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

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The pair-rule gene, odd-paired (opa), is essential for parasegmental subdivision of the Drosophila embryo. In addition to its previously defined role in the activation of wingless [wg] in odd parasegments, we find that opa is required for the timely activation of wg in the remaining parasegments and for the timely activation of engrailed (en) in all parasegments. opa encodes a zinc finger protein with fingers homologous to those of the Drosophila segment polarity gene ci, the human glioblastoma gene GLI, and the Caenorhabditis elegans sex determination gene tra-1. Previous work showed that opa activity was essential for the establishment of alternate parasegments, suggesting opa expression or activity would be spatially restricted like other pair-rule genes. Instead, opa mRNA and protein are found throughout all segment primordia. Thus, opa does not act in a spatially restricted manner to establish the position of en and wg expression. Rather, opa must cooperate with other spatially restricted proteins to achieve proper subdivision of the Drosophila embryo.

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A strategy common to many organisms during embryonic development is the subdivision of the body axis into small domains. Examples of such patterning units are the neuromeres that form in the diencephalon and hindbrain in the developing vertebrate nervous system (Lumsden and Keynes 1989; Figdor and Stem 1993) and the segmental divisions of the insect body (Garcia-Bellido et al. 1973, 1976; Morata and Lawrence 1975; Lewis 1978, Carroll et al. 1988b).

In fly embryos, subdivision along the anteroposterior axis begins with maternally deposited gene products and proceeds via a regulatory hierarchy of gene expression up to the time of cellular blastoderm formation (Nüsslein-Volhard and Wieschaus 1980; for reviews, see Nüsslein-Volhard et al. 1985; Ingham 1988). Genes within this hierarchy are expressed in increasingly narrow domains, reflecting the progressive subdivision of the body plan. Most of these genes encode known or presumed transcriptional regulators. Maternal gene products regulate the gap genes, which are expressed in broad domains. The gap proteins contribute to the control of three 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 and Ingham 1986; Frasch et al. 1987; Carroll et al. 1988b, Gergen and Butler 1988; Goto et al. 1989; Stanojevic et al. 1989; Pankratz et al. 1990). These pair-rule proteins, probably with assistance from the gap proteins, activate the other pair-rule genes, fushi tarazu [ftz], paired [prd], odd-skipped [odd], and sloppy-paired [slp] (Carroll and Scott 1986; Howard and Ingham 1986; Frasch and Levine 1987; Baumgartner and Noll 1990). Each of these genes is expressed in dynamic, striped patterns with each stripe encompassing two to four cells (Hafen et al. 1984; Kilchherr et al. 1986; Coulter et al. 1990; Grossniklaus et al. 1992). The last remaining pair-rule gene, odd-paired [opa], has been less well-characterized and is the subject of our analysis.

The body plan is further subdivided into small, repeating units, the parasegments (Martinez-Arias and Lawrence 1985), as the segment polarity genes wingless [wg] (Baker 1987) and engrailed [en] (Kornberg et al. 1985) are each activated in 14 single-cell-wide stripes. wg is expressed in the row of cells that defines the posterior edge of each parasegment (Ingham 1991; Dougan and DiNardo 1992), whereas en is expressed in the row of cells that defines the anterior edge of each parasegment (Lawrence et al. 1987; Carroll et al. 1988b). One function of en and wg is to maintain the parasegmental subdivisions of the body axis. The expression of both of these genes must be controlled with single-cell precision for this to occur (Babu 1977; Kornberg 1981; Nüsslein-Vol-
hard et al. 1985; Poole and Kornberg 1988; Noordermeer et al. 1985; Poole and Kornberg 1988; Noordermeer et al. 1992).

Several models suggest that combinatorial interactions among pair-rule proteins restrict the activation of en and wg to single-cell-wide stripes (Howard and Ingham 1986; DiNardo and O'Farrell 1987; Ingham et al. 1988; Morrissey et al. 1991; Manoukian and Krause 1992, 1993). For instance, both ftz and opa activity are required for normal en expression in the even parasegments (Howard and Ingham 1986; DiNardo and O'Farrell 1987; Ingham et al. 1988). The ftz protein is expressed in stripes several cells wide (Carroll and Scott 1985), but en is activated only in the anterior-most of these cells [Lawrence et al. 1987; Carroll et al. 1988a]. 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 parasegment [DiNardo and O'Farrell 1987, Ingham 1988]. en would then be activated only in cells expressing both proteins. Recent data have suggested that the repressor odd also limits the expression of en [Manoukian and Krause 1993]. The restricted expression of wg was also thought to involve the position-specific expression or activity of the opa gene [Ingham et al. 1988]. Here, we report that opa activity is essential for the appropriate level and timing of en and wg expression in all parasegments, but opa activity does not determine the restricted spatial domains of these genes.

Results

Body pattern defects

The segmental pattern of wild-type embryos is easily visible at the end of embryogenesis in the cuticle secreted by epidermal cells. There are three thoracic and eight abdominal segments [Fig. 1A]. The anterior of each segment has several rows of cuticular projections, or denticles, comprising a denticle belt. The remainder of each segment consists of smooth cuticle. In opa mutants, only half the number of denticle belts are present as a result of the deletion of alternate segments [Jürgens et al. 1984]. The same defects are found in embryos harboring molecularly null opa mutations, Df(3R) 63/Df(3R) 107 [Fig. 1B; see below]. These segmentation defects result from the failure to establish or maintain the parasegments early in embryogenesis.

opa activity is required for optimal levels of en and wg in all parasegments

At cellular blastoderm in wild type, wg is expressed in 14 single-cell-wide stripes defining the posterior borders of parasegments 0–12 [Baker 1987; Fig. 1C]. In opa null embryos, wg expression never initiates in the odd parasegments, as reported previously for ethylmethane sulfonate (EMS)-induced opa alleles [Ingham et al. 1988]. In the even parasegments, wg expression is severely delayed except in parasegments 0 and 2 [Fig. 1D]. As development proceeds, wg expression initiates in the even parasegments, 4–12, and approaches normal levels, although it remains spotty [e.g., see Fig. 4B, below]. We conclude that opa function is essential for the initiation of wg in the odd parasegments and is important for normal wg expression in the even parasegments. Similarly, opa is required for normal en expression in all parasegments. In wild type, en is expressed in 14 single-cell-wide stripes by the onset of gastrulation [Fig. 1E, Kornberg et al. 1985]. In opa null mutants at gastrulation, little en expression is visible [Fig. 1F]. en expression is severely delayed, but eventually appears in both the odd and even parasegments, indicating that opa is required in all parasegments for the timely activation of en. The defects in both wg and en expression demonstrate that opa is required in the cells that flank each parasegment border, and not only in the cells flanking alternate parasegments borders, as was thought previously [DiNardo and O'Farrell 1987, Ingham et al. 1988]. In the earlier analysis of EMS-induced opa alleles, some en expression was detected in the even parasegments. Although this was assumed to be attributable to residual opa activity, we have found that in opa null embryos, en is expressed in the correct position in these parasegments [Fig. 1H, P; see below]. This result indicates that although opa is required for the proper timing of en activation, it is not required for either activation or proper positioning of en expression in the even parasegments.

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

It has been suggested that, in the even parasegments, the position of en expression is defined by the presence of the activator ftz and the absence of odd, a presumed repressor of en [Manoukian and Krause 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 seven stripes approximately three cells wide, corresponding to the anterior-most cells of the even parasegments [Carroll and Scott 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 and Krause 1992]. By gastrulation, odd expression [blue color in Fig. 1K] is repressed in the anterior-most Ftz+ cells [brown color in Fig. 1I], 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 [Fig. 1E, Lawrence et al. 1987; Carroll et al. 1988a]. After cellularization, odd expression is initiated in secondary stripes within the odd parasegments [Fig. 1I, K, Coulter et al. 1990].

In opa mutants, the secondary odd stripes fail to appear [Fig. 1J], indicating that opa is required for the activation of odd expression in the odd parasegments. 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. 1K, L]. This suggests that the
delay in activation of en in even parasegments is attributable 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 germ-band extension in opa mutants, odd expression is lost from the anterior-most Ftz + cells of parasegments 4 and 8 [Fig. 1L]. About this time, en expression first appears in parasegments 4 and 8 [Fig. 1H]. 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-germ-band extension, the Ftz stripes become particularly broad in parasegments 4 and 8, and odd expression in these parasegments is no longer detectable [Fig. 1N]. Shortly thereafter, the en stripes in parasegments 4 and 8
broaden to about twice wild-type width [Fig. 1P] correlating with the presence of Ftz and the absence of odd expression 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 the odd gene product and Ftz [Manoukian and Krause 1993].

The effects on wg, en, ftz and odd expression demonstrate that opa activity not only is required at cellular blastoderm in the cells that flank each parasegment border but also in most cells within each parasegment. 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.

Localization and cloning of the opa gene

By generating deficiencies [Fig. 2, see Materials and methods (Letsou et al. 1991)] we refined the opa map position [Jürgens et al. 1984] to the interval 82D(3-8i); 82E(3-4) and used a phage clone that mapped to the 82D region to begin a chromosome walk. To identify putative sites in the region cloned from 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.

Figure 2. Deficiency maps and cDNA structure of opa. Deficiency chromosomes in the cytological interval 81F–83A. Solid lines indicate missing DNA. The SalI 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 SalI map, but are not drawn to scale.

Rescue of the opa mutant phenotype

Sequencing and primer extension experiments identified one of the clones, opaC, as a full-length cDNA (data not shown). Two complementation assays were used to test whether it was the opa gene. First, capped transcripts synthesized in vitro from opaC injected into embryos during the syncytial blastoderm rescued the cuticle phenotype of 8 of 16 embryos [Materials and methods; data not shown]. We then constructed transgenic flies in which the opaC cDNA was placed under the control of the hsp70 promoter (HSopaC). opaC homozygous embryos have only four complete abdominal segments [Fig. 3A], although occasionally the remnants of a fifth definitive belt are found. When transgenic embryos were exposed to a 20-min heat shock during cellularization, 16 of 27 (59%) and 22 of 48 (46%) opa mutants showed rescue as judged by the presence of at least five full definitive belts [Fig. 3B, C]. In some cases, rescue approximated the wild-type body pattern [Fig. 3C]. No rescue was found in heat-shocked embryos that did not harbor the transgene.

We then asked whether the rescue of body pattern correlated with the restoration of wg and en expression. In opa7 mutants, the wg stripes in the odd parasegments are missing (cf. Fig. 4, A and B). In opa mutants carrying the HSopaC transgene, wg expression was restored in odd parasegments 3-11 (Fig. 4C). 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 HSopaC transgene. About 1 hr after cellularization in opa mutants, en expression is usually absent from parasegment 2, is spotty and broad in parasegments 4 and 8, and fails to extend dorsally in all stripes (cf. Fig. 4, D and E). In contrast, 29 of 54 (54%) of the opa transgenic embryos exhibited en expression in parasegment 2, narrower en stripes in parasegments 4 and 8, and more extensive dorsal expression in all stripes [Fig. 4F]. Although en expression was partially restored, some defects remained in the positioning of the stripes such that odd parasegments appeared narrower than the even parasegments (Fig. 4F). 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, heat shock during cellularization may lead to translation of opa after its normal peak requirement. Second, there may be a postblastoderm requirement for opa that will not be fulfilled by a single heat pulse during cellularization. Third, we recently
**Figure 3.** Rescue of *opa* body pattern by the HSopaC transgene. Cuticles oriented anterior up, ventral to right, dark-field images. (A) *opa*<sup>SH</sup>/*opa*<sup>SH</sup>. 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 wild type in D. (B) HSopa; *opa*<sup>SH</sup>/*opa*<sup>SH</sup>. Partially rescued embryo with splitting of denticle belt fusions. (C) HSopa; *opa*<sup>SH</sup>/*opa*<sup>SH</sup>. Fully rescued embryo. All eight abdominal denticle belts are present. (D) Wild type. Eight abdominal denticle belts are present.

learned that the *opaC* cDNA contains a frameshift resulting in a truncated protein, which nevertheless rescues, but may have affected, rescue frequency [see Materials and methods].

**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 [Materials and methods]. The transcription unit spans a total of 14.5 kb of genomic sequence with introns of ~12 and 0.4 kb separating the three exons (Fig. 2). The *opa* transcript is 2959 bp in length, which corresponds well with the transcript size determined by Northern blotting [see below]. Sequence analysis at the 5’ end of the *opaC* cDNA and of the corresponding genomic region revealed a consensus cap site (GCAGTCCTGC) beginning 1 nucleotide upstream of *opa*. Primer extension experiments using a primer from +148 to +119 yielded two main products of 147 and 148 nucleotides in length and a minor product of 149 nucleotides [data not shown], indicating that *opaC* represents a full-length transcript. Three adjacent in-frame translation initiation codons are found at positions 294, 297, and 300 (Fig. 5A), and the sequence surrounding the first ATG matches the *Drosophila* translation start consensus [Cavener 1987]. Conceptual translation of the 1827-nucleotide open reading frame yields a protein of 609 amino acids (Fig. 5A). The predicted protein contains five
Figure 5. Nucleotide and protein sequence of *opa*, a GLI-Kr class zinc finger protein. (A) Conceptual translation of the *opa* cDNA. Noncoding nucleotides are indicated by an asterisk (*). (B) Amino acid sequence alignment of the *opa* protein with *GLI*, *Ci D*, and *TRA-1*. Identical residues are indicated by an asterisk (*), conservative substitutions by a caret (^), and residues corresponding to the GLI consensus zinc 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.

C3H2 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 (Kr) (Preiss et al. 1985). The predicted OPA protein also contains stretches of polyserine and polyglutamine repeats, motifs common to many transcription factors. The zinc finger region, from amino acid position 210 to 380 was the only domain exhibiting significant homology to other proteins. The most homologous zinc finger domain sequences found were *Drosophila* CI3, human GLI and GLI3, and *Caenorhabditis elegans* *Tra-1* (Fig. 5B, Eaton.
and Kornberg 1990; Orenic et al. 1990; Ruppert et al. 1990; Zarkower and Hodgkin 1992). Fingers 3–5 of Opa fit the GLI consensus sequence [Y/FXCX₃GCX₃(F/Y)]X₃HX₃-H, and the H/C link consensus [T/S]GEKP]. There is 42% (70/167) identity between Opa and all of 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 with the GLI, CiD, 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**

Northern blotting experiments with an opaC probe revealed a single mRNA of 3 kb with peak expression between 2 and 12 hr of embryogenesis [Fig. 6]. Expression of this transcript continues throughout the larval instars and during pupation, although at lower levels compared with 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.

**opa transcript** first appears at the beginning of cellularization (stage 5) (Campos-Ortega and Hartenstein 1985) in a stripe ~10 cells wide centered at 80% egg length [Fig. 7A]. Expression then extends posteriorly, and the level of expression increases, generating uniform expression between 20% and 80% egg length [Fig. 7B], with the posterior border of this domain more sharply defined than the anterior. The expression pattern does not change during gastrulation (stage 6). Once germ-band extension begins (stage 7), the level of ectodermal expression begins to decrease. Transcript fades more from particular cells within each parasegment, generating 14 weak stripes over a low background level of expression [Fig. 7C]. 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. Opa protein is present in a block from 20% to 80% egg length during cellularization, and protein levels peak late during cellularization and into gastrulation [Fig. 7D,E], paralleling expression of opa mRNA [cf. Fig. 7, B with D, and C with E]. 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. 7F], 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.

Opa accumulates in the nuclei, although some staining is visible in the basal cytoplasm of ectodermal cells [Fig. 7E, inset]. Throughout gastrulation Opa remains expressed in a solid block. During early germ-band extension, Opa expression levels decline compared with the peak at cellular blastoderm, although the protein re-

**Figure 7.** opa mRNA is expressed throughout the segmented region of the body. (A–C) Wild-type embryos probed in situ for opa RNA. (A) Early stage 5; opa RNA appears in a 10cell-wide patch at ~80% egg length. (B) Late stage 5; opa is expressed at peak levels in a broad domain from 20–80% egg length, with a sharp posterior edge and a fuzzier anterior edge. (C) Stage 7; 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. Wild type [D,E] and opa null [F] all labeled with rabbit polyclonal antibodies raised against the Opa protein. (D) Late stage 5; Opa expression extends from 20–80% egg length [cf. with B]. (E) Stage 6/7; Opa remains expressed in a broad domain, with no stripes visible. Magnified view of ventral ectoderm shows that Opa accumulates mostly in the nuclei. (F) opa null Df(3R)63/Df(3R)107 expresses no detectable Opa protein.
mains homogeneously expressed within the segmented region of the ectoderm. As germ-band extension continues, weak stripes evolve over a low level background of Opa expression (data not shown), similar to those of the transcript pattern.

Discussion

The loss of opa function leads to pair-rule defects in the body pattern. opa, however, is a ubiquitous factor expressed throughout all segment primordia and not spatially restricted as are other pair-rule proteins. Thus, the generation of parasegmental subdivisions requires that opa assists localized factors. We discuss how the molecular nature of the Opa protein may explain this cooperation. In addition, we consider how, in opa mutants, the early global defects in gene expression are partially resolved through subsequent cellular interactions such that only pair-rule defects are generated in the final body pattern.

Opa function may vary in the presence of other transcription factors

Strikingly, opa exhibits no periodicity in its early expression. In every cell at blastoderm for which a marker gene exists, we find an expression defect in opa mutants, suggesting that Opa is active in most and probably all cells of each segment primordium at blastoderm. Genetically, Opa positively regulates some genes while negatively regulating others. It is likely that Opa executes these genetic roles by directly regulating transcription, because several Drosophila Kr class finger proteins have been shown to act directly as transcriptional regulators [Sauer and Jäckle 1991; Zuo et al. 1991]. Opa has an 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]. Opa also has two polyglutamine repeats, which have been shown to activate transcription [Courey and Tjian 1988; Courey et al. 1989]. These motifs suggest that Opa may either activate or repress transcription upon interacting with a given target gene. Other ubiquitously expressed proteins have been shown to both activate and repress transcription in different contexts. The GLI-Kr class protein YY-1 [NF-E1/UCRBP/CF-1] either activates or represses transcription at the adenovirus-associated virus p5 promoter, depending on the presence or absence, respectively, of the E1A protein [Shi 1991]. In addition, YY-1 present throughout B-cell development [Kakkis 1989] can either activate transcription via a c-myc YY-1-binding site or repress transcription via the κ 3' enhancer in B cells [Park and Atchison 1991]. Presumably, this functional versatility results from the interaction of ubiquitous YY-1 with specific transcription factors at different promoters. The genetic analysis presented here suggests certain candidates for spatially localized factors with which Opa could cooperate.

In the even parasegment, our data support a combinatorial model wherein Ftz activates en expression and odd restricts this activation to the anterior-most Ftz-expressing cells [Fig. 8; Manoukian and Krause 1992, 1993]. An essential facet of this model is that Opa must repress odd transcription in a specific cell, the anterior-most Ftz-expressing cell, to allow induction of en by Ftz [Fig. 8]. The Run pair-rule protein is a candidate for a cofactor with which Opa may cooperate to specifically repress odd. The Ftz protein itself peaks in concentration in the anterior cell of its expression domain. Run is also expressed in these anterior-most Ftz * cells [Kania et al. 1990]. Perhaps an interaction between Opa and Run masks the Opa activator domain, and subsequent binding of this complex to the odd promoter represses odd transcription in these cells.

Opa acts as a positive regulator in other cells. Perhaps
in these cases, interactions with specific spatially restricted factors mask the repressor domain within Opa, allowing it to assist in transcriptional activation of target genes. Activation of en expression in the odd parasegment was thought to occur via a combination of Eve and Prd [Fig. 8; DiNardo and O’Farrell 1987; Morrissey et al. 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 and Levine 1987; Baumgartner and Noll 1990]. Perhaps in these parasegments, Opa interacts with Prd and/or Eve to form a complex that stimulates en transcription.

Opa sets the level, but not the position, of wg expression

ftz and eve have been genetically defined as negative regulators of wg expression, whereas prd and opa have been defined as activators [Ingham et al. 1988]. It was hypothesized that as ftz stripes narrowed, repression would be relieved at wg, allowing activation by prd in even parasegments [Fig. 8]. A similar narrowing of eve stripes in the odd parasegments was hypothesized to allow activation of wg by opa [Fig. 8]. Because the repressors ftz and eve eventually disappear, some mechanism must restrict wg activation to narrow stripes. In the even parasegments this role was ascribed to prd, because its expression domain is restricted [Fig. 8]. In the odd parasegments earlier models supposed a similar restriction in the domain of expression or function of opa. We have confirmed that opa function is essential for wg activation in the odd parasegments [Ingham et al. 1988] and have shown that opa is important for the timely activation of wg expression in the other parasegments. Thus, opa may directly activate wg in the odd parasegments and assist in the prd-dependent activation of wg in the even parasegments [Fig. 8]; however, because opa is expressed throughout all segment primordia and appears to act in all cells in which it is expressed, opa alone cannot determine the restricted position of wg expression in odd parasegments. The spatial information that restricts wg expression in the odd parasegments must reside with other pair-rule, or as yet uncharacterized, proteins.

Early global defects are partially resolved through cellular 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. The final pattern in opa mutants, however, is not surprising if one considers the cell signaling circuits that function during postblastoderm development. In wild-type embryos, after wg and en expression are initiated in adjacent cells, wg input to the neighboring cells maintains the expression of en in those cells [DiNardo et al. 1988; Martinez-Arias et al. 1988]. wg even appears to have the capacity to boost low levels of en expression [Bejovec and Martinez-Arias 1991]. Reciprocally, wg expression is reinforced by a signal from adjoining en-expressing cells [Martinez-Arias et al. 1988]. As a consequence of these interactions, the parasegment boundaries are stabilized and normal body pattern is generated. In opa mutants, although en expression eventually initiates in the even parasegments, the lack of wg expression in the anteriorly adjacent cell [in the odd parasegments] leads to premature decay of these en stripes. The boundaries between these parasegments are destabilized, leading to fusion of alternate parasegments and deletion of the odd denticle belts. At the other parasegment boundaries, wg expression is initiated, albeit at reduced levels, and en expression is severely delayed. The mutual positive feedback between adjacent wg- and en-expressing cells, however, stabilizes these parasegment boundaries, such that the final body pattern exhibits only pair-rule defects. Thus, the global defects observed in opa mutants at cellular blastoderm are partially compensated for later by the regulative properties of the en and wg cells flanking these parasegment boundaries.

Other proteins may be found that, like Opa, play a spatially restricted role but are not spatially restricted in their expression. Such proteins need not be zygotically expressed, but could be deposited maternally during oogenesis, because their restricted activity does not hinge on limited expression. The homeotic gene extradenticle is one such gene that is uniformly expressed in the embryo during the period when it modulates Ultrabithorax [Ubx] activity [Rauskolb 1993]. At least one other maternal effect lethal mutation that leads to patterned defects within the embryo has been identified [Perrimon and Mahowald 1986], and genetic screens are being conducted for other maternal factors. Current models for Drosophila segmentation are based on combinatorial interactions among patterned transcriptional regulators. These models will have to accommodate interactions with ubiquitous factors 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 Tübingen stock center. Deficiencies in Figure 1 were obtained from Steve Wasserman [Letsou et al. 1991], except for Df Z-I, which was generated as follows: rosy [ry] males, homozygous for the P[ta3; ry+] transgene, were irradiated with 4 krad and mated to MKRS ry/TM3 Sb, ry virgin females. The flies were allowed to lay for 4 days, and the F1 flies were screened for ry eye color. Of 9971 flies, 7 were identified as ry, 4 of which were recoverable. One of these, Df Z-I, was cytologically visible [Driever et al. 1989], provided by Ed Grell [University of California, San Francisco].

Chromosome walk

The EMBL3 genomic library [Frischauf et al. 1983] used was constructed and provided by John Tamkun [University of Arizona, Tucson]. The walk was initiated using a genomic phage clone [from Bill Kalionis and Rob Saint, University of Adelaide,
3 M sodium acetate, and 2 volumes of ethanol were added and ethanol precipitated twice to remove free nucleotides. The pellet was resuspended in 20 µl of water and injected at ~1 ng/ml into embryos from wy; opa +/TM6B P{y+} parents. Embryos were collected for 30 min, and aged for 60 min at 25°C, dechorionated with bleach, and injected at ~50% egg length under oil at 18°C [at ~2-2.5 hr after egg laying]. Injected embryos were placed at 18°C for ~40 hr, fixed overnight in 5% formaldehyde/PBTw, and devitellinized manually. Embryos were rinsed, rehydrated in PBTw for 1 hr at 37°C, mounted in Hoyer’s/lactic acid/water [1:1:1], and cleared overnight at 65°C.

Heat shock protocol For cuticle rescue, embryos from wy; HsopA/HsopA, opa +/TM6B P{y+} parents, or from control flies without the HsopA 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 and Hartenstein 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 hr, hatched and unhatched embryos were processed to observe cuticle pattern [Meer 1977]. Under bright-field optics wy; opa +/opa +HsopA embryos can be identified unambiguously because their ventral denticles lack pigment, whereas their wild-type siblings carry the P{y+} transgene on the TM6B balancer chromosome. For rescue of en and wg expression, embryos from HsopA/HsopA; opa2n/TM3 P{Pithb-lacZ} parents, or from control flies lacking the HsopA transgene, were collected and heat-shocked as above, returned to 25°C for 1 hr, 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, opaA, opaB, opaC, and opaD. Of these, opaC represented a full-length clone, with opaA, opaB, and opaD being 5' deletions thereof. Nested deletions of the opaC 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 [U.S. Biochemical] and [35S]dATP. Terminal deoxynucleotide transferase and deaza-substituted nucleotides [U.S. Biochemical] were used to resolve compressions. Recently, Cimbora and Sakonji have independently cloned the opa gene [D. Cimbora and S. Sakonji, in prep.]. During preparation of this manuscript, we exchanged sequence information and Dan Cimbora identified an extra nucleotide at position 1781 relative to our sequence. Sequencing and restriction analysis confirmed that this nucleotide was present in opaA, opaB, and opaD, but not in opaC. Therefore, opaC generates a carboxy-terminal truncated protein of 509 amino acids, which nevertheless rescues opa mutant embryos. The sequence reported here and to GenBank is the corrected one. Sequences were assembled using the PC/GENE ASSEMB- GEL and SEQIN programs. The PC/GENE TRANSPL program was used to conceptually translate the cDNA sequence. The PIR, SwissProt, and GenPept data bases were searched with the conceptual opa translation using the BLAST algorithm [Altschul et al. 1990]. Alignment comparison of the zinc finger do-
mains from the Opa, GLI, C10, 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 *opaC* cDNA further refined the location of individual exons within these fragments. The restriction fragments were sub-cloned into pKS + and used as templates for PCR and sequencing comparisons in parallel with similar reactions on the *opaC* cDNA to verify location of exons not confirmed by restriction analysis or Southern blotting. Exon 1 mapped to a genomic 6-kb BamHI fragment. The 5' and 3' end points 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 *opaC* cDNA constituted exon 3. All primers were obtained from Operon Technologies.

Poly(A)+ mRNA isolation
Total RNA was isolated according to Dorsett et al. [1989]. After resuspension of total mRNA in 0.4 M 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°C. Formaldehyde gels and Northern blotting procedures were as specified in Sambrook et al. [1989].

Antibody production
A BamHI fragment from *opaC* in NB40 was subcloned into BamHI-cut, phosphatased pAR3040, a T7 RNA polymerase expression vector [Rosenberg et al. 1987]. The 5' BamHI site is from NB40, whereas the 5' BamHI site is at position 591 in *opaC*. The construct fuses the first 10 amino acids of T7 gene 10 with the carboxy-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°C. Cells were pelleted, washed with lysis buffer [LB] (50 mM Tris at pH 8.0, 1 mM DTT, 1 mM PMSF, 0.01% NP-40), resuspended in LB plus lysozyme (1 mg/ml), and incubated for 30 min on ice. The mixture was frozen and thawed for three cycles, and sonicated three 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 three 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 milliliter 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 labeling for RNA and protein
In situ hybridization and protein staining of embryos was performed as in Dougan and DiNardo [1992]. 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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Note added in proof
The sequence data described in this paper have been submitted to the GenBank data library under accession number U04435.

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