zebra element contains binding sites for the Ttk/Ftz-F2 zinc finger protein and the Ftz-F1 steroid hormone receptor-like protein (Harrison et al., 1990; Ueda et al., 1990; Brown et al., 1991; Lavorgna et al., 1991). When the Ftz-F1 binding sites are mutated in these constructs, expression is reduced relative to wildtype

(Ueda et al., 1990). Repressors binding to other sites on the zebra element also contribute to the interstripe repression which generates the final striped pattern (Hiromi et al., 1985; Dearolf et al., 1989; Brown et al., 1991; Topol et al., 1991). In contrast, a zebra element-*lacZ* construct is expressed throughout the embryo when Ftz-F2/Ttk binding sites are mutated (Brown and Wu, 1993). Together, these autoregulatory and other *cis*- and *trans*-activating inputs serve to establish and maintain wildtype levels of *ftz* expression within all stripes of its seven striped domain.

Pair-rule genetic interactions restrict *en* and *wg* expression to single cell-wide stripes

After initial broad expression of the secondary pair-rule genes is established, mutual interactions refine this pattern into more restricted striped domains. Refinement of the initial pattern results in narrowing of the initial domains of expression. For instance, the *eve* and *ftz* stripes of expression are initially broad with indistinct borders (Frasch et al., 1987; Goto et al., 1989). Mutually repressive interactions restrict these stripes to narrow domains of seven alternating stripes with cells expressing neither gene in between (van den Heuvel et al., 1989).

The distribution of protein within individual stripes also changes during development. The initial bell-shaped distribution of mRNA and protein within a given stripe changes such that concentration peaks in the anteriormost cell of each stripe. This creates stripes with sharp anterior borders and fuzzy posterior edges (van den Heuvel et al., 1989). Throughout cellularization the stripes become more narrow and uniform such that by the end of cellularization the stripes are sharply defined and 1-2 cells wide. This sharp on-off periodicity of the stripes is generated by a combination of pair-rule genetic interactions and autoregulatory processes for a given pair-rule gene. Rapid changes in *ftz* transcription are seen in embryos in which *eve* is ectopically expressed, suggesting a direct effect on *ftz* transcription by Eve (Manoukian et al., 1992). Sharpening of the

initial *eve* striped pattern of expression also requires interactions from the other pair-rule genes, as late *eve* stripes do not form properly in *h* mutant embryos (Goto et al., 1989).

eve stripe expression is also reinforced by positive autoregulatory input from the Eve protein itself binding to sites within its own promoter (Goto et al., 1989; Harding et al., 1989; Jiang et al., 1991). When these sites are mutated in *eve* promoter-*lacZ* fusions, *lacZ* expression is greatly reduced (Jiang et al., 1991). Similarly, embryos containing mutations in the Eve homeodomain which disrupt DNA binding show abnormal *eve* expression (Frasch, 1988). These mutations as well as *ftz* mutations which result in abnormally perduring Ftz protein also disrupt the uniform spacing between *ftz* and *eve* stripes (Kellerman et al., 1990). Embryos with either of these mutations develop with abnormal spacing of segment primordia later in embryogenesis.

All of these mechanisms generate the refined pair-rule gene expression patterns required for precise activation of *wg* (Baker, 1987) and *en* (Kornberg et al., 1985) in fourteen single cell-wide striped domains at blastoderm. These *wg* and *en* domains subdivide and maintain the body plan into small, repeating units called parasegments (PS) (Martinez-Arias et al., 1985). The expression of both of these genes must be controlled with single-cell precision for this to occur (Poole et al., 1988; Noordermeer et al., 1992). *wg* is expressed in the row of cells that defines the posterior edge of each PS (Ingham, 1991b; Dougan et al., 1992), while *en* is expressed in the row of cells that defines the anterior edge of each PS (Lawrence et al., 1987; Carroll et al., 1988).

Gergen et al. (1986) first proposed that the pair-rule genes act in combination to specify cell position within the embryo. Several models suggest combinatorial interactions among pair-rule proteins restrict activation of *wg* and *en* to single cell-wide stripes (Howard et al., 1986; DiNardo et al., 1987; Ingham et al., 1988b; Morrissey, 1991; Manoukian et al., 1992; Manoukian et al., 1993). Fig. 1 summarizes the pair-rule genetic interactions originally thought to regulate the expression of *wg* and *en* at the PS border cells. For instance, *paired* (*prd*) is required for odd PS *en* expression, as in *prd* mutant

Fig. 1 Original model for expression and activity of *opa* at blastoderm.

Two complete PS are shown, with *en*-expressing cells (filled nuclei) at the anterior and wg-expressing cells (filled cytoplasm) at the posterior of each PS. Interactions (↓ = activating, ⊥ = repressive) involving *opa* are in boldface solely for emphasis and do not reflect magnitude of interaction. In the even PS, *ftz* is expressed in a gradient similar to *eve*. *ftz* eventually retracts to the anteriormost cell of the PS. *opa* was postulated to assist *ftz* in activation of *en* in the even PS (Ingham et al., 1988). The *prd* domain straddling the even/odd PS border activates *wg* (filled cytoplasm) in the even PS (Ingham et al., 1988). *eve* is expressed in a graded manner across the odd PS as it retracts to the anteriormost cell of the PS. The anteriormost *eve*-expressing cell overlaps with one domain of *prd* expression, and *eve* and *prd* together appear to activate *en* expression (filled nuclei) in these PS (Morrissey et al., 1991). *opa* activates *wg* in the odd PS (thick arrow) in a fashion analogous to that of prd in the even PS and was postulated to be expressed in a restricted pattern similar to that of *prd* (Ingham et al., 1988).

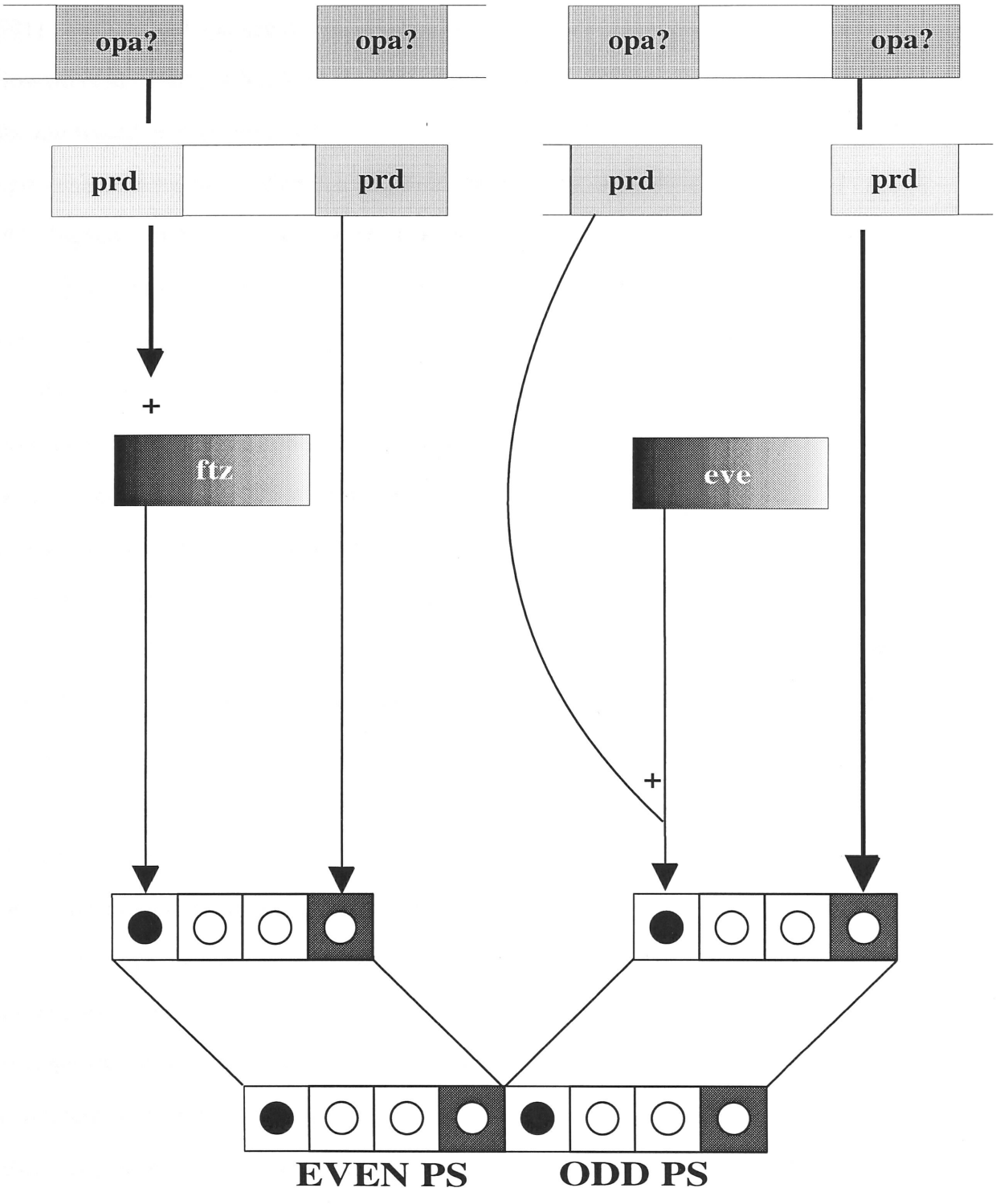


Fig. 1

embryos *en* fails to be expressed in the odd PS (DiNardo et al., 1987; Baumgartner et al., 1991). Both *ftz* and *opa* activity are required for normal *en* expression in the even PS (Howard et al., 1986; DiNardo et al., 1987; Ingham et al., 1988b). It was hypothesized that *opa* would be expressed or active in restricted domains, such that *ftz*- and *opa*-expressing cells would overlap in a single-cell wide stripe in each even PS (DiNardo , 1987; Ingham, 1988a). *en* would then be activated only in cells expressing both proteins.

The restricted expression of *wg* was also thought to involve the position-specific expression or activity of the *opa* gene (Ingham et al., 1988b). In *prd* mutant embryos, even PS *wg* expression is missing. In *opa* mutant embryos, *wg* expression is missing in the odd PS (Ingham et al., 1988b). This suggested a symmetrical, position-specific role for *opa* in generating odd PS *wg* expression analogous to that of *prd* in the even PS. In contrast, *ftz* and *eve* are genetic repressors of *wg*, as in either *ftz* or *eve* mutant embryos, *wg* expression is greatly expanded (Ingham et al., 1988b). Thus, it was thought that cells expressing *prd* or *opa* but not *ftz* or *eve* would express *wg*. Overall, these models pointed to *opa* having a functional domain restricted to the PS border cells and acting as the odd PS counterpart to *prd* with regard to its effects on *wg* expression.

BACKGROUND

Body pattern defects in *opa* mutant embryos

The segmental pattern of wildtype embryos is easily visible at the end of embryogenesis in the cuticle secreted by embryonic epidermal cells. Late in development, these cells undergo dramatic shape changes as a result of changes in cytoskeletal architecture (Lohs-Schardin et al., 1979; Pesacreta et al., 1989) . During this period, they secrete a mixture of chitin, various proteins, and phenolic compounds which hardens into the larval exoskeleton, itself textured to shape of underlying cells at the time of secretion (Hillman et al., 1970). At the end of embryogenesis the body pattern consists of 11 body segments. There are three thoracic (T1-T3) and eight abdominal segments

(A1-A8) (Fig. 2, panel A) visible on the ventral embryo surface. The anterior part of each segment has several rows of cuticular projections, or denticles, comprising a denticle belt. The remainder of each segment consists of smooth cuticle. Each segment has a pattern unique to its position on the AP axis, and for a given segment, this pattern is invariant from embryo to embryo.

In *opa* null mutant embryos, only half the number of denticle belts are present due to the deletion of portions of denticle bands from T2, A1, A3, A5, and A7 (the even PS) and portions of smooth cuticle from anteriorly adjacent segments T1, T3, A2, A4, A6, and A8 (the odd PS) (Jurgens et al., 1984). Fig. 2 shows the ventral cuticle patterns of wildtype (Fig. 2, panel A) and hypomorphic and null *opa* mutant embryos (Fig. 2, panels B, C.). Note the increasing loss of pattern elements (severity of denticle fusions) with increasing loss of *opa* activity. All *opa* mutant embryos have severe head defects.

opa activity is required for optimal levels of _en_ and _wg_ in all PS

At cellular blastoderm in wild type embryos, *wg* is expressed in 14 single-cell wide stripes defining the posterior borders of PS 0-13 ((Baker, 1987); Fig 3, panels A, C). In *opa* null embryos (for sources of all stocks, see Materials and methods), *wg* expression never initiates in the odd PS, as previously reported for EMS-induced *opa* alleles (Ingham et al., 1988b). In the even PS, *wg* expression is severely delayed except in PS 0 and 2 (Fig 3, panel B). As development proceeds, *wg* expression initiates in the even PS 4-12 and approaches normal levels, although it remains spotty (see Fig 3, panel D). We conclude that *opa* function is essential for the initiation of *wg* in the odd PS, and is important for normal *wg* expression in the even PS. Similarly, *opa* is required for normal *en* expression in all PS. In wild type embryos, *en* is expressed in 14 single-cell wide stripes by the onset of gastrulation ([Kornberg et al., 1985]; Fig 3, panel E). In *opa* null mutants at gastrulation, little *en* expression is visible (Fig 3, panel F). *en* expression is severely delayed, but eventually appears in both the odd- and even PS, indicating that *opa*

Fig. 2 Body pattern defects of *opa* mutant embryos.

A. Wild type cuticle, anterior up, ventral view. **B.** *opa* hypomorphic cuticle, opa^{13D}/Df (3R) 107. Note gaps between incompletely fused denticles. **C.** *opa* null cuticle, Df(3R) 63/Df(3R) 107, only half of the segments develop. Most of each odd abdominal denticle belt is deleted, e.g., compare denticle fusions of A4/A3 and A2/A1 with those in panel **B**. The anterior half of each even segment is fused to the posterior remnant of each odd denticle belt (Ingham et al., 1986).

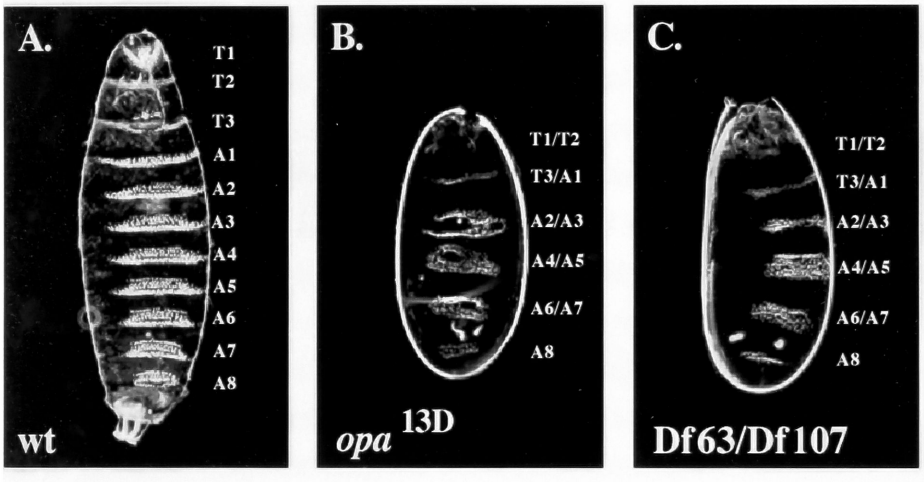

A.
T1
T2
T3
A1
A2
A3
A4
A5
A6
A7
A8
wt
B.
T1/T2
T3/A1
A2/A3
A4/A5
A6/A7
A8
opa 13D
C.
T1/T2
T3/A1
A2/A3
A4/A5
A6/A7
A8
Df63/Df107

Fig. 2

Fig. 3 The regulation of *wg*, *en*, *odd* and *ftz* expression by *opa*.

All embryos shown here and throughout are oriented anterior left, ventral down. All *opa* mutants shown are null unless otherwise indicated. Staging is after Campos-Ortega, et al., (1985). **A - D.** Embryos labelled *in situ* for *wg* and *lacZ* RNA. **A.** Wild type at cellular blastoderm, *wg* is expressed in PS 0-13, and *lacZ* is expressed anteriorly from the *hb-lacZ* transgene on the balancer chromosome. **B.** *opa* at cellular blastoderm, *wg* is expressed only in PS 0 and 2. The anterior patch of *wg* expression and the head "blob" (obscured by *lacZ* in **A**), and the posterior ring are established normally in *opa* mutants. **C.** Wildtype stage 8. **D.** *opa* stage 8, note only seven stripes are present and less intense than those in wildtype. **E-H.** labelled *in situ* for *lacZ* and En. **E.** Wild type stage 7, En is expressed in 14 stripes. **F.** *opa* stage 7, En is expressed only in PS 4. **G.** Wild type stage 8, En stripes are well-established. **H.** *opa* stage 8, En is just appearing, first in PS 4 and 8, then in PS 6 and 10. The previous analysis of *en* expression in *opa* mutants suggesting that these were the odd *en* stripes is incorrect (Inghamet al., 1986; DiNardoet al., 1987). *opa* embryos doubly labelled for *wg* and *en* expression confirm that these En stripes correspond to the even PS (see Fig. 4). **I - N.** Embryos doubly labeled for *odd* RNA (blue) and Ftz protein (brown). **I.** Wild type stage 8, *odd* stripes in the even PS have narrowed to single-cell width, while Ftz stripes are roughly 2 cells wide. The secondary *odd* stripes have appeared in the odd PS. **J.** *opa* stage 8, secondary *odd* stripes never appear, clearly marking the mutant embryos. *odd* expression persists at relatively high levels in PS 2, where *en* induction is lowest (see **H**). **K.** Magnified view of PS 4-10 of wild type in **I**. Each Ftz stripe extends 1-2 cell widths anterior to each primary *odd* stripe. **L.** Magnified view of PS 4-10 of *opa* embryo from panel **J**. Ftz remains strong and broad in PS 4, rather than narrowing (compare with **K**). *odd* expression is beginning to clear from anteriormost Ftz cells of PS 4 and 8, where *en* expression appears first in *opa* mutants (see **F.**). *odd* expression remains coincident with Ftz cells in PS 6, where *en* expression is more severely delayed (see **H**). **M.** Wild type stage 9, Ftz stripes have retracted to ~1 cell wide, just anterior to the primary *odd* stripes. **N.** *opa* stage 9, Ftz fades early in PS 2. High level *odd* persisting in PS 2 (see **J.**), may cause early loss of Ftz, since *odd* represses *ftz* expression in wild type (Janet Mullen and Steve DiNardo, in preparation). Early loss of Ftz in PS 2 could be the cause of very weak En expression in this PS (see **H.**). Ftz is quite broad in PS 4 and 8, and *odd* has faded from PS 4 and 8, correlating with the broadening of En in these PS at this stage (see **P.**). **O.** Wild type stage 9, En is expressed in 14 evenly spaced stripes.

P. *opa* stage 9, En is broad in PS 4 and 8. En expression in the odd PS is established, although all stripes are stronger ventrally than dorsally. In older *opa* embryos, *en* expression in the even PS decays, as previously reported (see Fig. 4 below) (Ingham et al., 1986; DiNardo et al., 1987).

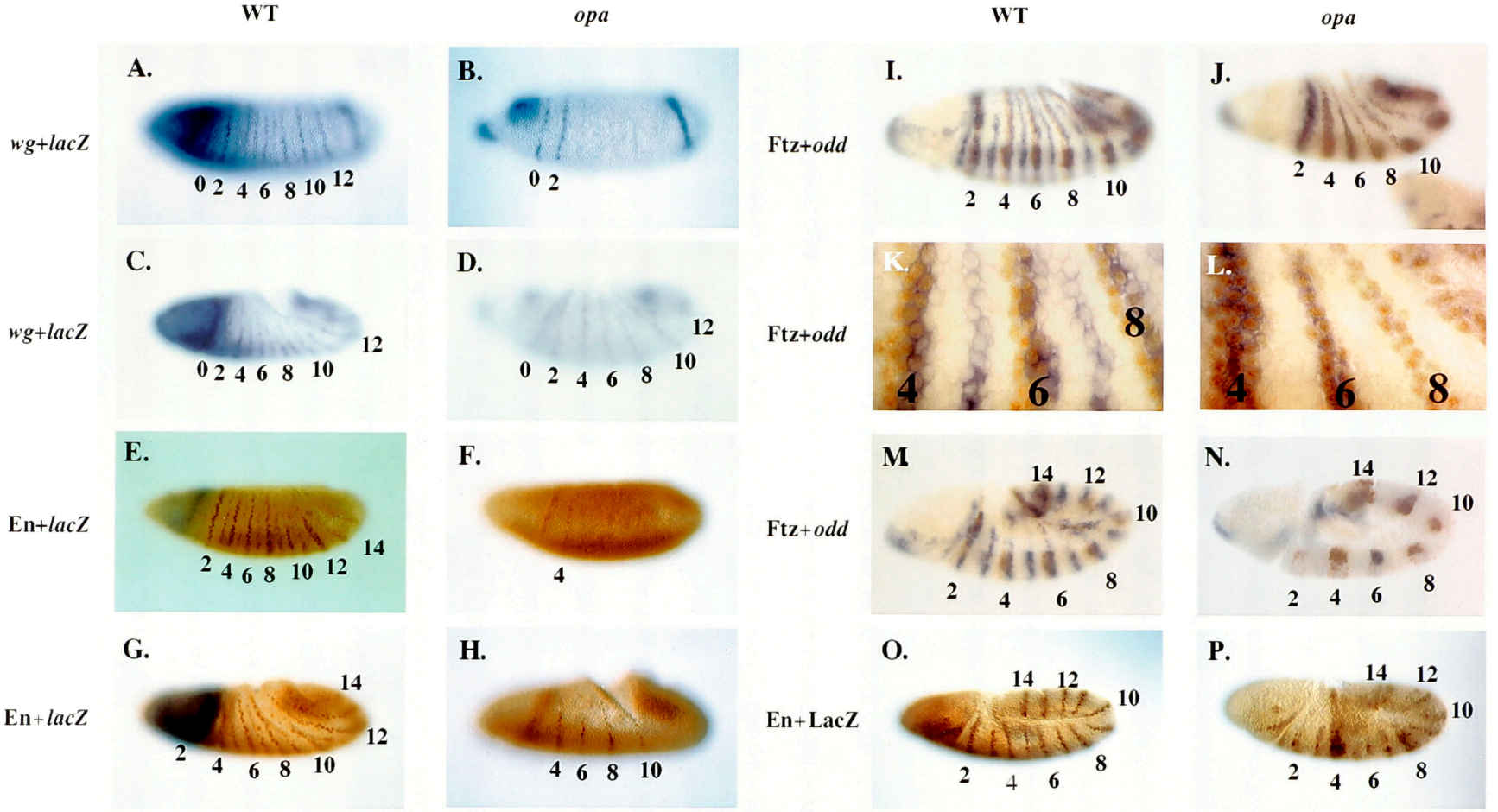

WT
opa
WT
opa
wg+lacZ
wg+lacZ
En+lacZ
En+lacZ
Ftz+odd
Ftz+odd
Ftz+odd
En+LacZ
A.
0 2 4 6 8 10 12
B.
0 2
I.
2 4 6 8 10
J.
2 4 6 8 10
C.
0 2 4 6 8 10 12
D.
0 2 4 6 8 10 12
K.
4 6 8
L.
4 6 8
E.
2 4 6 8 10 12 14
F.
4
M
14 12 10 2 4 6 8
N.
14 12 10 2 4 6 8
G.
14 12 2 4 6 8 10
H.
4 6 8 10
O.
14 12 10 2 4 6 8
P.
14 12 10 2 4 6 8

Fig. 3

is required in all PS for the timely activation of *en* (Fig 3, panel P). The defects in both *wg* and *en* expression demonstrate that *opa* is required in the cells that flank each PS border, and not only in the cells flanking alternate PS borders, as was previously thought (DiNardo et al., 1987; Ingham et al., 1988b). In the earlier analysis of EMS-induced *opa* alleles, some *en* expression was detected in the even PS. Although this was assumed to be due to residual *opa* activity, we have found that in *opa* null embryos, *en* is expressed in the correct position in these PS (see below and Fig 3, panels H, P). This result indicates that although *opa* is required for the proper timing of *en* activation, it is not required for either activation of, or proper positioning of *en* expression in the even PS.

The position of *en* expression in the even PS is determined by *ftz* and *odd*

It has been suggested that, in the even PS, the position of *en* expression is defined by the presence of the activator *ftz*, and the absence of *odd*, a presumed repressor of *en* (DiNardo et al., 1987; Howard et al., 1988; Manoukian et al., 1993). We investigated whether *opa* mutants exhibited changes in the expression of Ftz protein and *odd* mRNA that could account for the defects in *en* expression. During cellularization in wild type, Ftz is expressed in 7 stripes approximately three cells wide, corresponding to the anteriormost cells of the even PS (Carroll et al., 1985). At the same time, *odd* transcript appears in seven broad primary stripes that overlap completely with the cells expressing Ftz (Coulter et al., 1990; Manoukian et al., 1992). By gastrulation, *odd* expression (blue color in Fig. 3, panel K) is repressed in the anterior-most Ftz^+ cells (brown color in Fig. 3, panel I), leaving each Ftz stripe extending one or two cell diameters anterior to each primary *odd* stripe. It is these anterior-most Ftz^+ cells that activate *en* ([Lawrence et al., 1987; Carroll et al., 1988]; see[Fig 3, panel E]). After cellularization, *odd* expression is initiated in secondary stripes within the odd PS (Coulter et al., 1990); panels I, K).

In *opa* mutants, the secondary *odd* stripes fail to appear (compare Fig 3, panels I and J), indicating that *opa* is required for the activation of *odd* expression in the odd PS.

In addition, the expression of *odd* in its primary stripes remains coincident with the Ftz$^+$ cells at a stage where, in wild type, *odd* expression has been lost from these anterior-most Ftz$^+$ cells (Fig 3, panels K and L). Indeed, although the *odd* and Ftz expression patterns are somewhat variable, Janet Mullen observes *en* expression in *opa* mutant embryos strongly correlates with the presence of Ftz and the absence of *odd*. This suggests that the delay in activation of *en* in even PS is due to persistent *odd* expression. A continued correlation between coincident *odd* and Ftz expression and the delay in *en* activation in *opa* mutants supports this notion.

During early germband extension in *opa* mutants, *odd* expression is lost from the anteriormost Ftz$^+$ cells of PS 4 and 8 (Fig 3, panel L). About this time, *en* expression first appears in PS 4 and 8 (Fig 3, panel H). The close timing of these events suggests that Ftz can activate *en* in the anterior-most cells of its domain only after *odd* activity is removed. Around mid-germband extension, the Ftz stripes become particularly broad in PS 4 and 8, and *odd* expression in these PS is no longer detectable (Fig 3, panel N). Shortly thereafter, the *en* stripes in PS 4 and 8 broaden to about twice wild type width (Fig 3, panel P) correlating with the presence of Ftz and the absence of *odd* in these cells. This supports the model of Manoukian and Krause in which the position of *en* expression is determined by the offset of the expression domains of *odd* and Ftz (Manoukian et al., 1993).

Even PS *en* expression decays late in *opa* mutants

Given *opa* has functions in many cells across each PS, and not just in the PS border cells, how is the *opa* cuticle phenotype generated? Given its widespread function in the blastoderm embryo, one would predict that *opa* mutant embryos would lack all segmentation. The cuticle phenotype in *opa* mutant embryos can be explained if one takes into account post-blastoderm cell signalling interactions betweenen and wg expressing PS border cells. In wildtype embryos, the single cell-wide *en* and *wg*

Fig. 4 Even PS *en* expression decays late in *opa* mutant embryos.

All embryos are stained for *wg* RNA (blue stripes), *lacZ* RNA (head staining), and En protein (brown stripes). **A.** Germband elongated wildtype embryo. Note uniformity of En stripes apposed to adjoining *wg* stripes. **B.** Germband elongated *opa* null embryo of the same age as that in **A**. Although stripes are not consistent, all fourteen En stripes are clearly evident. **C.** Slightly older germband elongated *opa* null embryo than that in panel **B**. Compare En stripes 6 and 8 from this embryo with those from panels **A** and **B**.

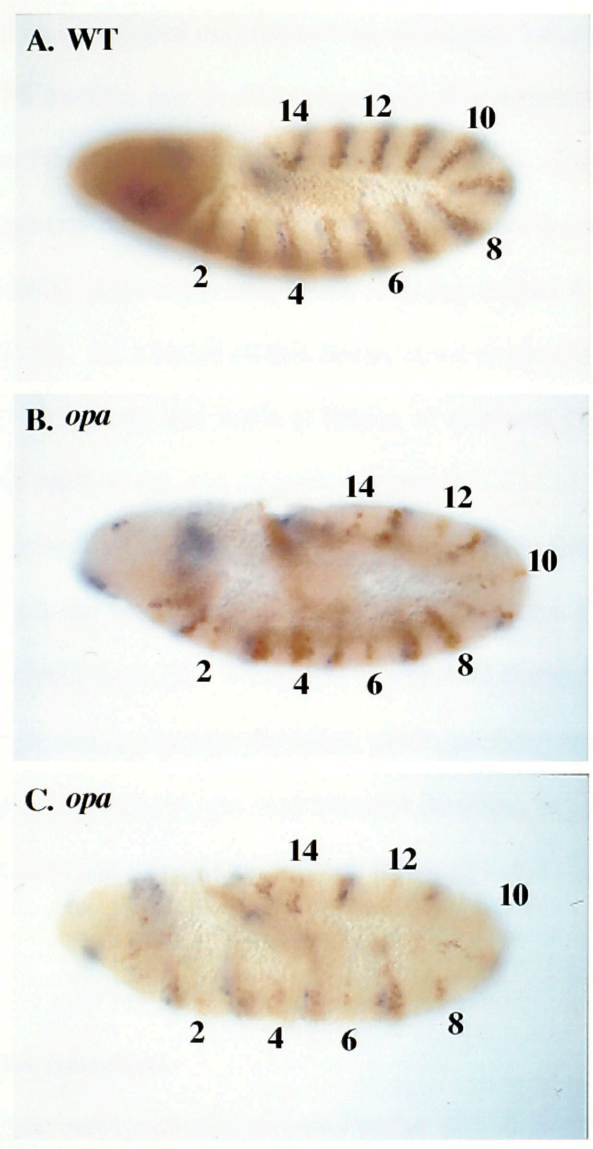


Fig.4

expression domains abut each other at the PS borders ([Lawrence et al., 1987; Carroll et al., 1988; Ingham, 1991b; Dougan et al., 1992], and Fig. 4, panel A). After the pair-rule genes initiate *wg* and *en* expression in PS border cells, mutual positive feedback maintains expression of each gene in its respective domain. In *opa* mutant embryos, pair-rule gene activation of *en* is delayed and somewhat deranged, but *en* is expressed in cells at both even and odd PS borders late in embryogenesis (Fig 4, panel B). In contrast, *wg* is expressed only in even PS border cells in *opa* mutant embryos. Even PS *en* expression fades later in embryogenesis due to loss of positive input from the adjoining odd PS border cells (Fig 4, panel C, note especially weak staining stripes 4 and 8. Compare with those from Fig 4, panel B). As a result of this decay in *en* expression, these PS border cells are destabilized. Ultimately this leads to fusion of alternate PS and subsequent deletion of odd denticle belts at the end of embryogenesis.

Overall, *opa*'s effects on *wg*, En, Ftz and *odd* expression demonstrate that *opa* activity is required at cellular blastoderm not only in the cells that flank each PS border, but also in most cells within each PS. Thus, *opa* is required throughout the segmented region of the embryo and not in discrete domains, distinguishing *opa* from all the other pair-rule genes. To understand how *opa* may execute its roles, we cloned and sequenced the *opa* gene, and analyzed the expression of both transcript and protein.

RESULTS

Localization of the *opa* mutation

The *opa* mutation was originally mapped to the 82A-E interval on the right arm of chromosome three (Jurgens et al., 1984). Steve DiNardo and Steve Wasserman further refined the *opa* position by generating deficiencies within this interval (Fig. 5, Letsou et al., 1990). Complementation tests between Df(3R) 6-7 and Df(3R) z-1 localized the *opa* mutation to the interval 82 $D_{3\text{-}8}$-82$E_{3\text{-}4}$. Fig. 5 depicts deficiencies used to localize *opa* as well as the chromosome walk. Df(3R)107 and Df(3R) 63, though not cytologically

Fig. 5 Deficiency maps and cDNA structure of *opa*.

Deficiency chromosomes in the cytological interval 81F - 83A. Solid lines indicate missing DNA. The Sal I sites in the region cloned from 82D/82E, are shown below. The chromosome walk, which was initiated to the left of Df(3R) 6-7, is represented by phage clones 1-8. The *opa* transcript spans phage clones 6-8. The exons depicted are located in the restriction fragments shown on the Sal I map, but are not drawn to scale.

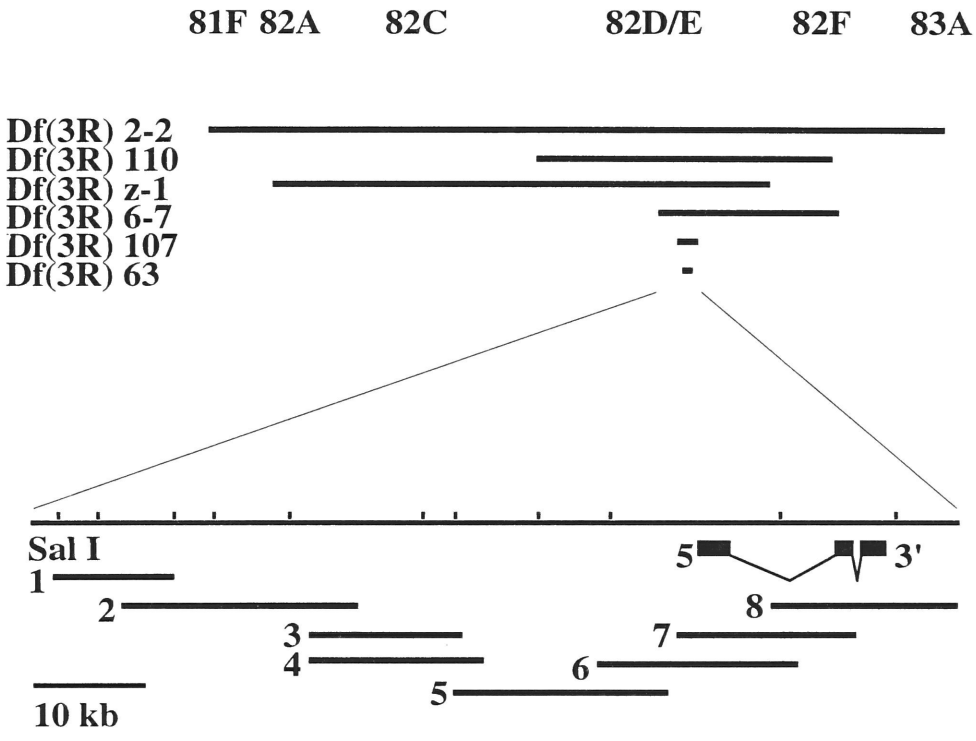

81F 82A 82C 82D/E 82F 83A
Df(3R) 2-2
Df(3R) 110
Df(3R) z-1
Df(3R) 6-7
Df(3R) 107
Df(3R) 63
Sal I
1
2
3
4
5
5
3'
8
7
6
10 kb

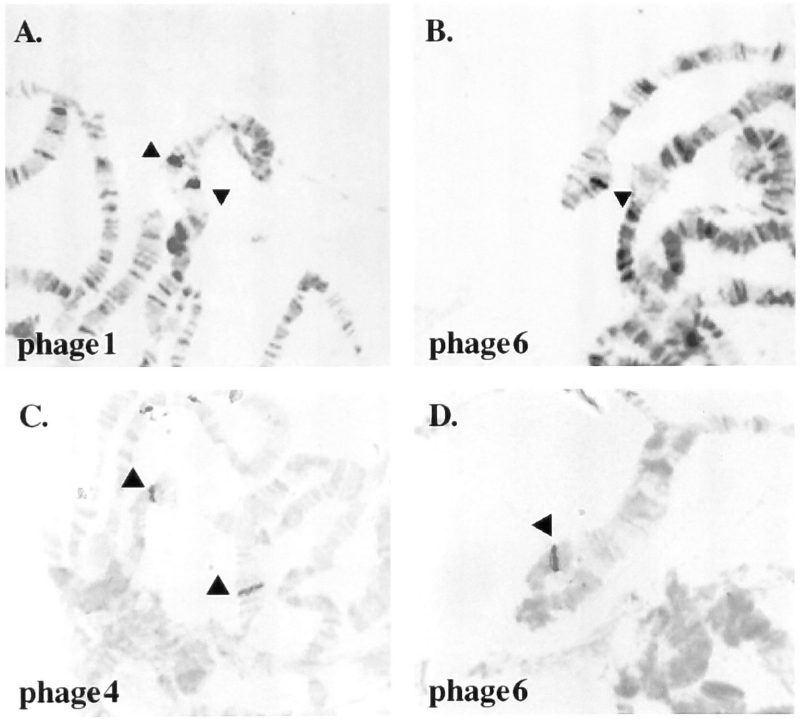
Fig. 5

visible, were useful later in isolating the gene using molecular experiments (see Fig.6 below). We obtained a phage containing DNA homologous to sequences on the centromere proximal border of Df(3R) 6-7 (see Fig. 5). This probe was used to initiate a chromosome walk towards the gene. I used polytene hybridization of phage DNA to Df(3R) 6-7 chromosomes to monitor progress of the walk into the deficiency. Fig. 6A shows a polytene chromosome prepared from larvae heterozygous for Df(3R) 6-7 hybridized to a probe prepared from phage 1 of the walk (Fig. 6A.) and a probe from phage 6 of the walk (Fig. 6B.). The chromosome preparations have been stained with Giemsa to show different banding patterns specific to different regions of the chromosomes. Poor pairing of the wildtype and deficiency homologs causes them to asynapse during preparation, as seen in each panel. The phage 1 probe in panel A. hybridizes to sequences on both homologs, indicating sequences within this phage lie outside of Df(3R) 6-7. In contrast, phage 6 lies within Df(3R) 6-7, as in this preparation, only one homolog, the wildtype, contains sequences homologous to those within the probe.

In addition, phage were assayed for sequences present within the region uncovered by the noncytologically visible deficiency Df(3R) 63. Fig. 6C shows sequences from phage 4 hybridizing to both homologs of a polytene preparation from a larva heterozygous for Df(3R) 63. In contrast, phage 6 lies within the deficiency (Fig 6D). Fig. 6E depicts quantitative Southern blots performed with probes prepared from phage 4, 6, and 8 used on genomic DNA blots prepared from Df(3R) 63 or Df(3R) 65 heterozygous flies. Df(3R) 65, used as a control, is a stock isogenic to Df(3R) 63 which contains a deletion on 3R outside of the interval containing *opa*. All lanes were genomic DNA digested with Bgl II and BamHI and the panels represent serial hybridizations of the same blot. Phage 4, which lies within Df(3R) 6-7 and does not lie within Df(3R) 63 hybridizes to identical bands of equal intensity in both the Df(3R) 63 and Df(3R) 65 lanes. In contrast, phage 6 lies within Df(3R) 63, hybridizing with ~1/2 intensity to

Fig. 6 Phage 6 and 8 lie within Df(3R) 63.

Panels **A-D**, polytene chromosome in situs stained with Giemsa and labelled *in situ* with various probes. See Fig. 5 for positions and relative sizes of deficiencies. **A.** Df(3R) 6-7/+ chromosome probed with phage 1 probe. Probe hybridizes to both homologs. **B.** Df(3R) 6-7/+ chromosome probed with phage 6 probe. Phage 6 sequences hybridize to only wildtype homolog. **C.** Df(3R) 63/+ chromosome probed with sequences from phage 4. Probe hybridizes to both homologs. **D.** Df(3R) 63/+ chromosome probed with sequences from phage 4. Only one homolog contains hybridization signal. **E.** Southern blot hybridized to whole phage sequences from (left to right) phage 4, 6, or 8. In each panel the left lane contains genomic DNA from flies wildtype for *opa*, the right, DNA from flies heterozygous for Df(3R) 63. Asterisks denote polymorphic bands missing from the mutagenized chromosome which lie beneath the probe.


A.
B.
C.
D.
Df(3R)6-7/+
probedwith:
phage1
phage6
Df(3R)63/+
probedwith:
phage4
phage6

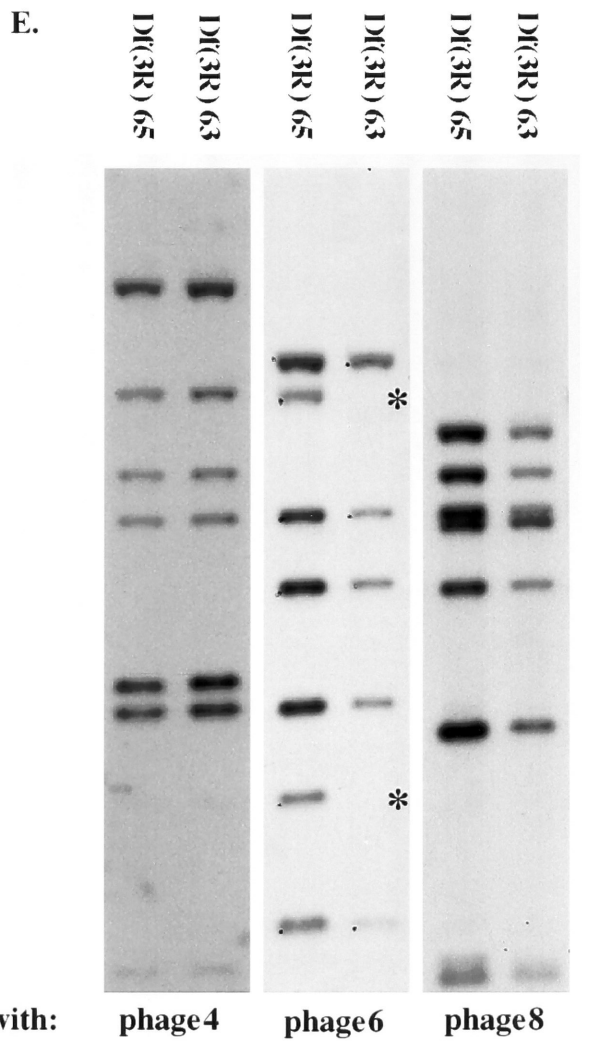

E.
Df(3R) 65
Df(3R) 63
Df(3R) 65
Df(3R) 63
Df(3R) 65
Df(3R) 63
probedwith:
phage4
phage6
phage8

Fig. 6

sequences within the Df(3R) 63 lane. In addition, polymorphisms on one homologue lie within the region deleted in the Df(3R) 63 (asterisks). Phage 8 which is contiguous with phage 6 also displays quantitative differences in hybridization indicating that it, too, lies underneath Df(3R) 63.

Isolation of *opa* genomic sequences

As contiguous phage 6 and 8 lie beneath Df(3R) 63, they were then assayed to see if they contained sequences expressed at blastoderm. DNA prepared from (whole) phage 1, 6, and 8 was individually digested with Sau 3A, individually labelled with digoxigenin and used as *in situ* hybridization probes on whole mount wildtype blastoderm stage embryos. Each probe contained 15-20 kb of genomic sequences and 29 kb of phage vector sequences (see Materials and methods). The results of these experiments are shown in Fig.7. Panel A shows an optical cross section of an embryo labelled with sequences from phage 1. No staining in the ectoderm is seen, though there is some background staining of the embryo interior. In contrast, embryos labelled with sequences from phage 6 or phage 8 show ectodermal staining in a domain encompassing ~20-80 % egg length (arrowheads, Fig.7,.panels C and D). The character of the signal is consistent with that of other pair-rule genes as it is localised to the apical cytoplasm of ectodermal cells (panel B, edge of embryo) (Davis et al., 1991). The nuclearly localized, punctate appearance of the signal is representative of hybridization to nascent transcripts (Fig.7., panel D.). To identify sequences within these phage responsible for this hybridization, DNA was prepared from phage 6 and 8, electrophoresed after restriction with Bam HI and BglII. Purified fragments were then individually used as templates for digoxigenin probes. Figure 8A is a map of the genomic region in the vicinity of phage 6 and 8 showing the locations of these fragments (denoted **a-g**) relative to the *opa* gene. Three fragments (asterisks) generated 20-80% egg length hybridization signals when used to label whole mount blastoderm stage embryos (data not shown). All fragments were then

Fig. 7 Phage 6 and 8 contain sequences expressed in blastoderm embryos.

All embryos shown are wildtype. **A.** Optical section of embryo hybridized *in situ* with whole phage DNA probe from phage 1. **B.**, **C.** Optical section of embryos hybridized with whole phage sequences from phage 6 and 8, respectively. Arrows denote boundaries of hybridization signal extending 20-80% egg length. Compare with embryo hybridized with sequences from phage 1 in panel **A**. **D.** Magnified surface view of embryo in panel **C**. Note punctate nature of signal, indicating hybridization of probe to nascent transcripts.

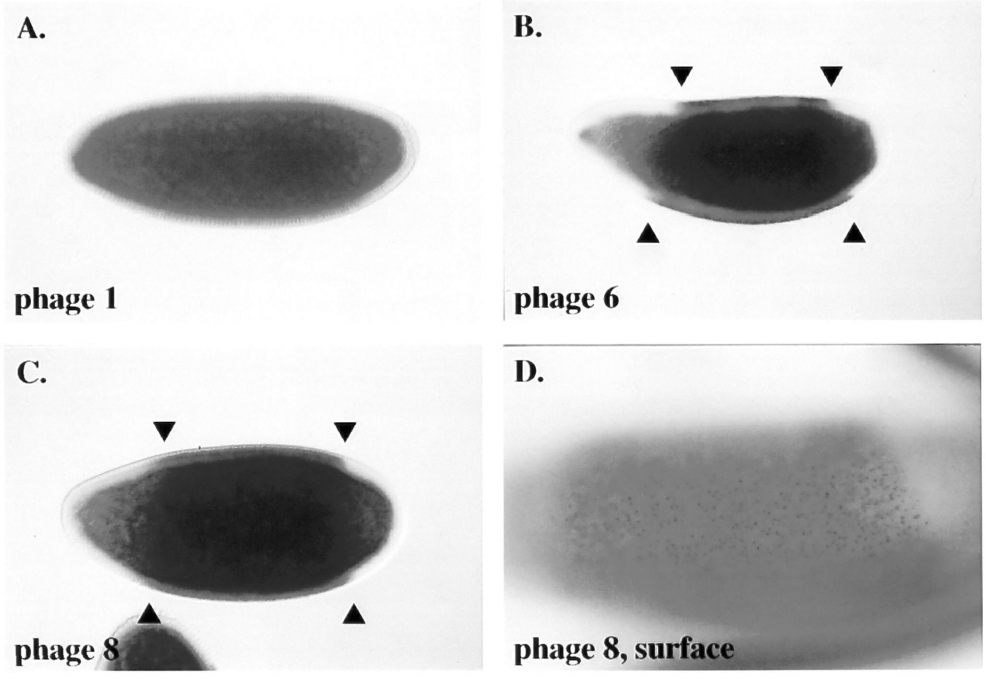
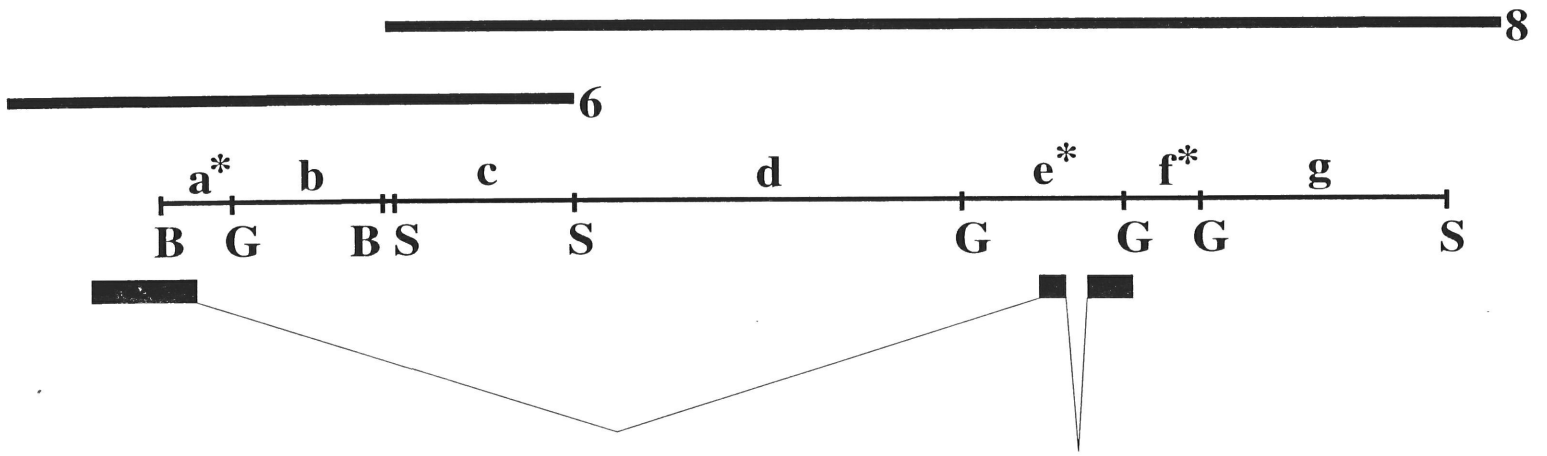


Fig. 7

Fig. 8 Sequences from phage 6 and 8 are homologous to a ~3 kb mRNA expressed at blastoderm.

A. Map of genomic probes from phage 6 and 8 and their location relative to *opa* coding sequences. Restriction enzymes are denoted as follows: B = Bam HI, G = Bgl II, S = Sal I. Each probe fragment was individually labelled with either digoxigenin or ^{32}P and used to screen both embryos (data not shown) and Northern blots, respectively, for expressed sequences. Probe fragments **a**, **e**, and **f** were homologous to sequences expressed from 20-80% egg length in blastoderm stage embryos (data not shown). **B.** Northern blot hybridization of poly A^+ mRNA isolated from 2.5-5.0 h old embryos hybridized with *opa*C (leftmost panel) or individual genomic restriction fragments from phage 6 and 8 in conjunction with a probe for the pair-rule gene *eve* (subsequent panels). Note hybridization of Fragments **a** and **e** to a ~3 kb message in these experiments. These fragments were subsequently pooled, labelled, and used as probes in the isolation of cDNA *opa*C (Materials and methods).



—— = 1 kb

Fig. 8A

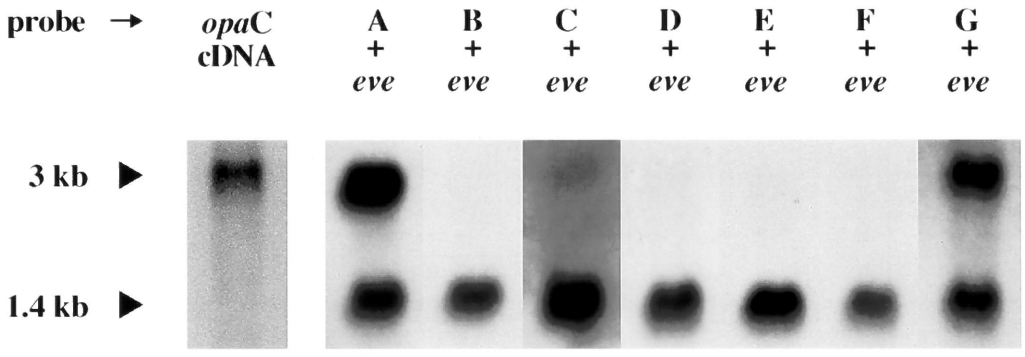


Fig. 8B

individually labelled with ^{32}P and used to separately probe Northern blots prepared with polyA$^+$ mRNA from 2.5-5.5 h old embryos. These were hybridized in parallel with a probe to another pair-rule gene, *eve*, generating a 1.4 kb signal. Fig.8B shows the results from this experiment. Two fragments, **a** and **e**, hybridized to an ~3kb message (Fig. 8B). The weak signal seen in the blot probed with fragment c was an artefact not seen upon further washing. Fragments **a** and **e** were subsequently pooled, labelled with ^{32}P and used to screen a plasmid cDNA library prepared from 0-4h embryos obtained from Nick Brown (Brown and Kafatos, 1988). 38 positives were obtained from a total of $2.5x10^5$ colonies screened. 4 of the 38 were colony purified. When DNA from these colonies was purified and used in *in situ* hybridization and Northern blotting experiments results identical to those obtained with the probe fragments **a** and **e** were seen (data not shown and see Fig.8B, blot probed with *opa*C).

_opa_C encodes a full length cDNA

Restriction and sequence analysis of the four cDNAs indicated that three of them (B,C,D) are 5' deletions of the longest cDNA, *opa*C (see below). Prior to testing whether *opa*C was functional *in vivo*, a primer extension experiment with a primer spanning nucleotides +148 to +119 of opaC was done to see if *opa*C encoded a full length cDNA. The primer was annealed to sequences within 2.5-5.5 h polyA$^+$ mRNA, extended and electrophoresed in parallel with sequence reactions as size standards. Fig. 9 shows the two major extension products of 147 and 148 nt as well as a low abundance product of 149 nt that resulted from this experiment. As the extended products were not sequenced, however, the 5' end of *opa*C may correspond to another alternatively spliced species from the same locus. Excepting this possibility, the primer extension and sequencing data indicate that the gene corresponding to the *opa*C transcript may initiate at more than one site, and suggest that *opa*C encodes a full length cDNA.

Fig. 9 Primer extension experiments using *opa*C.

Primer extension product generated using the primer **tttcgctcttagatatttcgcatgcgactt** (from positions $^{+}$148 to $^{+}$119 nt of *opa*C) annealed to sequences within 2.5-5.5 h polyA^{+} mRNA. A sequencing reaction was used as a sizing standard. The region between the nucleotides marked with asterisks represents products from 144 to 155 nucleotides in length. Two major products of 147 and 148 nt are visible. Upon further exposure, a low abundance 149 nt product is also visible (data not shown).

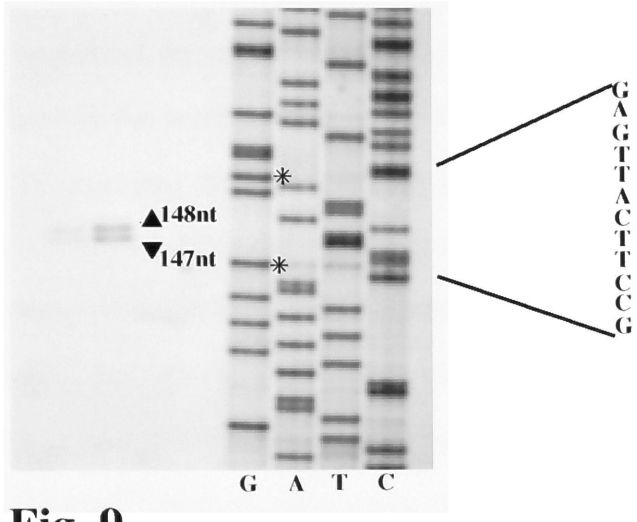


Fig. 9

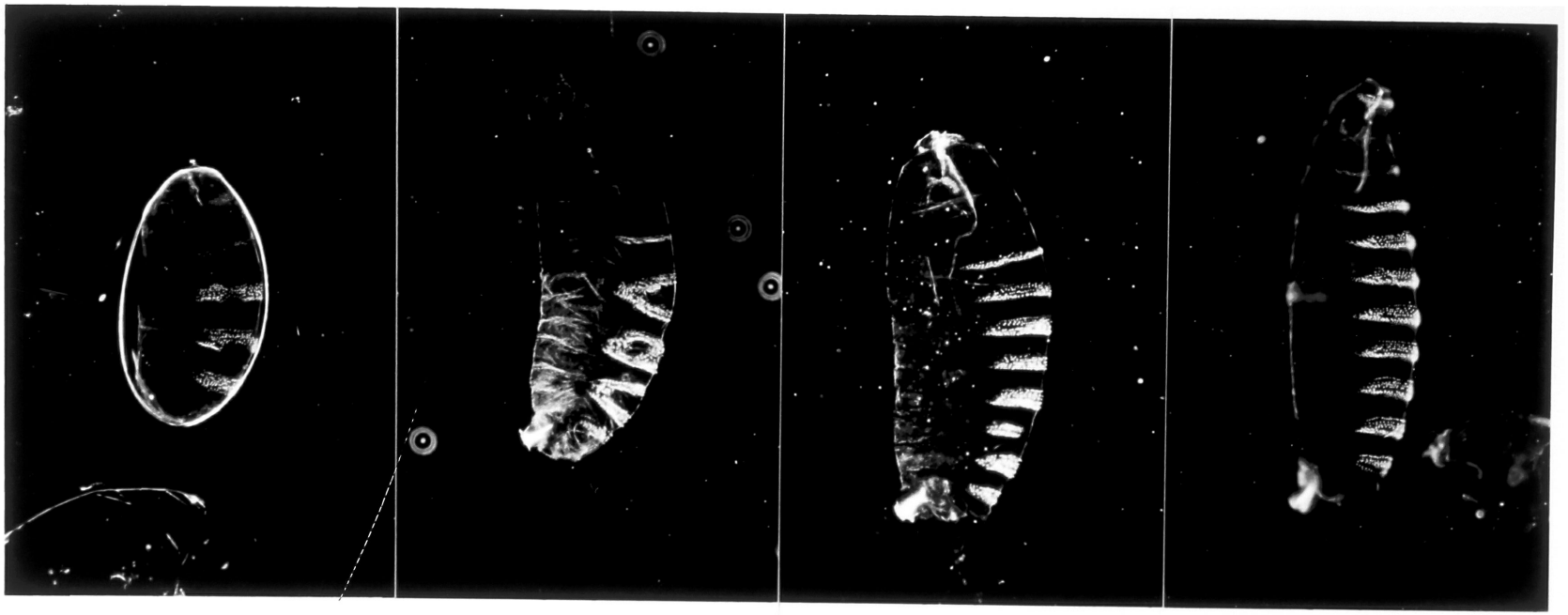
Rescue of the *opa* mutant phenotype

Two complementation assays were used to test whether *opa*C encoded a functional *opa* gene. First, capped transcripts synthesized *in vitro* from *opa*C injected into embryos during the syncytial blastoderm rescued the cuticle phenotype of eight of sixteen *opa* embryos (Janet Mullen performed these experiments). Janet Mullen then constructed transgenic flies in which the *opa*C cDNA was placed under the control of the hsp70 promoter. The stocks used were constructed such that cuticles of homozygous mutant embryos were unambiguously marked (see Materials and methods). *opa*^{5H} homozygous embryos have only four complete abdominal segments (Fig. 10A), although occasionally the remnants of a fifth denticle belt are found. When transgenic embryos were exposed to a 20 minute heat shock during cellularization, 38 of 75 (57%) of *opa* mutants showed rescue as judged by the presence of at least five full denticle belts (panels B, C). In some cases rescue approximated the wild type body pattern (panel C). No rescue was found in heat shocked embryos that did not harbor the transgene.

We next asked whether the rescue of body pattern correlated with the restoration of *wg* and *en* expression (Fig. 11). In this case, marked balancer chromosomes conferred β-gal staining in the cephalic region of wildtype embryos. Mutant embryos lack this stain (see Materials and methods). In *opa*^{7N} mutants, the *wg* stripes in the odd PS are missing (compare panels A and B). In *opa* mutants carrying the hs*opaC* transgene, *wg* expression was restored in odd PS 3-11 (Fig. 11,panel C). *wg* rescue was found in 25 of 40 (62%) of the *opa* mutant transgenic embryos assayed. No rescue was found in heat shocked embryos that did not harbor the transgene. *en* expression was also rescued in *opa* mutants carrying the hs*opaC* transgene. About one hour after cellularization in *opa* mutants, *en* expression is usually absent from PS 2, is spotty and broad in PS 4 and 8, and fails to extend dorsally in all stripes (compare Fig. 11, panels D, E). In contrast, 29 of 54 (54%) of the *opa* transgenic embryos exhibited *en* expression in PS2, narrower *en* stripes in PS 4 and 8, and more extensive dorsal expression in all stripes (Fig. 11, panel F). Although *en*

Fig. 10 Rescue of *opa* body pattern by the hs*opa*C transgene.

Cuticles oriented anterior up, ventral to right, darkfield images. **A.** *opa*^{5H}/*opa*^{5H}. Embryos have four completely fused abdominal denticle belts resulting from loss of alternate body segments; only three of these compound segments are visible in this photo. Compare with wildtype in **D**. **B.** hs*opa*; *opa*^{5H}/*opa*^{5H}. Partially rescued embryo with "splitting" of denticle belt fusions. Compare with hypomorphic *opa* embryo in Fig. 1B. **C.** hs*opa*; *opa*^{5H}/*opa*^{5H}. Fully rescued embryo. All eight abdominal denticle belts are present. **D.** Wildtype. Eight abdominal denticle belts are present.



A. +; opa 5H

B. hs-opa; opa 5H

C. hs-opa; opa 5H

D. wildtype

Fig. 11 Rescue of *en* and *wg* target gene expression by the hs*opa*C transgene.

A-C. Germband extended embryos labelled *in situ* for *wg* and *lacZ* RNA. **A**, Wild type. *wg* is expressed in PS 0-13 and *lacZ* is expressed in the anterior from the *hb*-lacZ transgene on the balancer chromosome. **B.** opa^{7N}/opa^{7N}. *wg* is not expressed in the odd PS, and is expressed weakly in the even PS. **C.** hs*opa*; opa^{7N}/opa^{7N} one hour after heat shock. *wg* expression is restored to the odd PS 3-13. **D-F.** Germband extended embryos labelled for En protein. **D.** Wild type. En is expressed in PS 1-14 and beta-galactosidase in the head due to presence of the TM3 *pthb-lacZ* balancer chromosome. **E.** opa^{7N}/opa^{7N}, En is expressed in PS 4 and 8 in broad stripes, and all En stripes are weak dorsally. **F.** hs*opa*; opa^{7N}/opa^{7N}, one hour after heat shock. En expression is narrower in PS 4 and 8, and is more robust and extends more dorsally in all PS.

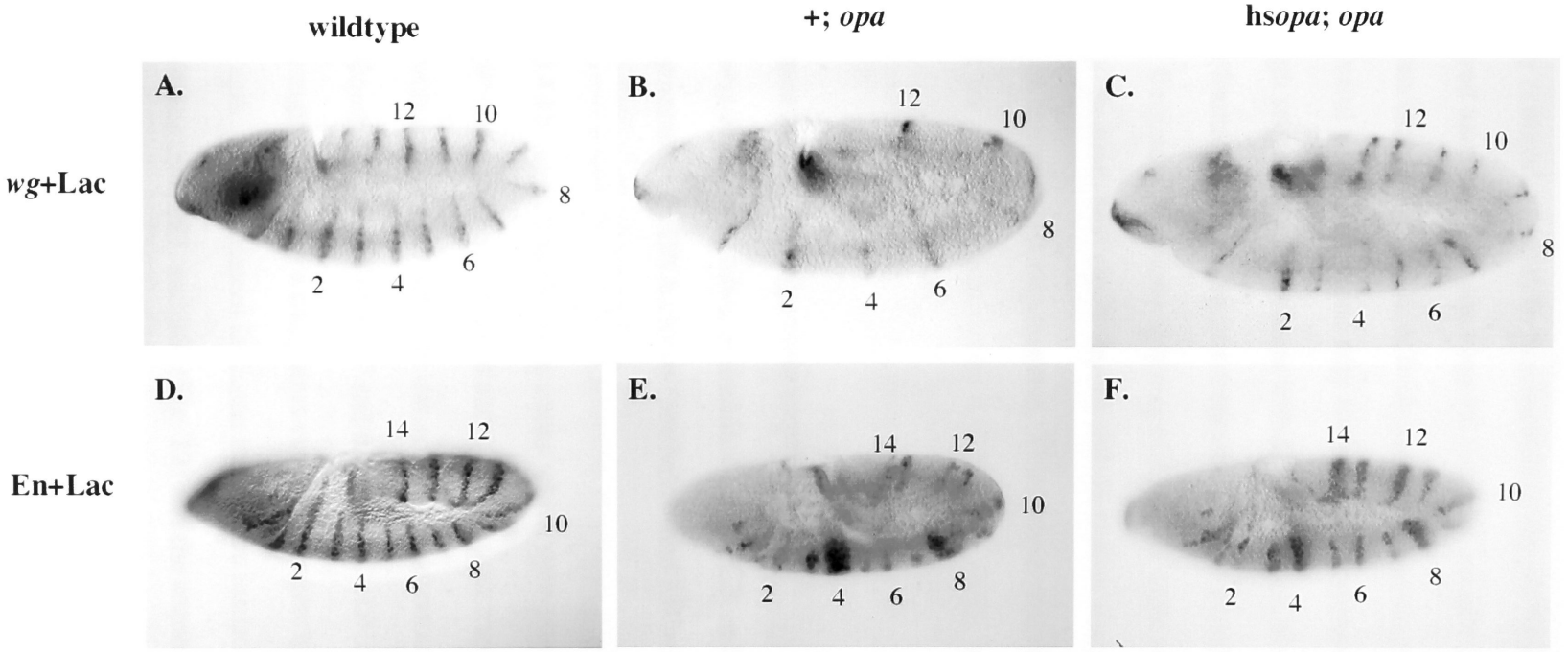


Fig. 11

expression was partially restored, some defects remained in the positioning of the stripes such that odd PS appeared narrower than the even PS (Fig. 11, panel F). The significant rescue of both target gene expression and final body pattern demonstrates that this is the *opa* gene. There are several possible reasons why rescue did not approach 100% efficiency. First, through sequence comparisons with Dan Cimbora, we learned that the *opa*C cDNA contains a frameshift resulting in a truncated protein which may have affected the degree and frequency of rescue in transgenic mutant embryos (see below and Materials and methods). Second, heat shock during cellularization may lead to translation of *opa* after its normal peak requirement. Third, there may be a post-blastoderm requirement for *opa* which was not be fulfilled by a single heat pulse during cellularization.

Structure of the *opa* gene and deduced protein sequence.

Gene structure was determined using a combination of PCR and sequencing comparisons between genomic and cDNA clones (see below and Materials and methods). The transcription unit spans a total of 14.5 kb of genomic sequence with introns of approximately 12 and 0.4 kb separating the three exons which span nucleotides 1-1243, 1244-1413, and 1413-2959, respectively (Figs. 4 and 12). The *opa* transcript is 2959 base pairs in length, which corresponds well with the transcript size determined by Northern blotting (see below). Sequence analysis at the 5' end of the *opa*C cDNA and of the corresponding genomic region revealed a consensus cap site (**gcagtcctgc**) beginning 1 nucleotide upstream of *opa*. Three adjacent in-frame translation initiation codons are found at nucleotide positions 294, 297, and 300 (Fig. 12) and the sequence surrounding the first ATG matches the *Drosophila* translation start consensus (Cavener, 1987). In addition, the 5' ends of the remaining partial cDNAs are indicated by underlined, boldface nucleotides at positions 423, 1009, and 1293. There are two polyadenylation signals at nucleotides 2859-2864 and 2920-2925. Conceptual translation of the 1827 nucleotide

Fig. 12 Nucleotide and protein sequence of *opa*, a GLI-*Kr* class zinc-finger protein.

Conceptual translation of the *opa*C cDNA. Non-coding nucleotides (nt) are in lower case. Start and stop codons are in boldface type at 294 and 2121 nt, respectively. The first ATG is a good match to the *Drosophila* consensus (Cavener, 1987). Starting nucleotides of cDNAs *opa*A, *opa*B, and *opa*D are shown underlined and in boldface at 403, 1009, and 1293 nt, respectively. Nucleotides flanking intron-exon borders are depicted in underlined, boldface type at nt positions 1243/1244 and 1413/1414. Polyadenylation signal sequences are present at 2858 nt and 2918 nt and are indicated by bold underlined type. Within the amino acid sequence, the polyserine and polyglutamine repeats are shown in single and double underlined type, repectively. Zinc finger domains are depicted in boldface.

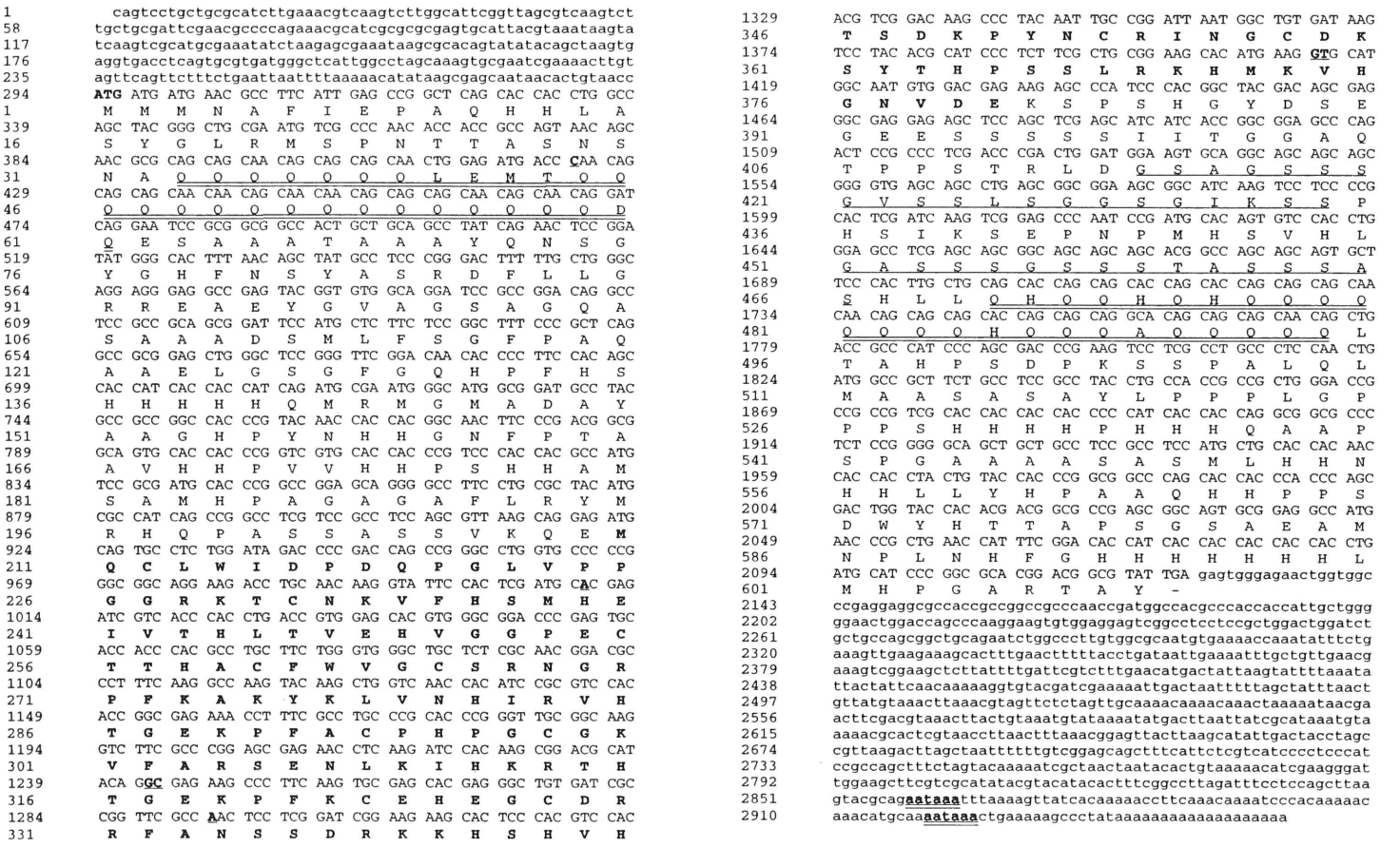


Fig. 12

Fig. 13 Amino acid alignment of zinc finger domains of Opa, GLI, Ci^D and Tra-1.

Interfinger linker regions are underlined. Identical residues are indicated by ' * ', conservative substitutions by ' ^ ', and residues corresponding to the GLI consensus finger sequence are indicated by boldface type. As the GLI and GLI3 proteins share 88% identity across their finger domains (Ruppert et al., 1990), only GLI is shown in the alignment.

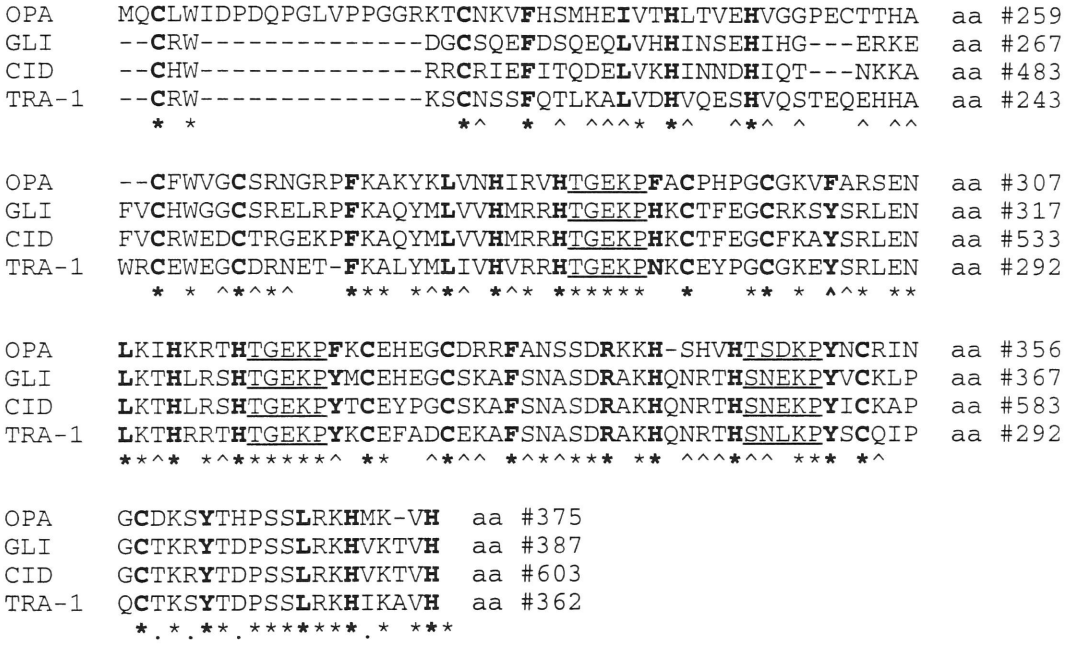

OPA MQCLWIDPDQPGLVPPGGRKTCNKVFHSMHEIVTHLTVEHVGGPECTTHA aa #259
GLI --CRW----------DGCSQEFDSQEQLVHHINSEHIHG---ERKE aa #267
CID --CHW----------RRCRIEFITQDELVKHINNDHIQT---NKKA aa #483
TRA-1 --CRW----------KSCNSSFQTLKALVDHVQESHVQSTEQEHHA aa #243
OPA --CFWVGCSRNGRPFKAKYKLVNHIRVHTGEKPFACPHPGCGKVFARSEN aa #307
GLI FVCHWGGCSRELRPFKAQYMLVVHMRRHTGEKPHKCTFEGCRKSYSRLEN aa #317
CID FVCRWEDCTRGEKPFKAQYMLVVHMRRHTGEKPHKCTFEGCFKAYSRLEN aa #533
TRA-1 WRCEWEGCDRNET-FKALYMLIVHVRRHTGEKPNKCEYPGCGKEYSRLEN aa #292
OPA LKIHKRTHTGEKPFKCEHEGCDRRFANSSDRKKH-SHVHTSDKPYNCRIN aa #356
GLI LKTHLRSHTGEKPYMCEHEGCSKAFSNASDRAKHQNRTHSNEKPYVCKLP aa #367
CID LKTHLRSHTGEKPYTCEYPGCSKAFSNASDRAKHQNRTHSNEKPYICKAP aa #583
TRA-1 LKTHRRTHTGEKPYKCEFADCEKAFSNASDRAKHQNRTHSNLKPYSCQIP aa #292
OPA GCDKSYTHPSSLRKHMK-VH aa #375
GLI GCTKRYTDPSSLRKHVKTVH aa #387
CID GCTKRYTDPSSLRKHVKTVH aa #603
TRA-1 QCTKSYTDPSSLRKHIKAVH aa #362

Fig. 13

open reading frame yields a protein of 609 amino acids. The predicted protein contains 5 C_2H_2 zinc finger domains. Between several of the fingers, there are conserved stretches of amino acids ([T/S]GEKP), known as H/C links, that were first identified in the *Drosophila* gap gene *Krüppel* (Preiss et al., 1985). The predicted *opa* protein also contains stretches of polyserine and polyglutamine repeats, motifs common to many transcription factors. In addition, the *opa* protein contains an alanine-rich region from amino acids 94-122 (34% alanine rich). The zinc finger region, from amino acid positions 210 to 380 was the only domain exhibiting significant homology to other proteins. The most homologous zinc finger domain sequences found were *Drosophila* Ci^D, human GLI and GLI3, and *C. elegans* Tra-1 (Fig. 13) (Eaton et al., 1990; Orenic et al., 1990; Ruppert et al., 1990; Zarkower et al., 1992). Fingers three through five of Opa fit the GLI consensus sequence (**Y/FXC**X_3**GC**X_3**[F/Y]**X_5**L**X_2**H**X_{3-4}**H**), and the H/C link consensus ([T/S]GEKP). There is 42% (70/167) identity between Opa and all the other proteins for both consensus and non-consensus residues within the zinc finger region. All four proteins lack a perfect consensus for the first finger and are missing consensus linker residues between the first and second fingers. Opa has 21 conservative substitutions compared to the GLI, Ci^D, and Tra-1 zinc finger sequences, indicating that these proteins are more similar to each other than to Opa. We found no significant homology between Opa and these proteins outside of the finger domains.

opa mRNA and protein expression during embryogenesis

Simon Kidd performed Northern blotting experiments with an *opa*C probe which revealed a single mRNA of 3 kb peaking in expression from two to twelve hours of embryogenesis (Fig. 14). Expression of this transcript continues throughout the larval instars and during pupation, although at lower levels compared to embryogenesis. Some transcript was found in unfertilized eggs, indicating that there may be a small maternal contribution. We focus here on the zygotic expression and function of the gene.

Fig. 14 Developmental expression of *opa* mRNA.

Developmental timepoints of lanes are as indicated. *opa* expression peaks between 2-12 hr after egg laying (upper panel). Expression of a ribosomal protein gene, *rp49*, was used as a loading control (lower panel).

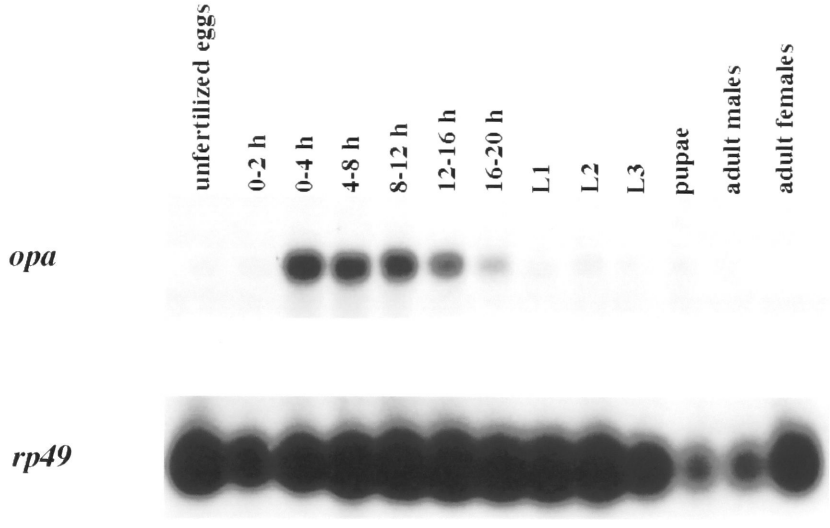

unfertilized eggs
0-2 h
0-4 h
4-8 h
8-12 h
12-16 h
16-20 h
L1
L2
L3
pupae
adult males
adult females
opa
rp49

Fig. 14

opa transcript first appears at the beginning of cellularization (stage 5) (Campos-Ortega et al., 1985), in a stripe about 10 cells wide centered at 80% egg length (Fig. 15, panel A). Expression then extends posteriorly and dorsally and the level of expression increases, generating uniform expression between 20-80% egg length (Fig. 15, panels B-C) with the posterior border of this domain more sharply defined than the anterior (Fig. 15, panel C). The expression pattern does not change during gastrulation (stage 6). Once germband extension begins (stage 7, Fig.15, panel D), the level of ectodermal expression begins to decrease. As germband elongation progresses, transcript fades more from particular cells within each PS, generating 14 weak stripes over a low background level of expression (Fig. 15, panels D-E). This is the first stage at which there is some periodicity to *opa* expression, but it does not approximate the sharp on-off expression patterns observed for other pair-rule genes. At the end of germband elongation *opa* expression has faded dramatically from ectodermal cells with the exception of some cells which may represent neuroblasts (Fig. 15, panel F.).

Janet Mullen prepared Opa protein for injection into rabbits and the resultant anti-Opa antisera was used to monitor expression of Opa during embryogenesis. Antibody specificity was determined by staining embryos heteroallelic for Df(3R)107 and Df(3R) 63, both of which delete *opa* coding sequences. The *opa* null embryos were devoid of signal (Fig. 16, panel A), indicating that the antibody is specific for Opa, or, if there are any cross-reacting antigens, they are under the control of the *opa* gene. The remaining panels depict wildtype embryos stained with the antibody.

Opa protein is also present in a block from 20-80% egg length during cellularization, and protein levels peak late during cellularization and into gastrulation (Fig. 16, panels C,D), paralleling expression of *opa* mRNA (compare Fig. 15B and 16D, 15C and 16E). Opa accumulates in the nuclei, although some staining is visible in the basal cytoplasm of ectodermal cells (Fig. 16, panel D, inset). Throughout gastrulation Opa remains expressed in a solid block (Fig. 16, panels C-E). During early germband

Fig. 15 *opa* mRNA is expressed throughout the segmented region of the body.

All embryos shown are wild type embryos probed *in situ* for *opa* RNA. **A.** Early syncytium, note faint staining in region at ~80-60% egg length. **B.** Embryo just prior to cellularization (stage 5[3]), *opa* RNA appears in a ten cell wide patch at ~80% EL. **C.** Fully cellularized embryo, *opa* is expressed at peak levels in a broad domain from 20-80% EL, with a sharp posterior edge and a fuzzier anterior edge. **D.** Early gastrula, *opa* is still expressed throughout all segment primordia, but a faint periodicity in expression is appearing. Expression levels decrease dramatically in the ectoderm after this stage. **E.** Germband (gb) extended embryo , optical cross section showing weak periodic staining in ectoderm. **F.** Germband extended embryo slightly older than that in **E.** Embryo shows punctate staining which may correspond to neuroblasts.

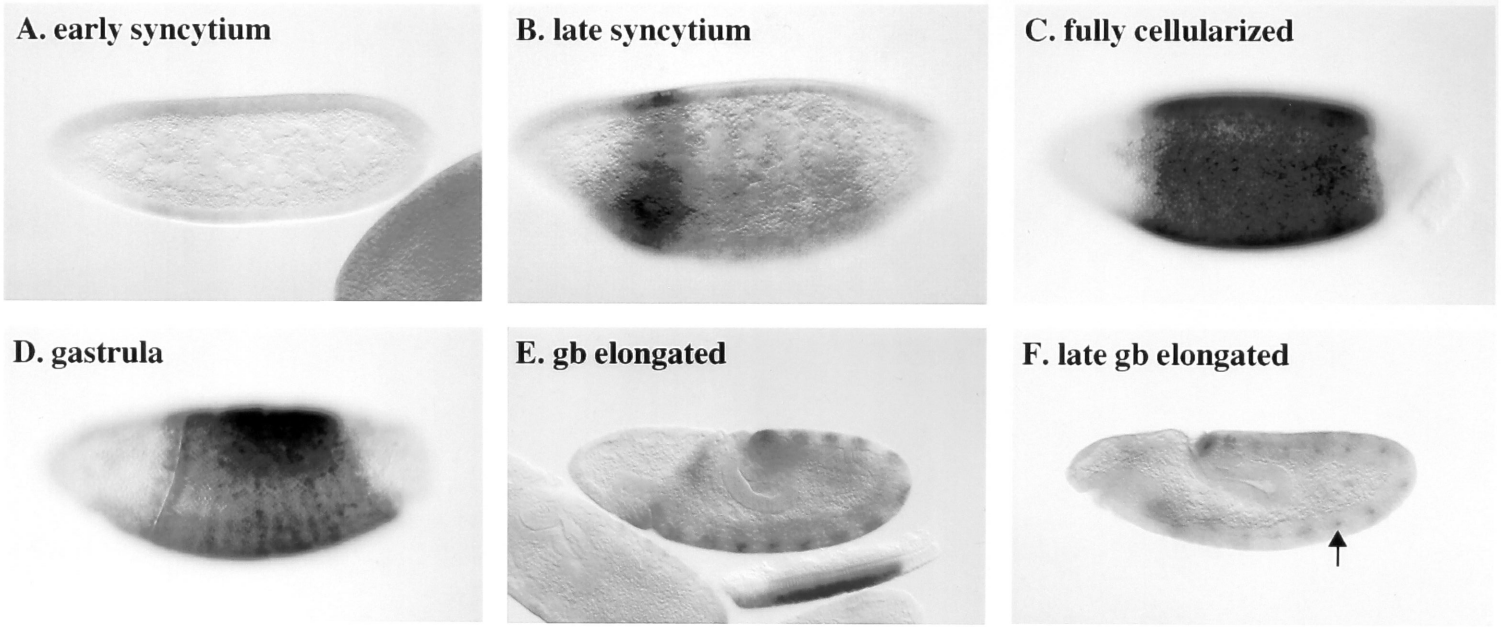

A. early syncytium
B. late syncytium
C. fully cellularized
D. gastrula
E. gb elongated
F. late gb elongated

Fig.15

Fig. 16 Opa protein is expressed throughout the segmented region of the body.

All embryos are stained with rabbit anti-Opa antisera. **A.** Optical section through Df(3R) 63/Df(3R) 107 *opa* null embryo at cellularization. **B-J.** Wildtype (wt) embryos. **B.** Optical section through early syncytium. Note light staining from 20-80% egg length. **C.** optical section through cellularized embryo. Peak expression occurs at this stage. Compare with *opa* null embryo in panel **A**. **D.** Surface view of early gastrula. Inset, magnified view of ventral ectoderm shows that Opa accumulates mostly in the nuclei. **E.** Early germband elongated embryo. Staining intensity has begun to fade. **F.** Surface of germband elongated embryo. Faint periodic expression can be seen above a low level of expression. **G.** Slightly older germband elongated embryo, ventral surface view. Note staining in individual cells corresponding to neuroblasts along the ventral midline. **H.** Lateral (lat.) surface view, late germband retracted embryo. Staining occurs at the base of the gnathal bud and in a scallopped pattern in ventrolateral ectoderm (see inset). **I.** Ventral (vent.) surface view of the embryo in **H**. Note staining in cells along ventral midline and ventrolateral scalloped expression pattern.

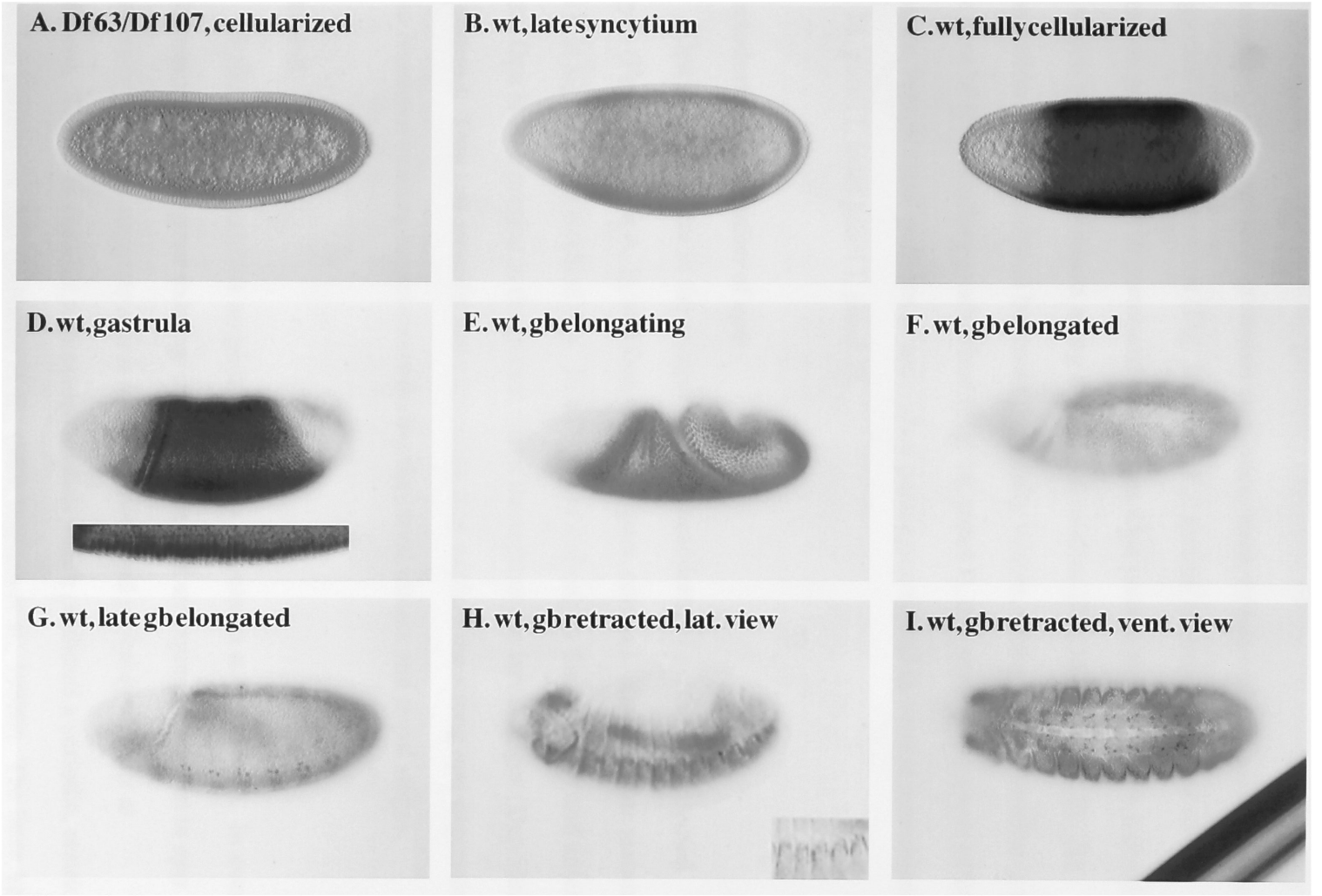

A. Df63/Df107, cellularized
B. wt, late syncytium
C. wt, fully cellularized
D. wt, gastrula
E. wt, gb elongating
F. wt, gb elongated
G. wt, late gb elongated
H. wt, gb retracted, lat. view
I. wt, gb retracted, vent. view

Fig. 16

extension, Opa expression levels decline compared to the peak at cellular blastoderm, although the protein remains homogeneously expressed within the segmented region of the ectoderm. As germband extension continues, weak stripes evolve over a low level background of Opa expression (Fig. 16, panel F.), similar to those of the transcript expression pattern. Late in germband elongation, Opa protein is also seen in individual cells which may be neuroblasts along the ventral midline (Fig.16, panels G, I). As segmentation of the germband becomes physically visible, strong Opa expression is seen at the base of the maxillary gnathal bud (Fig. 16, panel I), in a scalloped pattern extending across the ventrolateral portions of individual parasegments and in ventral neuroblasts (Fig. 16, panel I, inset panel I, panel J).

Nonectodermal expression of *opa*

Opa is also present in the midgut visceral mesoderm of germband retracted embryos (Fig. 17, panels A, B, and Dan Cimbora, pers. comm.). The punctate staining in panel B is signal in the tracheal pits. In addition to staining in the posterior and anterior midgut, Opa is also present in the gastric caeca throughout the larval stages (Deb Hursch, pers. comm.). This late mesodermal expression may explain the strong *opa* expression seen in Northern blotting experiments during the larval instars and pupal stages.

Discussion

Strikingly, *opa* exhibits no periodicity in its expression at blastoderm. In every cell for which a marker gene exists at this stage, we find an expression defect in *opa* mutant embryos. This suggests *opa* is active in most, and probably all cells at blastoderm. This is in contrast to previous models which predicted a periodic, restricted expression pattern for *opa* (DiNardo et al., 1988; Ingham et al., 1988b). The later, striped *opa* expression program occurs after the stage at which target genes are affected in *opa*

Fig. 17 Nonectodermal expression of *opa* protein.

A. Optical section through late germband retracted embryo staining in the anterior and posterior midgut (arrows). **B.** Cross section of same embryo, ventral view. Peak expression occurs cells in the anterior and posterior midgut (arrows) and tracheal pits.

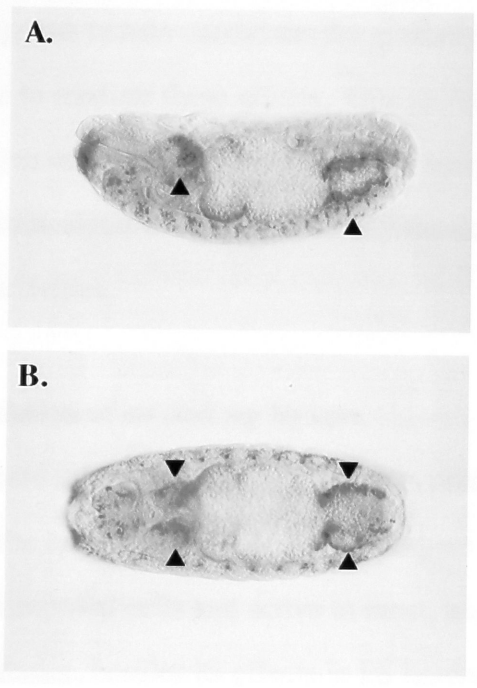


Fig. 17

mutants. Thus, *opa* is unique among the pair-rule genes in that its expression and function are not restricted to a subset of cells in each PS at blastoderm.

Genetically, *opa* can either activate or repress different genes in discrete domains when it is ubiquitously expressed at blastoderm. Most likely this is not due to any posttranslational modification of Opa, but to interactions with other factors, themselves restricted in expression. It is likely that *opa* executes some of these genetic roles by directly regulating target gene expression, since several *Drosophila* Kr class zinc finger proteins have been shown to act directly as transcriptional regulators. The genetic analysis presented here suggests certain candidates for spatially localized factors with which *opa* could cooperate to mediate these effects. Opa probably interacts with such factors, forming transcription complexes with different net transcriptional activities in different cells. A molecular analysis of Opa will identify the domains involved in generating some of these activities.

A revised model for regulation of *en* and *wg* by *opa*

The data reported here substantially revise previous predictions for the extent of expression and activity of the *opa* expression domain (compare Figs.1 and 18). *opa* is expressed in all segment primordial cells and active in most, and probably all cells in every PS (Fig. 18). For instance, besides its effects in PS border cells (see below), *opa* represses *prd* in even PS, as *prd* stripes fail to split in *opa* mutant embryos (Fig.18 and (Baumgartner et al., 1991); Steve DiNardo, pers. comm.). In addition, *opa* is an activator of *oddskipped* (*odd*) in anterior cells of the odd PS, as secondary *odd* stripes do not form in *opa* mutant embryos (Fig.18 and Janet Mullen, pers. comm.).

In PS border cells, *opa* has effects on both *wg* and *en* expression. In the even PS, our data support a combinatorial model wherein Ftz activates *en* expression, and *odd* restricts this activation to the anteriormost Ftz-expressing cells (Fig. 8) (Manoukian et al., 1992; Manoukian et al., 1993). Although Ftz protein is expressed in stripes several

Fig 18. Pair-rule genetic interactions regulating *en* and *wg* at cellular blastoderm.

Two complete PS are shown, with *en*-expressing cells (filled nuclei) at the anterior and wg-expressing cells (filled cytoplasm) at the posterior of each PS. Interactions (↓ symbol = activating, ⊥ symbol = repressive) involving *opa* are in boldface solely for emphasis and do not reflect magnitude of interaction. *eve* is expressed in a graded manner across the odd PS as it retracts to the anteriormost cell of the PS. The anteriormost *eve*-expressing cell overlaps with one domain of *prd* expression, and *eve* and *prd* together appear to activate *en* expression (filled nuclei) in these PS (Morrissey et al. 1991). This *prd* domain straddles the even/odd PS border and activates *wg* (filled cytoplasm) in the even PS (Ingham et al. 1988). To a much lesser extent than *prd*, *opa* contributes to *wg* activation here, too, since these *wg* stripes are weaker than normal in *opa* mutants. In the even PS, *ftz* is expressed in a gradient similar to *eve*. *ftz* eventually retracts to the anteriormost cell of the PS. Initially, *odd* is expressed in a broad domain coinciding with broad *ftz* stripes. *opa* represses *odd* in the anterior cells of the *ftz* domain. *odd* represses *ftz*-dependent activation of *en,* allowing activation of *en* in only this anteriormost cell (Manoukian and Krause 1993). *opa* may also act directly to activate *en* in this cell. *opa* also activates *wg* in the odd PS (Ingham et al., 1988). The *prd* stripes that straddle the odd/even PS boundary express *prd* at a lower level than the other stripes. This *prd* domain probably helps *opa* to activate *wg*, since in *prd* mutants these *wg* stripes are weaker than wild type stripes (Janet Mullen, unpub.). *opa* acts to repress interstripe *prd* in the even PS, as stripes fail to split in *opa* mutant embryos (Baumgartner, 1991).

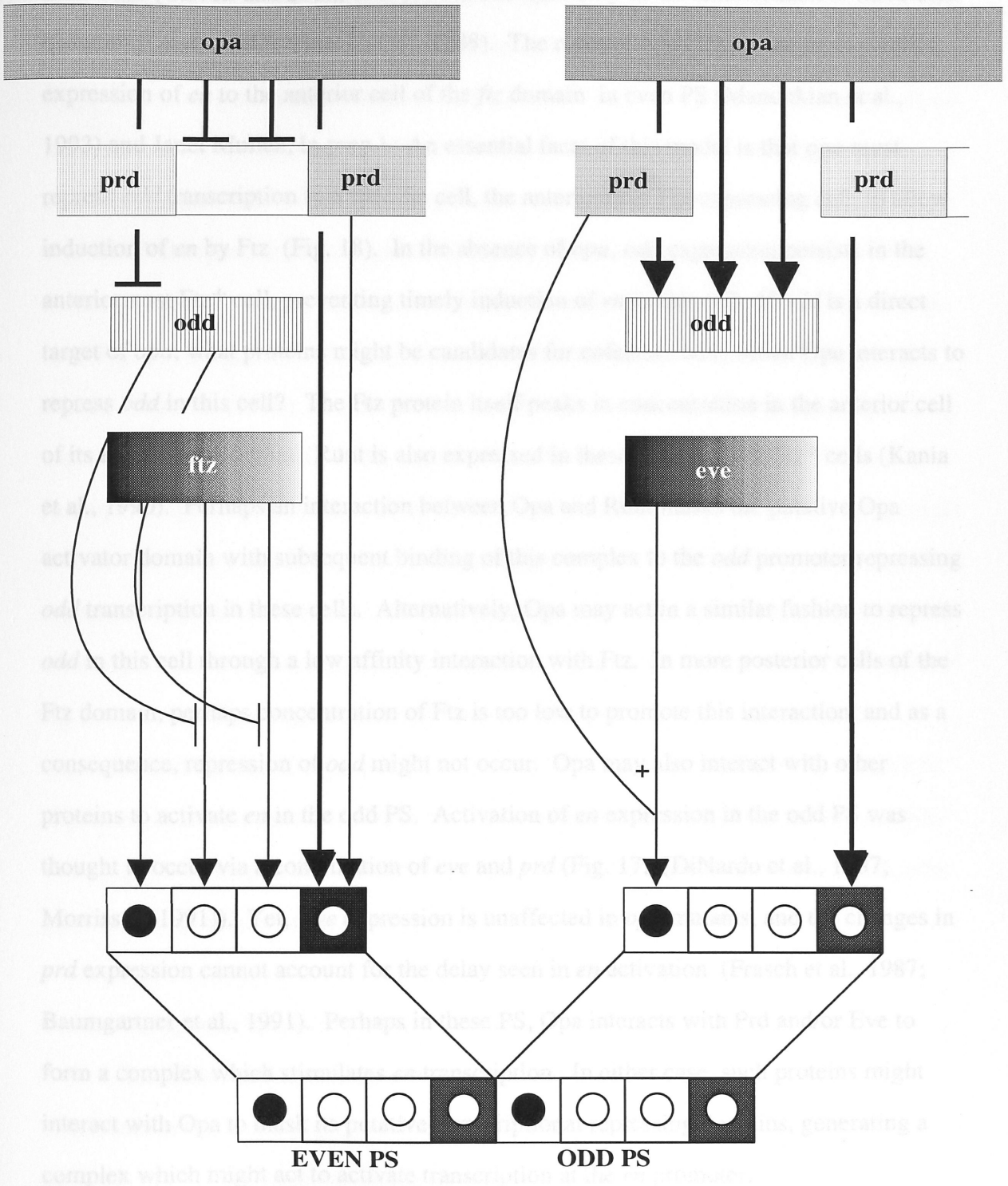


Fig. 18

cells wide (Carroll and Scott, 1985), *en* is activated only in the anteriormost of these cells (Lawrence et al., 1987; Carroll et al., 1988). The repressor *odd* limits the *ftz*-dependent expression of *en* to the anterior cell of the *ftz* domain in even PS (Manoukian et al., 1993) and Janet Mullen, in prep.). An essential facet of this model is that *opa* must repress *odd* transcription in a specific cell, the anteriormost Ftz-expressing cell, to allow induction of *en* by Ftz (Fig. 18). In the absence of *opa*, *odd* expression persists in the anteriormost Ftz^+ cell, preventing timely induction of *en* in this cell. If *odd* is a direct target of *opa*, what proteins might be candidates for cofactors with which Opa interacts to repress *odd* in this cell? The Ftz protein itself peaks in concentration in the anterior cell of its expression domain. Runt is also expressed in these anteriormost Ftz^+ cells (Kania et al., 1990). Perhaps an interaction between Opa and Runt masks the putative Opa activator domain with subsequent binding of this complex to the *odd* promoter repressing *odd* transcription in these cells. Alternatively, Opa may act in a similar fashion to repress *odd* in this cell through a low affinity interaction with Ftz. In more posterior cells of the Ftz domain, perhaps concentration of Ftz is too low to promote this interaction, and as a consequence, repression of *odd* might not occur. Opa may also interact with other proteins to activate *en* in the odd PS. Activation of *en* expression in the odd PS was thought to occur via a combination of *eve* and *prd* (Fig. 17; [DiNardo et al., 1987; Morrissey, 1991]). Yet, *eve* expression is unaffected in *opa* mutants, and the changes in *prd* expression cannot account for the delay seen in *en* activation (Frasch et al., 1987; Baumgartner et al., 1991). Perhaps in these PS, Opa interacts with Prd and/or Eve to form a complex which stimulates *en* transcription. In either case, such proteins might interact with Opa to mask its putative transcriptional repressing domains, generating a complex which might act to activate transcription at the *en* promoter.

Similar interactions may play a role in Opa-dependent activation of *wg*. However, the restriction of these effects to a specific stripe is problematic. Specifically, *ftz* and *eve* have been genetically defined as negative regulators of *wg* expression, while *prd* and *opa*

have been defined as activators (Ingham, 1988a). It was hypothesized that as *ftz* stripes narrowed, repression would be relieved at *wg*, allowing activation by *prd* in even PS (Figs. 1, 18). A similar narrowing of *eve* stripes in the odd PS was hypothesized to allow activation of *wg* by *opa* (Fig. 1). Since the repressors *ftz* and *eve* eventually disappear, some mechanism must restrict *wg* activation to narrow stripes. In the even PS this role was ascribed to *prd*, since its expression domain is restricted (Fig. 18). In the odd PS, earlier models supposed a similar restriction in the domain of expression or function of *opa* (Fig. 1). We have confirmed that *opa* function is essential for *wg* activation in the odd PS (Ingham, 1988a), and shown that *opa* is important for the timely activation of *wg* expression in the other PS. Thus, *opa* may directly activate *wg* in the odd PS, and assist in the *prd*-dependent activation of *wg* in the even PS (Fig. 18). In the odd PS, Opa might form a homodimer through hydrophobic interactions between its putative transcriptional repressing alanine-rich domains. This might generate a transcription complex with a net transcriptional activating activity. Opa may contribute to the *prd*-dependent activation of *wg* in the even PS through a similar interaction with Prd. However, since Opa is expressed throughout all segment primordia and appears to act in all cells in which it is expressed, it alone cannot determine the restricted position of *wg* expression in odd PS. The spatial information that restricts *wg* expression in the odd PS must reside with other pair-rule, or as yet uncharacterized, proteins.

Control of zygotic *opa* expression

In contrast to the periodic expression patterns of other pair-rule genes, *opa* is expressed uniformly throughout all segment primordia at blastoderm. The *runt* (*run*) pair-rule gene is also expressed ubiquitously at blastoderm, but this expression is involved in sex determination and not segmentation (Duffy et al., 1991). What might generate expression of *opa* throughout all the segment primordia? Such expression might be formed through activation of intercalated domains of *opa* stripes by periodic patterns

of other pair-rule activators. Yet, no underlying periodicity of *opa* transcription is seen at any time during cellularization. Thus, this seems unlikely, unless expression of such activators were tightly synchronized. Similarly, it is difficult to envision how various gap proteins might uniformly activate *opa* transcription, especially since most pair-rule promoters respond to discrete concentrations of a given gap protein (Howard et al., 1988; Warrior et al., 1990; Riddihough et al., 1991; Small et al., 1991; Small, 1992).

Many segmentation genes are expressed very early during embryogenesis. Edgar, et al. (1986) showed that the genome acquires a general competence for transcription as early as nuclear cycle 10 (Edgar et al., 1986a). At this stage, low level transcription of several segmentation genes, including the pair-rule genes *eve, run, h*, and *ftz* can be detected throughout the embryo using *in situ* hybridization (Weir, 1985; Edgar et al., 1987). If protein synthesis is blocked by injection of cycloheximide in cycle 11-14, polar repression of these pair-rule genes does not occur (Edgar, 1988). This suggests that subsequent to their global activation, (a) repressive interaction(s) at the embryo poles restrict pair-rule gene expression to the embryo midsection.

Some of this polar repression is due to interactions with different gap proteins. For instance, *runt* stripe #7 is shifted anteriorly in *hkb* mutant embryos (M. Klingler and P.Gergen, unpubl). Perhaps, like the other pair-rule genes, *opa* may be expressed at low levels throughout the embryo during cycles 10-13. Genes expressed at the embryo termini might then restrict *opa* expression to the embryo midsection. The anterior and posterior edges of the *opa* expression domain are at 80 and 20 % egg length, respectively, implying similarly situated gap proteins might set these borders through repressive interactions at the *opa* promoter. The gap gene *tll* is expressed prior to cellularization in terminal domains corresponding to 0-20% egg length in the posterior and 80-100% egg length in the anterior (Pignoni et al., 1990). Thus, the timing and complementary nature of its expression in the early embryo make it a good candidate for a repressor of *opa* at

the embryo termini. *In situ* hybridization will reveal a shift in the borders of the *opa* expression domain in *tll* mutant embryos if this is the case.

Other segmentation genes with simple expression patterns in central regions of the embryo may be regulated by similar mechanisms. For instance, the *Kr* domain is unchanged in zygotic effect mutants except where such genes are normally expressed in embryonic regions adjacent to the *Kr* domain. In these cases, the *Kr* domain is shifted anteriorly or posteriorly as compared to wildtype (Gaul et al., 1987; Gaul et al., 1989). Similarly, no maternal mutant results in loss of *Kr* expression. Instead, the domain is again shifted in certain maternal mutant embryos such as *bcd* or *osk* (Gaul et al., 1987). This suggests that *Kr* is not under the control of a restricted activator. Instead, perhaps *Kr* expression depends upon general factor(s) which may activate *Kr* throughout the embryo in the absence of other organisers which might restrict this activation to the central expression domain. In the case of *opa* perhaps similar such activators may activate *opa* at low levels throughout the embryo prior to its restriction to the segmental anlagen by repressors at the embryo termini.

By the end of cellularisation, *opa* is transcribed at high levels throughout its central domain. What mechanisms might generate this enhanced transcription? Positive autoregulatory interactions are thought to generate the high levels of transcription of other segmentation genes seen at the end of cellularization. For instance, positive autoregulation is involved in generating high levels of transcription of *eve, ftz*, and *runt*, at the end of cycle 14 (Hiromi et al., 1987; Frasch, 1988; Gergen et al., 1988; Jiang et al., 1991). Positive autoregulation also generates high levels of *Kr* transcription at the end of cellularisation (Hoch et al., 1990). After the establishment of restricted domains of these genes at the start of cycle 14, positive autoregulation is thought to heighten their expression to create the focussed, periodic patterns seen at the very end of cycle 14. Perhaps autoregulatory interactions at the *opa* promoter give rise to the high levels of *opa* transcription seen at the end of cycle 14 as well after its expression has been restricted to

the segment primordia. Such a mechanism is thought to generate restricted expression of the *Xenopus* gene *MyoD*. Similar to some pair-rule genes, *MyoD* is expressed throughout the early *Xenopus* embryo at low levels. Mesoderm induction is thought to stabilize transcription in the marginal zone, with positive autoregulation generating the high levels of restricted transcription seen later (Rupp, 1991) in this domain. Thus, similar combinations of both repressive and positive autoregulatory interactions may be a general mechanism for generating broad patterns of transcription in a variety of different systems. Autoregulation of pair-rule gene expression has been demonstrated using two techniques. For some genes, transcription is reduced in those amorphic alleles expressing transcript (Jiang et al., 1991), suggesting positive autoregulation is necessary for wildtype levels of expression. The three *opa* null alleles 9C, IIP, and IIC each express transcript (author, unpublished observ.), but none has been examined in detail in marked balanced stocks. *In situ* hybridization will reveal reduced *opa* transcription in 9C, IIP, or IIC homozygous null embryos, if such autoregulation is necessary for wildtype levels of *opa* transcription. However, it is formally possible that reduced *opa* expression in these lines may be due to mutation(s) at the *opa* promoter. An alternative experimental approach to identifying autoregulatory processes involved in segmentation gene expression has been shown for *eve*, *en*, and *ftz* using promoter/*lacZ* constructs . When fused to a *lacZ* reporter gene and transformed into flies, pair-rule promoter sequences give rise to β gal expression patterns coinciding with the native patterns of the specific pair-rule genes *eve*, *ftz*, and *h*, (Hiromi et al., 1987; Goto et al., 1989; Harding et al., 1989). Reduced expression of such constructs in null mutant backgrounds demonstrates that positive autoregulatory feedback contributes to wildtype expression levels of a given gene. Construction of similar *opa* promoter/*lac Z* fusion constructs is feasible as *opa* promoter sequences have been cloned in the chromosome walk. In addition, promoter dissection experiments using such constructs have helped identify the *cis*-regulatory elements necessary for positive autoregulation of *h*, *eve* and *ftz* (Hiromi et al., 1985; Hiromi et al., 1987; Goto et al.,

1989). Similar experiments could identify the promoter sequences involved in possible *opa* positive autoregulation as well as those elements involved in the repressive interactions which might restrict *opa* to its central domain of expression.

opa mesodermal expression and function

Just prior to gastrulation, *opa* expression intensifies in the presumptive mesodermal cells adjoining the ventral furrow (data not shown). Cimbora, et al. have cloned *opa* using a P-element enhancer trap construct which inserted into a midgut enhancer of *opa*. Specifically, they found that *opa* transcription is regulated in the midgut visceral mesoderm by genes of the Bithorax complex. In particular, *opa* is activated by *AbdA* and repressed by *AbdB* in the posterior midgut. In the anterior midgut, *opa* is repressed by *Ubx*, and may also be regulated by the *Scr* and *Antp* homeotic genes (Dan Cimbora, pers. comm.).

The morphological counterpart to this genetic regulation is seen in the midgut phenotype in *opa* mutant embryos. During stages 15 and 16, three constrictions appear consecutively in the midgut wall leading to the formation of four intestinal compartments in the developed wildtype In contrast, *opa* mutant embryos lack all midgut constrictions even though the expression patterns of the homeotic genes *AbdA*, *Ubx*, and *Antp* are all normal in the midgut mesoderm of *opa* mutant embryos (Dan Cimbora, pers. comm.). Overall, these data show that *opa* is downstream of the homeotic selector genes in these tissues and demonstrate a role for *opa* in regulating midgut mesoderm morphogenesis late in development.

Late ectodermal expression

At gastrulation and throughout germband elongation, there is an overall dampening of *opa* transcription throughout the ectoderm. As germband elongation progresses, a pattern of 14 roughly defined stripes appears against a background of low

level *opa* expression. This periodic pattern is visible for transcript at the beginning of germband elongation, but not visible in the protein expression pattern until later in embryogenesis. Other pair-rule genes are expressed in single segment periodical patterns after blastoderm (Frigerio et al., 1986; MacDonald et al., 1986a; Gergen and Butler, 1988), but the *opa* striped expression pattern does not correspond to the sharp on-off periodic pattern seen for these genes. Instead, the post-blastoderm *opa* expression pattern is more reminiscent of the indistinct striped patterns of the *ciD* and *ptc* segment polarity genes. These genes are also expressed in broad patterns at blastoderm which evolve into roughly periodic patterns later in embryogenesis (Hooper et al., 1989; Nakano et al., 1989; Eaton et al., 1990; Orenic et al., 1990; Ingham et al., 1991a). Steve DiNardo has shown that the *opa* stripe domains lie within the interstripe regions of the *engrailed* domain (unpub.). As *en* has been shown to act as a repressor in cell culture (Jaynes et al., 1991), it is a good candidate for an interstripe repressor of *opa*. Indeed, *in vivo*, *en* has been shown to be a repressor of *ciD*, as *ciD* stripes are broadened in *en* mutants (Eaton et al., 1990). *en* expression peaks during germband elongation, corresponding to the time interstripe repression is seen in the *opa* protein expression pattern. If *en* is a repressor of *opa* post-blastoderm, then *opa* mRNA and protein expression will be uniform throughout gastrulation and germband elongation in *en* mutant embryos.

Alternatively, *opa* transcription might drop off to low levels across all segment primordia, but simultaneously be weakly activated or maintained in stripes. This may be due to periodic expression of an activator or a factor which antagonizes such general repression. *ciD* encodes another GLI-Kr protein required to maintain *wg* expression late in embryogenesis which is also expressed in roughly defined stripes out of register with *en* ([Eaton et al., 1990], and see below). Perhaps *ciD* also maintains or activates *opa* expression in its striped domain in germband elongated embryos, as both the registration and the quality of ciD expression correspond to that of *opa* late in embryogenesis. If this is the case, then *opa* should be expressed uniformly at low, interstripe levels in germband

elongated ciD mutant embryos. Given that both positive and negative regulatory mechanisms are involved in regulation of many of the other pair-rule genes, it is likely that the mechanisms described above might act in combination to generate the *opa* striped pattern late in embryogenesis.

Identifying roles for the late, periodic *opa* expression program

Although the other pair-rule genes also show periodic expression late in embryogenesis, the function of these later, restricted pattern is obscure. They may have roles in specifying neuroblast segmental identity, as many pair-rule genes are expressed in neurectoderm after germband elongation has initiated (Carroll et al., 1985; Doe et al., 1988). Alternatively, they may play roles in regulating segment polarity gene expression. The fact that this later expression depends on the blastoderm expression pattern complicates analysis of the late program. One way to to find a role for the late pattern of *opa* expression is to perturb or abolish it without affecting the blastoderm expression program necessary for correct segmentation. If the hs-*opa* construct is crossed into an *opa* null background and expressed at blastoderm under the appropriate heatshock regime, *opa* activity could be supplied at blastoderm in the absence of later *opa* function. This was not the case in the heatshock rescue experiments we conducted for two reasons. First, these experiments were performed in hypomorphic *opa* backgrounds. Second, we did not monitor post-blastoderm *opa* protein expression in treated embryos in our experiments. Thus, in either case we supplied some *opa* activity to the embryo after cellularization. If supplied at blastoderm using the correct heatshock regimen it is likely expression will fade after cellularization. Most pair-rule proteins are relatively labile due to the presence of one or more PEST sequences which target them for rapid degradation (Laughon et al., 1984; Frigerio et al., 1986; Rogers et al., 1986; MacDonald et al., 1986a; Kania et al., 1990). Opa also contains a PEST sequence (amino acids 386-410), and most likely cycles rapidly as well. *In situ* hybridization of germband elongated embryos might

then reveal effects of loss of *opa* expression late on target gene expression. Conversely, the heatshock construct could be expressed after blastoderm in a wildtype background to abolish periodic expression late in embryogenesis prior to monitoring changes in target gene expression. Similar experiments in which *eve* and *runt* were ectopically expressed identified distinct stage specific functions for these pair-rule genes (Manoukian et al., 1992; Manoukian et al., 1993). As *opa* may have subtle effects late in embryogenesis, these experiments should be conducted with hs-*opa* constructs containing wildtype *opa* coding sequences, and not the frameshift mutant used in our rescue experiments.

Opa may share targets with Ci^D

The strong homology to the Ci^D GLI-Kr zinc finger region (42% identity) and its parallel pattern of expression to that of *opa* suggest the two proteins may share the same targets. At blastoderm, *ci^D* is expressed in three broad domains across the AP axis. As germband elongation proceeds the *ci^D* expression pattern changes into 14 roughly defined stripes overlaid upon a weaker background of expression (Eaton et al., 1990; Orenic et al., 1990). The poor definition of the stripes is reminiscent of the late periodic pattern of *opa* expression seen during germband elongation.

ci^D maintains *wg* expression in all PS late in embryogenesis, after initiation of *wg* expression at blastoderm by the pair-rule genes *opa* and *prd* (Hidalgo, 1991). Perhaps expression of a wildtype hs-*opa* construct during germband elongation could partially rescue late *wg* expression in a *ci^D* mutant embryo. In addition, given their similar expression patterns and zinc finger domains, putative DNA binding motifs, perhaps cross-regulatory interactions exist between Opa and Ci^D as well. For instance, I argue above that positive autoregulation at the *opa* promoter might generate *opa*'s pattern of blastoderm expression. Later, perhaps Ci^D acts at the *opa* promoter as well during germband elongation, given the strong homologies between the two proteins' zinc finger regions. Monitoring *ci^D* or *opa* expression in germband elongated *opa* or *ci^D* mutant

embryos, respectively, will demonstrate whether such mutual interactions exist. If they do, it would be interesting to see if providing *opa* expression during germband elongation with the hs-*opa* construct could restore ci^D expression in non-expressing mutants (or in ci^D-*lacZ* embryos, see [Eaton et al., 1990]). If so, this would point to one possible regulatory function for the later striped program of *opa* expression (see above).

Possible function for maternally supplied Opa

Hypomorphic *opa* mutant embryos have cuticle defects that are more severe ventrolaterally than in the ventralmost portions of the embryo (Fig. 2B). In addition, in those *opa* embryos exhibiting partial rescue (see Fig. 11), pattern elements along the ventral midline are rescued before ventrolateral elements. Consistent with this, domains of *en* and *wg* are ventrally restricted in *opa* mutant backgrounds (this work). Thus, it appears that ventrolateral tissues have a stricter requirement for *opa* compared to the most ventral regions of the embryo. This graded requirement for pair-rule gene activity is also seen in hypomorphic alleles of *h* and *run*, for example (Ingham et al., 1985; Gergen et al., 1986b). For these genes, there is no explanation as to how this mosaic requirement might arise in the early embryo. How then might requirements for *opa* vary for different cells within its uniform domain of expression? It is formally possible that the *opa* promoter might respond to different dorsoventral positional cues within its expression domain. For instance, there is a dorsoventral asymmetry in the development of the *opa* blastoderm expression pattern, as expression across the segment anlagen proceeds in a ventral to dorsal fashion, with a mediodorsal patch the last to express *opa* (Fig. 15, compare panels B, C and Fig. 16, compare panels B, C). There is a dorsoventral asymmetry in the development of the Prd and *ftz* expression patterns as well ([Gutjahr et al., 1993], and Leslie Pick, pers. comm.). Alternatively, perhaps ventrally localized maternal *opa* activity might supplement the ventralmost tissues in the early embryo. *opa* mRNA is present in unfertilised eggs (Fig. 13) and in ovaries (Louise O'Keefe, pers. comm),

indicating that there is a maternal contribution to *opa* expression in the early embryo. These experiments are highly preliminary, but perhaps this maternally supplied *opa* activity supplements the zygotic supply of *opa* in the ventralmost portions of the embryo. It is possible that in hypomorphic mutants a threshold level of *opa* is reached in ventral cells which can ameliorate loss of *opa* activity in these cells. More ventrolateral cells might lack this activity and thus be more sensitive to the loss of zygotic *opa* as a result.

Early global defects are partially resolved through post-blastoderm cell signalling interactions

Given the global effects on gene expression at cellular blastoderm in the absence of *opa*, one might expect defects more severe than pair-rule deletions in body pattern. However, the final body pattern in *opa* mutants is not surprising if one considers the cell signalling circuits that function during post-blastoderm development. After the pair-rule genes initiate *wg* and *en* expression in PS border cells in wild type embryos, mutual positive feedback maintains expression of each gene in its respective domain. Specifically, after the pair-rule genes initiate *en* expression in its striped domain, *wg* input to *en* expressing cells maintains this expression, as *en* expression is initiated, but fades prematurely in *wg* mutant embryos (DiNardo et al., 1988; Martinez-Arias et al., 1988a). *wg* encodes a secreted glycoprotein homologous to the mammalian protein Wnt-1 (Cabrera et al., 1987; Rijsewijke et al., 1987). This signalling pathway involves the *dsh*, *zw3*, and *armadillo* proteins acting within the *en*+ cell to transduce the *wg* signal (Noordermeer et al., 1994; Siegfried et al., 1994). Reciprocally, after *wg* expression is initiated at blastoderm, it is maintained later in embryogenesis by input from the adjacent *en*-expressing cells, probably by a signal encoded by the *hedgehog* protein (*hh*) (Martinez-Arias et al., 1988a). *hh* expression is under the control of *en* and in either *en* or *hh* mutant embryos, *wg* expression decays after its initiation at blastoderm by the pair-rule genes (Hidalgo et al., 1990; Hidalgo, 1991; Lee et al., 1992; Mohler et al., 1992; Tabata

et al., 1992; Tabata et al., 1994). *hh* encodes a protein produced in both membrane-bound and secreted forms (Lee et al., 1992; Mohler et al., 1992; Taylor et al., 1993). It is thought that *hh* might render certain cells competent to express *wg* at the posterior PS border (Ingham, 1991b).

As a consequence of these interactions, the PS boundaries are stabilised in wildtype embryos, generating wildtype body pattern at the end of embryogenesis. However, in *opa* mutants, although *en* expression initiates eventually in the even PS, *wg* expression is never initiated in the adjoining odd PS border cells. The lack of *wg* input from these anterior cells causes even PS *en* expression to fade later in embryogenesis. Consequently, the boundaries between these PS are destabilised, leading to fusion of alternate PS and deletion of the odd denticle belts. At the other PS boundaries, *wg* expression is initiated, albeit at reduced levels relative to wild type, and *en* expression is severely delayed in the adjoining odd PS *en*-expressing cell. However, *wg* has been shown to boost *en* expression in adjoining cells (Bejsovec et al., 1991), and similar positive input to the adjoining *en*-expressing cell stabilizes these PS boundaries, such that the final body pattern exhibits only pair-rule defects. Thus, the global defects observed in *opa* mutants at blastoderm are partially compensated for later in development by the regulative properties of the *en*- and *wg*- expressing cells flanking alternate PS boundaries.

Identifying direct target genes of *opa*

Our genetic analysis provides information about *opa*'s role in the pair-rule network, but does not reveal which interactions are direct. As *opa* encodes a GLI-Kr zinc finger protein and other similar proteins have been shown to act as direct transcriptional regulators, it is likely Opa acts at some target gene promoters directly. A relevant *in vivo* approach to identifying direct target genes of a pair-rule protein has been demonstrated by Manoukian et al. (Manoukian et al., 1992). They used a hs-*eve* construct to conditionally express *eve* ectopically at different timepoints during cellularisation. Using *in situ*

hybridization, they then monitored changes in the expression of different segmentation genes in response to this treatment. Two tiers of response times distinguished direct from indirect genetic targets of Eve. Specifically, they noticed changes in target gene expression occurring within 30 minutes of heat shock for those genes which were direct targets of the Eve protein. In addition, their technique allowed for quantitative measurement of target gene transcription to identify subtle changes in target gene expression. Such an approach has been successfully used to identify direct targets of Runt as well (Manoukian et al., 1993), and appears feasible for use in identifying direct targets of Opa. In this case, the construct used should contain wildtype *opa* sequences and not the frameshift mutant used in our rescue studies. Certain data from the hs-*opa* rescue experiments indicate, however, that the original protocol must be modified in the case of *opa*. I observe no gross defects in either *wg* and *en* expression or body pattern in heatshocked wildtype embryos containing the hs *opa* construct. Thus, excess *opa* in certain cells may have no function where these cofactors are tied up with endogenous levels of *opa,* suggesting that this assay for direct *opa* targets should be conducted in an *opa* null background. These experiments will also identify candidates for putative cofactors with which Opa may interact (see below), as such cofactors will be expressed in domains coincident with direct targets.

Experimental evidence that Opa acts as a cofactor

Opa has different restricted effects within its homogeneous domain at blastoderm. Some of these are bound to be direct, and most likely due to Opa's interactions with other restricted factors at target gene promoters. In addition, I observed no gross defects in either body pattern or *en* or *wg* expression in heatshocked wildtype embryos containing the hs-*opa* construct. This also is consistent with a model in which Opa acts as a cofactor with other proteins, themselves perhaps limiting for the formation of transcription complexes containing Opa. Thus, excess Opa in certain cells may have no function

where these restricted cofactors are sequestered with wildtype levels of Opa. Mechanistically, how might these interactions affect target gene regulation? Ubiquitous Opa may form complexes with other factors at a specific *cis* regulatory element, with complex activity being determined by the accessibility of different regulatory domains of component proteins. Alternatively, perhaps interactions with different proteins may alter the specificity of Opa's putative DNA binding domain, and Opa may generate its effects on transcription at different promoter elements.

Other ubiquitous factors have been shown to either activate or repress transcription in different contexts, in some cases, via an identical *cis* regulatory element. For instance, the GLI-Kr class protein YY-1 (NF-E1/UCRBP/CF-1) either activates or represses transcription at the adeno-associated virus p5 promoter depending on the presence or absence, respectively, of the E1A protein (Shi et al., 1991). The Kr zinc finger protein bound to the K element as a dimer acts as a repressor. In contrast, Kr bound to this sequence as a monomer acts as a transcriptional activator (Sauer et al., 1991). For instance, YY-1 present throughout B cell development (Kakkis et al., 1989) can either activate transcription via a *c-myc* YY-1 binding site, or repress transcription via the kappa 3' enhancer in B cells (Park et al., 1991). Overall, YY1 acts at a wide variety of different promoters containing the motif CAT (Hahn, 1992). A ubiquitous factor in yeast, MCM1, can also generate different effects from the same promoter element (Jarvis et al., 1989; Ammerer, 1990). Each yeast cell type, **a** and α produces its own distinct mating pheromone and receptor. Pheromone-receptor binding generates a signal transduction cascade with discrete effects on transcription in each cell type. MCM1 is a MADS box protein which mediates some of these transcriptional effects at a motif known as the P box element (Johnson et al., 1985; Jarvis et al., 1989; Ammerer, 1990). MCM1 interacts with the α2 protein at P element promoter sequences to repress **a**-specific transcription in α cells (Tan and Richmond, 1990). Reciprocally, in **a** cells, MCM1 acts alone at P box elements to activate transcription at **a**-specific promoters (Bender and Sprague, 1987; Tan

et al., 1988). Presumably, a conformational change in MCM1 upon binding certain **a**-specific promoters plays a role in this regulation (Tan and Richmond, 1990). Mechanistically, all of these different effects arise through different transcriptional activities generated by interactions with other factors (YY1), homodimeric interactions (Kr) or conformational changes upon DNA binding in conjunction with other factors (PRTF). It is possible that Opa's different effects may be generated by some of these above described mechanisms

Opa-cofactor interactions at target promoters: a molecular analysis

Once direct targets of Opa are identified, promoter-*lac Z* transgenic lines to can be used to define promoter elements at which Opa and other cofactor(s) may interact. Truncated promoter sequences fused to reporter constructs have been useful in identifying transcription factor binding sites within both the *ftz* and *eve* promoters (Hiromi et al., 1985; Dearolf et al., 1989; Goto et al., 1989; Ueda et al., 1990; Lavorgna et al., 1991; Han et al., 1993a). A truncated promoter construct which fails to express a particular target gene domain, indicates missing sequences may contain an Opa-interacting site. Restoration of the site(s) should restore expression of the domain if the sequences are necessary for Opa to act at them.

Further resolution of the *cis* sites at which Opa acts can be accomplished using molecular techniques. As Opa binding to such elements may be influenced by interactions with cofactors, an identification of cofactor(s) should precede any binding studies. Interactions with Opa could be demonstrated *in vitro* using immunoprecipitation studies. For instance, our genetic analysis suggests either Runt or Ftz may act with Opa to repress *odd* in a specific cell. If such repression were shown to be direct, Runt and Ftz could be individually *in vitro* translated in the presence of ^{35}S methionine and incubated with Opa. Immunoprecipitation with anti-Opa antisera, followed by autoradiography could then detect the presence of labelled cofactor in the immunoprecipitate. In addition,

Louise O'Keefe in our lab is creating GST-Opa fusion proteins. Such proteins can be produced in *E. coli* and bound to glutathione-Sepharose beads (Smith et al., 1988) to form an Opa affinity column to test for Opa-cofactor physical interactions. Similarly, ^{35}S labelled cofactors should also be specifically retained by such a column. Both approaches have been successfully used to identify cofactors interacting with the GLI-Kr zinc finger protein YY1 (Lee et al., 1993).

Once such cofactors are identified, the target gene *cis* regulatory regions at which they act will have to be identified. The promoter-*lac Z* experiments described above will lead to a rough definition of Opa(-cofactor) binding sites within target gene promoters. The anti-Opa antibody can be used to further pinpoint such sites. Fragments from promoter sequences can be radiolabelled and incubated with Opa and putative cofactor protein(s). Fragments containing binding sites will be immunoprecipitated with anti-Opa and can be revealed by detection of radioactivity in the immunoprecipitate. Such fragments should also be shifted by Opa and cofactor protein(s) in a gel shift assay. Further definition of relevant *cis* elements can be obtained using DNAse footprinting and methylation experiments. Such an approach has been useful in defining FtzF1- and Ttk-binding sites on the Ftz promoter (Brown and Wu, 1993; Han et al., 1993a). The *in vivo* function of such elements can be further demonstrated by creating transgenic flies with mutated sites within a promoter construct fused to *lacZ*. Such flies should exhibit a concomitant target gene defect similar to that seen in an Opa mutant for that target. This approach has been used to demonstrate function of Tramtrack binding sites within the Ftz promoter (Brown and Wu, 1993) as well as *cis* regulatory sites within the *ftz* and *eve* promoters (Hiromi et al., 1985; Dearolf et al., 1989; Goto et al., 1989; Pankratz et al., 1990; Ueda et al., 1990; Lavorgna et al., 1991; Han et al., 1993a).

Opa biochemistry

The Opa protein sequence contains information about which domains may be involved in mediating its varied effects on target gene transcription. I have aready mentioned how possible interactions between Opa and other segmentation proteins may generate transcription complexes with different net effects on target gene transcription. Opa has a Gli-Kr zinc finger region, from amino acid positions 210 to 380 which most likely is a sequence specific DNA binding domain, as has been shown for other GLI-Kr. proteins (Ruppert et al., 1990). In addition, zinc finger domains in many cases have been shown to mediate heterodimeric interactions, in some cases with other zinc finger proteins (Glass et al., 1989; Schule et al., 1990; Yang-Yen et al., 1990; Seto et al., 1993). In addition to the zinc finger region, Opa has an helical, alanine-rich region (residues 94-122, 34% alanine-rich), a motif shown to be required for transcriptional repression in cultured cells (Licht et al., 1990). Similar domains are present in the segmentation genes *eve* and *en* (Kornberg et al., 1985; MacDonald et al., 1986a), themselves repressors in cell culture (Jaynes et al., 1991; Han et al., 1993b). Opa also has two polyglutamine repeats (poly Q residues 33-61, 470-494), domains which have been shown to activate transcription (Courey et al., 1988; Courey et al., 1989). Such polymeric domains along with Opa's glycine- and serine-rich regions may be involved in facilitating protein-protein interactions (Pontius, 1993). Like all other pair-rule proteins, Opa also contains a PEST sequence, (residues 386-410), a motif which regulates protein turnover (Rogers et al., 1986). Opa also contains polyhistidine stretches clustered within its C-terminal half, but the function of these repeats is obscure.

Experimentally, how might these domains be shown to be functional? Of interest are the domains responsible for transcriptional activation, transcriptional repression, and interactions with other cofactors. Truncated versions of GST-Opa proteins can be used to produce individual affinity columns such as those described above. Those proteins which do not bind cofactor define regions of the Opa protein responsible for protein-protein

interactions. This approach has been used to demonstrate the first 1.5 zinc fingers of YY1 bind the factor Sp1 (Lee et al., 1993). In addition, truncated versions of Opa expressed in cell culture can be assayed for the ability to regulate transcription of Opa binding sites fused to a reporter gene such as CAT when transiently expressed in Schneider cells. This assay has been useful in defining transcriptional repressor domains of Eve (Han et al., 1993b), and transcriptional regulatory domains of the *Kr*, *en*, and *prd* segmentation genes (Licht et al., 1990; Jaynes et al., 1991). It must be mentioned that inadvertently, we have shown *in vivo* that the C-terminal 100 amino acids of Opa are not required for either expression of *wg* or *en* or formation of wildtype body pattern.

In addition to such direct molecular approaches, sequencing of the 3 hypomorphic EMS alleles of *opa* may yield more information about functions of particular domains. The null EMS alleles may contain N-terminal stop codons or other mutations which abolish DNA binding. In contrast, the three hypomorphic alleles exhibit partial function, and most likely bind to target promoters to some degree. Such alleles may have mutations in a particular transcriptional regulatory domain with concomitant allele-specific effects on a specific target gene. A careful analysis of direct target gene transcription in *opa* hypomorphic backgrounds may reveal such effects, providing information about how Opa acts at a specific target promoter. Instead of sequencing all of the exons, exon amplification of sequences from mutant and wildtype embryos followed by rapid SSCP analysis can be used to detect point mutations in specific regions of a given exon. Such an approach has been useful in identifying single base mismatches in human rhodopsin and cystic fibrosis genes (Keen et al., 1991). The amplification products from a mutant embryo can then be directly sequenced to average out any mutations introduced by amplification. A similar sequence analysis of EMS-induced mutations in the *dor*, *eve*, and *Kr* genes identified domains and/or residues with different *in vivo* functions (Frasch, 1988; Redemann et al., 1988; Isoda et al., 1992).

Conclusion

Data from several experiments indicate that ratios of pair-rule proteins determine cell identity at blastoderm. For instance, in some pair-rule double mutant combinations, body pattern defects are less severe than the sum of each mutation taken individually. For example, the cuticle phenotype in *odd* mutants is phenotypically suppressed in double mutant combinations with *eve*, *prd*, *slp*, and *en* (Coulter, 1988). Consistent with this, the *en* expression pattern in *eve*, *odd* double mutant embryos is closer to wildtype than that of embryos mutant for *eve* or *odd* alone (DiNardo et al., 1988). This suggests loss of *odd* can be partially compensated for by loss of activity at other loci, implying that the relative ratios and not absolute amounts of each pair-rule product are important in patterning. In addition, ectopic expression of the pair-rule genes *eve*, *run*, *h*, or *ftz* generates body patterns complementary to those seen in embryos mutant for any of these respective genes (Struhl, 1985; Ish-Horowicz et al., 1987). This implies that both on and off states of a given pair-rule gene provide input to blastoderm patterning. Thus, both stripe and interstripe regions of the periodic pair-rule genes contribute positional information to the blastoderm embryo. Increasing levels of *runt* in its native expression domain also results in an 'anti-runt' phenotype complementary to that seen in *runt* mutants (Gergen et al., 1986b). These data suggest that for most pair-rule genes, only wildtype concentrations of pair-rule proteins provided in periodic domains can convey proper positional identities to blastoderm cells. Thus, the generation of periodic pair-rule gene expression is a mechanism by which the embryo titers specific ratios of pair-rule activities to different cells at blastoderm.

In contrast, *opa* is expressed and functional in most, if not all segment primordial cells at blastoderm. This suggests exclusion of *opa* activity from certain cells is not crucial for patterning, as is true for all other pair-rule genes. In addition, wildtype embryos in which *opa* was induced at blastoderm using hs-*opa* constructs showed both normal body pattern and normal expression of *wg* and *en*. Thus, increasing *opa* dosage

above wildtype does not affect patterning. It is likely that some of these embryos received a pulse of *opa* after blastoderm, as induction was carried out at several points during cellularization, and translation of the *opa* message lags behind transcription (this work and data not shown). Again, no heatshocked + ; hs*opa* embryos showed gross segmentation defects. In contrast, mutant embryos in which *ftz* expression perdures show segmentation defects (Kellerman et al., 1990), indicating blastoderm expression patterns of pair-rule genes late may disrupt patterning. These data suggest *opa* is different from the other pair-rule genes not just in its expression pattern but also in the way it interacts with other members of the pair-rule network to determine patterning.

Overall, the results described above are consistent with a model in which Opa acts as a cofactor with other pair-rule proteins at blastoderm. All the pair-rule proteins encode transcriptional regulators (Laughon et al., 1984; Frigerio et al., 1986; MacDonald et al., 1986a; Rushlow et al., 1989; Coulter et al., 1990; Grossniklaus et al., 1992). The one pair-rule protein not directly shown to be a transcription factor, Runt, contains sequences with high similarity to the DNA binding domain of the human transcription factor PEBP2 (Ogawa et al., 1993). Thus, it too is likely to be a transcription factor. In addition, all pair-rule proteins contain a PEST sequence , and decay rapidly after peak expression during cellularization (Laughon et al., 1984; Frigerio et al., 1986; Rogers et al., 1986; MacDonald et al., 1986a). Opa probably interacts with the other restricted, labile pair-rule proteins to form transcription complexes at target gene promoters. As concentrations of these restricted factors may be limiting for complex formation, excess Opa may not be able to form such complexes at blastoderm. Thus, increasing *opa* dosage at blastoderm may have no effect on target gene transcription. Similarly, providing ectopic Opa to the embryo just after cellularization may have no effect, as expression of other pair-rule proteins has decayed by this time. In either case, Opa must act in concert with other factors to effect changes on target gene transcription. If such factors are not available, Opa has no effect on patterning.

Does this imply *opa* has only a permissive and not an instructive role in determining blastoderm cell identities? For, instance, one might argue that Opa may be a basal transcription factor with different effects on targets reflecting different requirements in basal transcription factors at various promoters. Promoters do vary in their requirement for different basal factors (Parvin et al., 1992). In addition, there are proteins which contact both basal- and promoter-specific factors in *Drosophila* (Goodrich et al., 1993). Mutations in some basal factors and RNA pol II proteins mimic *Ubx* mutations (Francois Karch, pers. comm., [Mortin et al., 1981; Mortin et al., 1988; Mortin et al., 1992]), thus, this locus seems highly sensitive to dosage changes in basal factors at its promoter. However, it seems unlikely that Opa is involved in basal transcription, as it has such different effects on so many target loci. In addition, its expression is restricted to the segment primordia at blastoderm, with peak expression quickly fading thereafter. In contrast, other basal factors such as GAGA , NTF-1, and certain ribosomal proteins are expressed throughout the entire embryo at blastoderm (Dynlacht et al., 1989; Soeller et al., 1993). It is formally possible that Opa may have some effects on basal transcriptional regulation as well as acting as a traditional transcriptional regulator. The GLI-Kr. protein YY1 is such a protein. YY1 can serve as an initiator protein as well as affect transcription at different enhancers (Seto et al., 1993), most of which contain the sequence CAT (Hahn, 1992). In addition, the pair-rule protein Eve has been shown to affect basal transcription by inhibiting RNA pol II preinitiation complex formation *in vitro* (Johnson et al., 1992). Whether Opa is capable of acting similarly remains to be tested.

There is growing genetic and molecular evidence that other ubiquitous factors with restricted effects contribute to pattern formation in *Drosophila*. For instance, the zinc finger protein Ftz F2/Ttk is required to repress *ftz* expression in cleavage stage embryos (Brown et al., 1991; Brown et al., 1993). Ttk binds to specific sequences in the *ftz* zebra element, and mutations in these sites causes derepression of *ftz-lacZ* reporter

constructs as early as the third nuclear division cycle (Brown et al., 1991). Reciprocally, ectopic expression of Ttk at blastoderm results in global repression of *ftz*, as well as repression of specific stripes of the pair-rule genes *run*, *eve*, *h*, and *odd* (Read et al., 1992; Brown et al., 1993). Consistent with these functions, maternal *ttk* decays in wildtype embryos at blastoderm when *ftz* expression peaks (Brown et al., 1993). Perrimon et al. have also isolated another maternal mutation, *hop*, which has a restricted effect on segmentation (Perrimon et al., 1986). In *hop* embryos, although gap gene expression is normal, there are defects in specific stripes of *eve*, *ftz*, and *run* which give rise to specific body pattern defects after embryogenesis. *hop* encodes a non-receptor Jak tyrosine kinase, which may generate restricted effects by phosphorylation and subsequent activation of a restricted substrate transcription factor (Binari et al., 1994). Indeed, as other early segmentation proteins are phosphorylated (Ollo et al., 1987; Krause, 1989), such kinases may provide inputs to the pair-rule network during cellularization.

Another ubiquitous factor with restricted effects is extradenticle (*exd*). *exd* is globally expressed at the period in development when it modulates *Ubx* activity (Rauskolb et al., 1993). It does not do this by altering *Ubx* expression. Rather, it modifies Ubx activity via an interaction with the C-terminal region of Ubx, as activity of an ectopically expressed N-terminal deleted form of Ubx can still be affected by *exd* (Rauskolb et al., 1993). *exd* encodes a Pbx-1-like homeoprotein, suggesting Exd may participate with Ubx in the formation of a transcription complex. This model is somewhat analogous to how Opa may act with restricted pair-rule proteins. Chou et al, are conducting a search for other maternally encoded genes with restricted effects on segmentation (Chou et al., 1993). Most likely, these genes will also be ubiquitously expressed throughout the embryo. Current models for *Drosophila* segmentation are based upon combinatorial interactions among patterned transcriptional regulators. These models will have to accomodate interactions with other ubiquitous factors like *opa* as more of them are identified.

Materials and methods

Fly stocks

Flies were maintained on standard cornmeal-molasses-agar, at 25°C or 18°C. The EMS-derived *opa* alleles were obtained from the Tubingen stock center. The deficiency chromosomes depicted in Figure 1 were obtained from Steve Wasserman (Letsou et al., 1991), except for Df(3R) z-1 which was generated as follows: *ry* males, homozygous for the P(*ta3; ry*$^+$) transgene, were irradiated with 4-krads and mated to MKRS *ry* / TM3 *Sb, ry* virgin females. The flies were allowed to lay for four days, and the F1 flies were screened for *ry* eye color. Out of 9,971 flies 7 were identified as *ry*, 4 of which were recoverable. One of these, Df Z-1, was cytologically visible (82A; $82E_{3-4}$) and failed to complement the *opa* EMS alleles.

Two different balancer chromosomes were used to identify *opa* mutant cuticles and earlier stage embryos. TM3 *Sb* P(*pthb-lacZ*) (Driever et al., 1989a), provided by M. Weir, allowed for identification of homozygous mutant embryos in experiments in which embryos were stained with either antibody or *in situ* probes to *lac Z* products. In either case, homozygous mutant embryos lack cephalic staining , while wildtype embryos carrying the balancer chromosome are prominently marked. The TM6B *Tb* P(y^+) chromosome, provided by Ed Grell, allowed for the identification of homozygous mutant cuticles resulting from different crosses. Under bright field optics mutant embryos can be identified unambiguously because their ventral denticles lack pigment. Their wildtype siblings carry the P(y^+) transgene on the TM6B balancer chromosome and subsequently can be distinguished from mutant cuticles by their heavily pigmented denticles.

Chromosome walk

The EMBL3 genomic library (Frischauf, 1983) used was constructed and provided by John Tamkun. The walk was initiated using a genomic phage clone (from Bill Kalionis

and Rob Saint) that mapped to the centromere proximal edge of Df (3R)6-7. Whole phage DNA was individually isolated from each phage mapping under Df (3R)6-7 and digested with Sau3A. 100 ng from each phage prep was labelled with digoxigenin (Boehringer Mannheim Biochemical) in a reaction volume of 20 µl according to manufacturer's instructions, except the reaction took place in a silated Eppendorf tube for 10 hrs at room temperature. The reactions were stopped by heating to 65°C for 10 minutes and 20 µg glycogen, 1/10 volume of 3M sodium acetate, and 2 volumes ethanol were added and the reaction products precipitated on dry ice for 30 min. The reaction products were pelleted for 10 min in an Eppendorf centrifuge, aspirated and washed with 1 ml 70% ethanol, dried and resuspended in 50 µl of hybridization buffer. 4, 8 or 12 µl of this was used to hybridize fixed preparations of embryos (see below). Similarly treated DNA from a phage containing the first exon of the *engrailed* gene and our starting phage, which mapped outside of the *opa* deficiency, were used as positive and negative controls, respectively. 38 cDNAs were obtained by screening 2 x 10^5 colonies of a 0-4h cDNA plasmid library obtained from Nick Brown (Brown et al., 1988). 4 were analyzed in detail (see Sequence analysis below).

Primer extension

The primer **tttcgctcttagatatttcgcatgcgactt** extending from +148 to +119 on the noncoding strand of *opa*C was used for primer extension experiments. Primer extension was performed using a modification of a protocol described in (Ausubel et al., 1987), and was generously provided by John McKinney. Briefly, the primer was labelled with ^{32}P, annealed to embryonic mRNA, and extended with AMV reverse transcriptase for 1 hour. The reaction was stopped with EDTA and RNase A, exhaustively extracted with phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol, and precipitated. Prior to gel electrophoresis the reaction product was boiled for 3 min in sequencing buffer (80% formamide, 0.5X TBE, 0.1% xylene cyanol and bromphenol blue) and placed on

ice. The sample was run on a 6% polyacrylamide sequencing gel with sequencing reactions from two different primers as size standards. Reaction products were detected by autoradiography.

Construction of transgenic hs*opa* line

The *opa* cDNAs were originally isolated in pNB40 (Brown et al., 1988). A 2.8Kb HindIII fragment from *opa*C was cloned into pKS+ (Stratagene), yielding pKS-*opa*dIII. The 5' HindIII site comes from pNB40, while the 3' HindIII site resides at position 2795 of *opa*C. pKS-*opa*dIII was cut partially with HincII, and completely with Xba1. The resulting fragment was cloned into Hpa1, Xba1 cut pCaSpeR-hs (Mohler et al., 1989), to yield pCahs*opa*. Transgenic lines were constructed in the standard manner, using "wings-clipped" as a helper at 150µg/ml (Karess et al., 1984). A transgenic line that mapped to chromosome II was used to establish the experimental stock: *yw*; hs*opa*/hs*opa*; *opa*^{5H}/TM6B Tb P(y^+) (see Fly stocks above). From the crosses carried out to establish this stock, an isolate of *opa*^{5H}/TM6B Tb P(y^+)in the same genetic background as the experimental stock, but without the hs*opa* transgene was used as a negative control.

Rescue experiments

Injection of *in vitro* synthesized mRNA: A 2.8 kb EcoRI fragment from *opa*C was subcloned into pKS+ (Stratagene), yielding pKS-*opa*RI. Both EcoRI sites were derived from the NB40 vector. This EcoRI fragment included the SP6 RNA polymerase promoter from NB40, allowing the *in vitro* transcription of *opa* mRNA. 10 µg of pKS-*opa*RI was linearized with XbaI and incubated at 37°C with 1 mM each rATP, rCTP, rUTP, GpppGTP, 0.1 mM rGTP, 1.6 units/µl RNasin (Promega) and 40 units SP6 RNA polymerase in a total volume of 100 µl in the appropriate buffer (Promega). After 1.5 hours, 0.05 mM rGTP and 40 units of SP6 RNA polymerase were added and the incubation was continued. One hour later, 60 units of RNase-free DNase I (Boehringer

Mannheim Biochemical) were added and the mixture was incubated 20 minutes at 37°C. The mixture was diluted with an equal volume of water, phenol: chloroform: isoamyl alcohol extracted, and ethanol precipitated twice to remove free nucleotides. The pellet was resuspended in 20 µl of water and injected at ~1 mg/ml into embryos from *wy*; *opa*^{5H}/TM6B Tb P(*y*$^{+}$) parents. Embryos were collected for 30' and aged for 60' at 25°C, dechorionated with bleach and injected at ~50% EL under oil at 18°C (at about 2-2.5 hours AEL). Injected embryos were placed at 18°C for about 40 hours, fixed overnight in 5% formaldehyde/PBTw and de-vitellinized manually. Embryos were rinsed, rehydrated in PBTw for 1 hour at 37°C, mounted in Hoyer's:lactic acid:water (1:1:1) and cleared overnight at 65°C.

Heatshock protocol

For cuticle rescue, embryos from *yw*; hs*opa*/hs*opa*; *opa*^{5H}/TM6B P(*y*$^{+}$) parents, or from control flies without the hs*opa* construct were collected on grape agar plates and aged at 25° C. Chorions were cleared with 27S halocarbon oil and cellularizing embryos, representing stage 5 of development (~50 min) (Campos-Ortega et al., 1985), were transferred to a fresh plate. The plate was floated in a 37° C water bath for 20 min, and returned to 25° C. After ~30 hrs, hatched and unhatched embryos were processed to observe cuticle pattern (Meer, 1977). Under bright field optics *yw*; *opa*^{5H}/*opa*^{5H} embryos can be identified unambiguously because their ventral denticles lack pigment, while their wildtype siblings carry the P(*y*$^{+}$) transgene on the TM6B balancer chromosome. For rescue of *en* and *wg* expression, embryos from hs*opa*/hs*opa*; *opa*^{7N}/TM3 P(*pthb-lacZ*) parents, or from control flies lacking the hs*opa* transgene, were collected and heatshocked as above, returned to 25°C for one hour, fixed, and processed for *in situ* hybridization or immunohistochemistry as detailed below. The absence of *lacZ* expression identified the *opa* homozygous mutant embryos.

Sequence analysis

Four different cDNAs were isolated, *opa*A, *opa*B, *opa*C, and *opa*D. Of these, *opa*C represented a full length clone, with *opa*A, B, D being 5' deletions thereof. Nested deletions of the *opa*C subclone in both orientations in pKS+ were made using the Erase-A-Base kit (Promega) according to manufacturer's instructions. Sequencing was performed using the double stranded chain termination method (Sanger et al., 1977), the Sequenase 2.0 sequencing kit (US Biochemical) and ^{35}S-dATP. Terminal deoxynucleotide transferase and deaza-substituted nucleotides (US Biochemical) were used to resolve compressions. Cimbora and Sakonju have independently cloned the *opa* gene, and in comparing sequence information with them, (Cimbora and Sakonju, in preparation) Dan Cimbora identified an extra nucleotide at position 1781 relative to our sequence. Sequencing and restriction analysis confirmed that this nucleotide was present in *opa*A, B, and D, but not present in *opa*C. Therefore *opa*C generates a C-terminal truncated protein of 509 amino acids, which nevertheless rescues *opa* mutant embryos. The corrected sequence encodes a 609 amino acid protein. The *opa* nucleotide and protein sequences are available in the GenBank data library under accession number UO4435. Sequences were assembled using the PC/GENE ASSEMGEL and SEQIN programs. The PC/GENE TRANSL program was used to conceptually translate the cDNA sequence. The PIR, SwissProt, and GenPept databases were searched with the conceptual *opa* translation using the BLAST algorithm (Altschul et al., 1990). Alignment comparison of the zinc finger domains from the Opa, GLI, CiD and Tra-1 proteins was performed using the PC/GENE CLUSTAL program.

Exon mapping

cDNA probes on Southern blots of phage clones 6, 7, and 8 were used to map exons to individual genomic restriction fragments. Restriction site comparisons between these fragments and the *opa*C cDNA further refined the location of individual exons within

these fragments. The restriction fragments were subcloned into pKS+ and used as templates for PCR and sequencing comparisons in parallel with similar reactions on the *opa*C cDNA to verify location of exons not confirmed by restriction analysis or Southern blotting. Exon 1 mapped to a genomic 6 kb Bam HI fragment. The 5' and 3' endpoints of the exon within the fragment were determined by sequencing the 6 kb BamHI template with primers from the cDNA sequence. Exon 1 is 1243 bp and is completely within the 6 kb BamHI genomic fragment. Exons 2 and 3 lay within a 4.5 kb BamHI-SalI genomic fragment. Bases 1414-2959 of the *opa*C cDNA constituted exon 3. All primers used were obtained from Operon Technologies.

poly A$^+$ mRNA isolation

Total RNA was isolated according to (Dorsett et al., 1989). After resuspending total mRNA in 0.4M NaCl, 10 mM Tris pH 7.5, 5 mM EDTA and 0.5% SDS, the solution was passed over an oligo-dT cellulose column (Collaborative Research) which was then washed exhaustively with the same solution prepared without SDS. The poly A$^+$ fraction was eluted with TE and stored at -70^{o}C. Formaldehyde gels and Northern blotting procedures were as specified in (Sambrook et al., 1989).

Antibody production

A BamHI fragment from *opa*C in NB40 was subcloned into BamHI cut, phosphatased pAR3040, a T7 RNA polymerase expression vector (Rosenberg et al., 1987). The 3' BamHI site is from NB40, while the 5' BamHI site at position 591 in *opa*C. The construct fuses the first 10 amino acids of T7 gene 10 with the C-terminal 410 amino acids of *opa*. The construct was transfected into BL21(DE3) cells and induced with 0.4 mM IPTG for 2 hr at 37^{o} C. Cells were pelleted, washed with lysis buffer (LB) (50 mM Tris pH 8.0 /1 mM DTT /1 mM PMSF /0.01% NP-40), resuspended in LB plus lysozyme (1 mg/ml), and incubated 30 min on ice. Mixture was frozen and thawed for 3 cycles,

then sonicated 3 times for 10 sec. Inclusion bodies were pelleted at 10,000 rpm in a JS-13.1 rotor for 20 min at 4°C. Inclusion bodies were washed 3 times in LB plus 0.1% NP-40 (washing means resuspension, sonication and pelleting), once in LB, and once in PBS. Inclusion bodies were then resuspended in PBS at 4 mg/ml, and dispersed through progressively finer gauge needles. One ml of inclusion bodies was mixed 1:1 with Freund's complete adjuvant and injected into rabbits. Boosts were identical except they were mixed 1:1 with incomplete adjuvant.

In situ labelling for RNA and protein

In situ hybridization and protein staining of embryos was performed as in (Dougan and DiNardo, 1992). Prior to use in staining, the antisera was diluted 1:200 with PBS/1% Tween-20 and exhaustively preadsorbed against 7-10 h old embryos to remove cross-reactive antibodies. Preadsorption against blastoderm stage embryos resulted in substantial dampening of staining signal. The anti-Ftz antibody was a gift from H. Krause (Krause et al., 1988) and was used at a dilution of 1:500 (Kellerman et al., 1990). The *odd* probe was a gift from D. Coulter (Coulter et al., 1990).

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