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

Mark J. Benedyk,¹ Janet R. Mullen,¹ and Stephen DiNardo²

The Rockefeller University, New York, New York 10021-6399 USA

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^P*, 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.

[Key Words: *Drosophila*; pair-rule genes; zinc finger protein; segmentation]

Received September 3, 1993; revised version accepted November 22, 1993.

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 hind-brain in the developing vertebrate nervous system (Lumsden and Keynes 1989; Figdor and Stern 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 et al. 1986; Frasch et al. 1987; Car-

roll et al. 1988; 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-

¹These authors contributed equally to the work.

²Corresponding author.

hard 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 0–13 (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

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-germband 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₍₃₋₈₎; 82E₍₃₋₄₎ and used a phage clone that mapped to the 82D region to begin a chromosome walk. To identify putative candidate phage from within the walk, each clone was used as an in situ probe on blastoderm embryos. Only clones 6, 7, and 8 (Fig. 2) exhibited strong hybridization, detecting message expressed from ~20% to 80% egg length (not shown, but identical to Fig. 7B, below). These three phage clones also covered the DNA missing from two small deletions that failed to complement *opa*, *Df(3R) 107* and *Df(3R) 63* (data not shown and Fig. 2). Subfragments from each of these phage clones were iso-

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.

lated, individually labeled, and hybridized to embryos. Two of the fragments exhibited the same pattern on embryos as the parent phage. These two fragments identified a ~3-kb message on Northern blots of 2.5- to 5.0-hr embryonic mRNA. Four cDNA clones obtained using these fragments each exhibited hybridization results identical to those obtained with the genomic clones (see Figs. 6 and 7, below).

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 *opa* 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*). *opa*^{5H} homozygous embryos have only four complete abdominal segments (Fig. 3A), although occasionally the remnants of a fifth denticle 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 denticle 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 *opa*^{7N} 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*^{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 wild type in D. (B) *HSopa*; *opa*^{5H}/*opa*^{5H}. Partially rescued embryo with splitting of denticle belt fusions. (C) *HSopa*; *opa*^{5H}/*opa*^{5H}. 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 4. Rescue of *en* and *wg* target gene expression by the *HSopaC* transgene. (A–C) Stage 9 embryos labeled in situ for *wg* and *lacZ* RNA. (A) Wild type. *wg* is expressed in parasegments 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 parasegment, and is expressed weakly in the even parasegments. (C) *HSopa*; *opa*^{7N}/*opa*^{7N} 1 hr after heat shock. *wg* expression is restored to odd parasegments 3–13. (D–F) Stage 9 embryos labeled for En protein. (D) Wild type. En is expressed in parasegments 1–14 and β -galactosidase in the head because of the presence of the TM3 *pthb-lacZ* balancer chromosome. (E) *opa*^{7N}/*opa*^{7N}. En is expressed in parasegments 4 and 8 in broad stripes, and all En stripes are weak dorsally. (F) *HSopa*; *opa*^{7N}/*opa*^{7N}, 1 hr after heat shock. En expression is narrower in parasegments 4 and 8, and En is more robust and extends more dorsally in all parasegments.

A

1	cagtcctcgtcgcgcattcttgaacgcgtcaagtccttggcattcggttagcgtcaagtcct																															
58	tctcgtcgattcagacgccccagaaacgcgatcgccgcgagtgatattacgtataaataagtat																															
117	tcaagtcgttcgcgaataattctaaagcagcgaataaagcgcgcagcagatatacacgcaatg																															
176	aggtgacctcagtcgctgattggcctcatttggcctcagaagtcgcgaatacgaaaactgac																															
235	attcctcagttctctctcgaattaatcttataaacatataagcagcagataaacactgtaccc																															
294	ATG ATG ATG ATG AAC GCC TTC ATT GAG PCG GCT CAG CAC CAG CTG CGA																															
353	M M M M N A F F I E P A Q H H L L A																															
399	AGC TAC GGG CTG CGA ATG TCG CCC AAC ACC ACC GCC AGT AAC AGS																															
46	S Y G G L R M S T P N T T A S N N																															
384	AAC GCG CAG CAG CAA CAG CAG CAG CAA CTG GAG ATG ACC CAA CAG																															
31	N A Q Q O O O O O O O O L E M T O O																															
429	CAG CAG CAA CAA CAG CAA CAA CAG CAG CAG CAA CAG CAA CAG CAG																															
46	Q O O O O O O O O O O O O O O O																															
474	CAG GAA TCC GCG GCG GCG ACT GCT GCA GCC TAT CAG AAC TCC GGA																															
61	Q E S A A A T A A Y Q N S G																															
519	TAT GGG CAC TTT AAC AGC TAT GCC TCC CGG GAG TTT TTG CTG GCG																															
76	Y G H F N S Y A S R D F L L G																															
564	AGG AGG GAG GCC GAG TAC GGT GTG GCA GGA TCC GCC GGA CAG GCG																															
91	R R E A E Y G V A G S A G Q C A																															
609	TCC GCC GCA GCG GAT TCC ATG CTG TTC TCC GGC TTT CCC GCT CAG																															
106	S A A A D S M L F S G F P C A																															
654	GCC GCG GAG CTG GCG TCC GGS TTT GGA CAA CAC CCC TTT CAC AGG																															
121	A A E L G S G F G G Q H P F H S																															
699	CAC CAT CAC CAC CAT CAG ATG CGA ATG GCG ATG GCG GAT GCC TAC																															
136	H H H H H H Q M R M G M A D A																															
744	GCC GCC GGC CAC CCG TAC AAC CAC CAC GGC AAC TTC CCG AGC																															
151	A A G G H P Y N H H G N F P T C																															
789	GCA GTG CAC CAC CCG GTC GTG CAC CAC CCG TCC CAC GCC ATC																															
166	A V H H P V V H H C P S H H A M																															
834	TCC GCG ATG CAC CCG GCC GGA GCA GGG GCC TTC CTG CGC TAC AT																															
181	S A M H P A G A G G A F L R Y M																															
879	CGC CAT CAG CCG GCC TCG TCC GCC TCC AGC GTT AAC CAG GAG																															
196	R H Q P A S S A S S V K Q G E M																															
924	CAG TGC CTG TGG ATA GAC CCC GAC CAG CCG GGC CTG GTG CCC																															
211	Q C L W I D P D Q P G G L V P C																															
969	GGC GCG AGG AAG ACC TGC AAC AAG GTA TTC CAC TCG ATG CAG																															
226	G G R K T C N K V F F H S M H E																															
1014	ATC GTC ACC CAC CTG ACC GTG GAG CAC GTG GGC GGA CCC GAG TGC																															
241	I V T H L T T V E H V V G G P E C																															
1059	ACC AAC CAC GCC TGC TTC TGG VTG GGC TGC TCT CGC AAC GGA CGC																															
256	T T H A C F W V G C S R N G G R																															
1104	CCT TTC AAG GCC AAG TAC AAG CTG GTC AAC CAC ATC CCG GTC CAG																															
271	P F K A C K Y K L V N H I R V H																															
1149	ACC GGC GAG AAA CTT TTC GCC TGC CCG CAG CCG GGT TGC GGC CAG																															
286	T G E K P F A C P H P G C G K																															
1194	GTC TTC GCC CCG AGC GAG AAC CTC AAG ATC CAC AAG CCG AC																															
301	V F A R S E N L K I H K R T C																															
1239	ACA GGC GAG AAG CCC TTC AAG TGC GAG CAC GAG GGC TGT GAT CG																															
316	T G E K P F K C E H E G D R																															
1284	CGG TTC GCC AAC TCC TCG GAT CGG AAG AAG CAC TCC CAG GTC CAG																															
331	R F A N S S D R K K A H S H V																															

1329	ACG	TCG	GAC	AAG	CCC	TAC	AAT	TGC	CGG	ATT	AAT	GGC	TGT	GAT	AAG
1346	T	S	D	C	P	Y	N	C	R	I	N	G	C	D	K
1374	TCC	TAC	ACG	KAT	CCC	TCT	TCG	TCG	CGG	AGI	CAC	ATG	AGG	TGT	CAT
361	S	Y	T	H	P	S	S	L	R	K	H	M	K	V	H
1419	GGC	AAT	GTG	GAC	AGG	AGC	CCA	TCC	CAC	GGC	TAC	GAC	AGC	GAG	GAG
376	G	N	V	D	E	K	S	P	S	H	G	Y	D	S	E
1464	GGC	GAG	AGC	AGC	TCC	AGC	TCG	AGC	ATC	ATC	ACC	GGC	GGG	GCC	CAC
391	G	E	E	S	S	S	S	S	I	I	T	G	G	A	Q
1509	ACT	CCG	CCC	TCG	ACC	CGA	CTG	GAT	GGA	AGT	GCA	GGC	AGC	AGC	AGC
406	T	P	F	S	T	R	L	D	G	S	A	G	S	S	S
1554	GGG	GTG	AGC	AGC	CTG	AGC	GGC	GGA	AGC	GGC	ATT	AAG	TCC	TCC	CCG
421	G	V	S	S	L	S	G	G	S	G	G	I	K	S	P
1599	CAC	TCG	ATG	AKG	TCG	GAG	CCC	AAT	CCG	ATG	CAC	AGT	GTC	CAC	CTG
436	H	S	I	K	S	E	P	N	P	M	H	S	V	H	L
1644	GGA	GCC	TCG	AGC	AGC	GGC	AGC	AGC	AGC	AGC	GCC	AGC	AGC	AGT	GCT
451	G	A	S	S	S	G	S	S	S	T	A	S	S	S	A
1689	TCC	CAC	TTG	CTG	CAG	CAC	CAG	CAG	CAC	CAG	CAC	CAG	CAG	CAG	CAA
466	S	H	L	L	O	H	O	O	H	O	H	O	O	O	O
1734	CAA	CAG	CAG	CAG	CAC	CAG	CAG	CAG	GCA	CAG	CAG	CAG	CAA	CAG	CTG
481	O	O	O	O	H	O	O	O	A	O	O	O	O	O	L
1779	ACC	GCC	CAT	CCC	AGC	GAC	CGC	AAK	TCC	TCG	CCT	GCC	CTC	CAA	CTG
496	T	A	H	P	S	D	P	K	S	S	P	A	L	O	L
1824	ATG	CGC	GCT	TCT	GCC	TCC	GCC	TAC	CTG	CCA	CCG	CCG	CTG	GGG	CAG
511	M	A	A	S	A	S	A	Y	L	P	P	P	L	G	P
1869	CCG	CGC	TCG	CAC	CAC	CAC	CCC	ATC	CAC	CAC	CAG	GCG	GCG	CGC	AGC
526	P	P	S	H	H	H	H	P	H	H	O	A	A	P	
1914	TCT	CCG	GGG	GCA	GCT	GCT	GCC	TCC	GCC	TCC	ATG	CTG	CAC	CAC	AAC
541	S	P	G	A	A	A	A	S	A	S	M	L	H	H	N
1959	CAC	CAC	CTA	CTG	TAC	CAC	CGC	GCG	GCC	CAG	CAC	CAC	CCA	CCC	AGC
556	H	H	L	L	Y	H	P	A	A	Q	H	H	P	P	S
2004	GAC	TGG	TAC	CAC	ACG	ACG	GCG	CCG	AGC	GCG	AGT	GGG	GAG	GCG	ATG
571	D	W	Y	H	T	T	A	P	S	G					

Figure 5. Nucleotide and protein sequence of *opa*, a GLI-Kr class zinc finger protein. (A) Conceptual translation of the *opa*C cDNA. Noncoding nucleotides are in lowercase. Start and stop codons are in boldface type at nucleotides 294 and 2121, respectively. The first ATG matches the *Drosophila* consensus (Cavener 1987). Polyadenylation signal sequences are present at 2858 and 2918 nucleotides and are indicated by bold underlined type. The polyserine and polyglutamine repeats are shown in single and double underlined type, respectively. Zinc finger domains are depicted in boldface type. (B) Amino acid sequence alignment of the zinc finger domains of Opa, GLI, CiP, and Tra-1. Interfinger linker regions are underlined. Identical residues are indicated by an asterisk (*), conservative substitutions by a caret (^), 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.

B

OPA	MQCLWIDPDQGLVPPGGRKTCNKVFHSMHEIVTHLTVHHVGGEPTHTHA	aa #299
GLI	--CRW-----DGCSEFDSQEQLVHHINSEHIHG---ERKE	aa #267
CID	--CHW-----RRCRIEFITQDELVKHHNNDHIQT---NKKA	aa #483
TRA-1	--CRW-----KSCNSSPFTKLVDHVQHSESHVQSSEQEHHHA	aa #243
	* * ^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^	
OPA	--CFWVGCSRNGRPFKAQYMLVNHIRVHTGEKFPACPHPGCGKVFAENSEN	aa #307
GLI	FVCHWGCGSRELRPFFKAQYMLVVHMRRHTEGKPKHKCTFECCRKSYSRLEN	aa #317
CID	FVCRWEDCTRGEKPFKAQYMLVVHMRRHTEGKPKHKCTFECCFKAYSRLEN	aa #533
TRA-1	WRCEWEGCDNRNET-FKALYMLIVHVRRTHEGKPNKCEYPGCGKEYSRLEN	aa #292
	* * ^ ^ ^ ^ * * ^ ^ ^ ^ ^ ^ * * ^ ^ ^ *	
OPA	LKINKRTHTEGKPKFCKEHEGGCDDRFANSSDRKKH-SHVHTSDKPEYCRIN	aa #356
GLI	LKTHLRSHTEGKPYMCEHEGCSKAFSNASDRAKHQNRTHSNEKPYVCKLP	aa #367
CID	LKTHLRSHTEGKPYTCEYPGCSKAFSNASDRAKHQNRTHSNEKPYICAP	aa #583
TRA-1	LKTHRRTHTGKPYKCEFADCEKAFSNASDRAKHQNRTHSNLKPYSQCIP	aa #292
	** ^ * ^ ^ ^ ^ ^ ^ * * ^ ^ ^ ^ ^ ^ ^ * * ^ ^	
OPA	GCDKSYTHPSSLRKHKMK-VH	aa #375
GLI	GCTKRYTDPSSLRKHKVKTVH	aa #387
CID	GCTKRYTDPSSLRKHKVKTVH	aa #603
TRA-1	GCTKSYTDPSSLRKHLKAVH	aa #362
	* * ^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^	

C₂H₂ 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 com-

mon 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* Ci^D, 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)X₅LX₂HX₃₋₄H], 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, 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

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 par-

Figure 6. Developmental expression of *opa* mRNA. Lanes are as indicated. *opa* expression peaks between 2 and 12 hr after egg laying (top). Expression of a ribosomal protein gene, RP49, is used as a loading control (bottom).

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 10-cell-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.

ticular 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-

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 (NFE1/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 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-express-

ing 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

Figure 8. Pair-rule genetic interactions regulating *en* and *wg* at cellular blastoderm. Two complete parasegments are shown, with *en*-expressing cells (filled nuclei) at the anterior and *wg*-expressing cells (filled cytoplasm) at the posterior of each parasegment. *eve* is expressed in a graded manner across the odd parasegments as it retracts to the anterior-most cell of the parasegments. The anterior-most *eve*-expressing cell overlaps with one domain of *prd* expression, and *eve* and *prd* together appear to activate *en* expression (filled nuclei) in these parasegments (Morrissey et al. 1991). This *prd* domain straddles the even/odd parasegment border and activates *wg* (filled cytoplasm) in the even parasegments (Ingham et al. 1988). *opa* assists in *wg* activation here, too (thin arrow), because these *wg* stripes are weaker than normal in *opa* mutants. In the even parasegments, *ftz* is expressed in a gradient similar to *eve*. *ftz* eventually retracts to the anterior-most cell of the parasegments. Initially, *odd* is expressed in a broad domain coinciding with broad *ftz* stripes. *opa* represses *odd* in the anterior cells of the *ftz* domain ($\perp$). *odd* represses *ftz*-dependent activation of *en* ($\perp$), allowing activation of *en* in only this anterior-most cell (Manoukian and Krause 1993). *opa* may also act directly to activate *en* in this cell. *opa* activates *wg* in the odd parasegments (thick arrow) (Ingham et al., 1988). The *prd* stripes that straddle the odd/even parasegments boundary express *prd* at a lower level than the other stripes. This *prd* domain probably helps *opa* to activate *wg*, because in *prd* mutants these *wg* stripes are weaker than wild-type stripes (J.R. Mullen, unpubl.).

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 (Bejsovec and Martinez-Arias 1991). Reciprocally, *wg* expression is reinforced by a signal from adjoining *en*-ex-

pressing 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 (Letson et al. 1991), except for *Df Z-1*, 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-1*, was cytologically visible (82A; 82E_{3,4}) and failed to complement the *opa* EMS alleles. Balancer chromosomes used were TM3 *Sb* *P{pthb-lacZ}* (Driever et al. 1989), provided by M. Weir, and TM6B *Tb* *P{y⁺}*, 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,

Australia) that mapped to the centromere proximal edge of *Df* 3R(6-7). Whole phage DNA was isolated individually from each phage mapping under *Df* 3R(6-7) and digested with *Sau*3A. One hundred nanograms from each phage preparation was labeled with digoxigenin (Boehringer Mannheim Biochemical) in a reaction volume of 20 μ l according to manufacturer's instructions, except the reaction took place in a silated Eppendorf tube for 10 hr at room temperature. The reactions were stopped by heating to 65°C for 10 min, and 20 μ g of glycogen, $\frac{1}{10}$ volume of 3 M sodium acetate, and 2 volumes of 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 of 70% ethanol, dried, and resuspended in 50 μ l of hybridization buffer. Of this buffer, 4, 8, or 12 μ l was used to hybridize fixed preparations of embryos (see below). Similarly treated DNAs from a phage containing the first exon of the *en* gene and our starting phage, which mapped outside of the *opa* deficiency, were used as positive and negative controls, respectively. Thirty-eight cDNAs were obtained by screening 2×10^5 colonies of a 0- to 4-hr cDNA plasmid library obtained from Nick Brown (Brown and Kafatos 1988). Four clones were analyzed in detail [see Sequence analysis below].

Primer extension

The primer Pext (TTTCGCTCTTAGATATTTTCGCATGCGA-CTT), extending from +148 to +119 on the noncoding strand of *opaC*, was used for primer extension experiments. Primer extension was performed according to Ausubel et al. (1987). Prior to gel electrophoresis the reaction product was boiled for 3 min in sequencing buffer (80% formamide, 0.5 $\times$ 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.

Construction of transgenic HSopa line

The *opa* cDNAs were originally in pNB40 (Brown and Kafatos 1988). A 2.8-kb *Hind*III fragment from *opaC* was cloned into pKS+ (Stratagene), yielding pKS-*opad*III. The 5' *Hind*III site comes from pNB40, whereas the 3' *Hind*III site resides at position 2795 of *opaC*. pKS-*opad*III was cut partially with *Hinc*II and completely with *Xba*I. The resulting fragment was cloned into *Hpa*I/*Xba*I-cut pCaSpeR-HS (Mohler et al. 1989), to yield pCaHSopa. Transgenic lines were constructed in the standard manner, using "wings-clipped" as a helper at 150 μ g/ml (Karess and Rubin 1984). A transgenic line that mapped to chromosome II was used to establish the experimental stock: *yw*; HSopa/*HSopa*; *opa*^{5H}/TM6B P(y⁺). From the crosses carried out to establish this stock, an isolate of *opa*^{5H}/TM6B P(y⁺) in the same genetic background as the experimental stock, but without the HSopa transgene, was used as a negative control.

Rescue experiments

Injection of in vitro synthesized mRNA A 2.8-kb *Eco*RI fragment from *opaC* was subcloned into pKS+ (Stratagene), yielding pKS-*opa*RI. Both *Eco*RI sites were derived from the NB40 vector. This *Eco*RI fragment included the SP6 RNA polymerase promoter from NB40, allowing the in vitro transcription of *opa* mRNA. Ten micrograms of pKS-*opa*RI was linearized with *Xba*I and incubated at 37°C with 1 mM each rATP, rCTP, rUTP, and GpppGTP, 0.1 mM rGTP, 1.6 U/ μ l RNasin (Promega), and 40 units of SP6 RNA polymerase in a total volume of 100 μ l in

the appropriate buffer (Promega). After 1.5 hr, 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) was added, and the mixture was incubated for 20 min 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 μ l of water and injected at $\sim$ 1 mg/ml into embryos from *yw*; *opa*^{5H}/TM6B P(y⁺) parents. Embryos were collected for 30 min, and aged for 60 min at 25°C, dechorionated with bleach, and injected at $\sim$ 50% egg length under oil at 18°C (at $\sim$ 2–2.5 hr after egg laying). Injected embryos were placed at 18°C for $\sim$ 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 *yw*; HSopa/HSopa; *opa*^{5H}/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 ($\sim$ 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 $\sim$ 30 hr, 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, 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; *opa*^{7N}/TM3 P(*pthb-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, Cimborra and Sakonju have independently cloned the *opa* gene (D. Cimborra and S. Sakonju, in prep.). During preparation of this manuscript, we exchanged sequence information and Dan Cimborra 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 ASSEMBLY and SEQIN programs. The PC/GENE TRANSL 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, Ci^D, 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 subcloned 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 *Bam*HI fragment. The 5' and 3' end points of the exon within the fragment were determined by sequencing the 6-kb *Bam*HI template with primers from the cDNA sequence. Exon 1 is 1243 bp and is completely within the 6-kb *Bam*HI genomic fragment. Exons 2 and 3 lay within a 4.5-kb *Bam*HI-*Sal*I 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 *Bam*HI fragment from *opaC* in NB40 was subcloned into *Bam*HI-cut, phosphatased pAR3040, a T7 RNA polymerase expression vector (Rosenberg et al. 1987). The 3' *Bam*HI site is from NB40, whereas the 5' *Bam*HI 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).

Acknowledgments

We are grateful to the laboratory, especially to Scott Dougan and Monica Boyle, along with an anonymous reviewer for critical readings of this paper, and acknowledge the stimulating environment provided by Desplan–DiNardo laboratory interactions. We are deeply indebted to Dan Cimbora and Shigeru Sakonju for providing information prior to publication. We thank Eric Wieschaus, the Tübingen stock center, Doug Coulter, John Postlethwait, Nick Brown, Ed Grell, Michael Weir, Bill Kalionis, Rob Saint, and Henry Krause for providing various fly stocks and reagents; Simon Kidd for the Northern blot; Gregg Thomas and Jim Cleaver of the Laboratory of Radiation Biology at the University of California at San Francisco for use of the X-ray source; Kevin Moses for advice about the mutagenesis, and Louise O'Keefe. We are especially grateful to Steve Wasserman and members of his laboratory for deficiencies, γ -ray mutants, clones, and free exchange of ideas and strategies. This work began while S.D. was a postdoctoral fellow in Pat O'Farrell's laboratory at the University of California at San Francisco, and he is grateful for Pat's support, advice, and continued encouragement. M.J.B. was supported by the Lucille P. Markey Charitable Trust. J.R.M. was partially supported by the National Institutes of Health (GM-13838). S.D. is a Lucille P. Markey Scholar, and this work was supported by the Markey Charitable Trust, and the NIH (GM-45747).

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

Note added in proof

The sequence data described in this paper have been submitted to the GenBank data library under accession number U04435.

References

- Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**: 403–410.
- Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1987. *Current protocols in molecular biology*. Wiley Interscience, New York.
- Babu, P. 1977. Early developmental subdivisions of the wing disc in *Drosophila*. *Mol. Gen. Genet.* **151**: 289–294.
- Baker, N.E. 1987. Molecular cloning of sequences from *wingless* a segment polarity gene in *Drosophila*: The spatial distribution of a transcript in embryos. *EMBO J.* **6**: 1765–1773.
- Baumgartner, S. and M. Noll. 1990. Network of interactions among pair-rule genes regulating *paired* expression during primordial segmentation of *Drosophila*. *Mech. Dev.* **1**: 1–18.
- Bejsovec, A. and A. Martinez-Arias. 1991. Roles of *wingless* in patterning the larval epidermis of *Drosophila*. *Development* **113**: 471–485.
- Brown, N.H. and F.C. Kafatos. 1988. Functional cDNA libraries from *Drosophila* embryos. *J. Mol. Biol.* **203**: 425–437.
- Campos-Ortega, J.A. and V. Hartenstein. 1985. *The embryonic development of Drosophila melanogaster*. Springer-Verlag, New York.
- Carroll, S.B. and M.P. Scott. 1985. Localization of the *fushi tarazu* protein during *Drosophila* embryogenesis. *Cell* **43**: 47–57.
- . 1986. Zgotically active genes that affect the spatial expression of the *fushi tarazu* segmentation gene during early *Drosophila* embryogenesis. *Cell* **45**: 113–126.
- Carroll, S.B., A. Laughon, and B.S. Thalley. 1988. Expression

- function and regulation of the *hairy* segmentation protein in the *Drosophila* embryo. *Genes & Dev.* **2**: 883–890.
- Carroll, S.B., S. DiNardo, P.H. O'Farrell, R.A.H. White, and M.P. Scott. 1988b. Temporal and spatial relationships between segmentation and homeotic gene expression in *Drosophila* embryos: Distributions of the *fushi tarazu*, *engrailed*, *Sex combs reduced*, *Antennapedia*, and *Ultrabithorax* proteins. *Genes & Dev.* **2**: 350–360.
- Cavener, D.R. 1987. Comparison of the consensus sequence flanking translational start sites in *Drosophila* and vertebrates. *Nucleic Acids Res.* **15**: 1353–1361.
- Coulter, D.E., E.A. Swaykus, M.A. Beran-Koehn, D. Goldberg, E. Wieschaus, and P. Schedl. 1990. Molecular analysis of *odd-skipped* a zinc finger encoding segmentation gene with a novel pair-rule expression pattern. *EMBO J.* **8**: 3795–3804.
- Courey, A.J. and R. Tjian. 1988. Analysis of Sp1 *in vivo* reveals multiple transcriptional domains including a novel glutamine-rich activation motif. *Cell* **55**: 887–898.
- Courey, A.J., D.A. Holtzman, S.P. Jackson, and R. Tjian. 1989. Synergistic activation by the glutamine-rich domains of human transcription factor Sp1. *Cell* **59**: 827–836.
- DiNardo, S. and P.H. O'Farrell. 1987. Establishment and refinement of segmental pattern in the *Drosophila* embryo: Spatial control of *engrailed* expression by pair rule genes. *Genes & Dev.* **1**: 1212–1225.
- DiNardo, S., E. Sher, J. Heemskerk-Jongens, J.A. Kassis, and P.H. O'Farrell. 1988. Two-tiered regulation of spatially patterned *engrailed* gene expression during *Drosophila* embryogenesis. *Nature* **332**: 604–609.
- Dorsett, D., G.A. Viglianti, B.J. Rutledge, and M. Meselson. 1989. Alteration of *hsp82* gene expression by the gypsy transposon and suppressor genes in *Drosophila melanogaster*. *Genes & Dev.* **3**: 454–468.
- Dougan, S.T. and S. DiNardo. 1992. *wingless* generates cell type diversity among *engrailed* expressing cells. *Nature* **360**: 347–350.
- Driever, W., G. Thoma, and C. Nüsslein-Volhard. 1989. Determination of spatial domains of zygotic gene expression in the *Drosophila* embryo by the affinity of binding sites for the bicoid morphogen. *Nature* **340**: 363–367.
- Eaton, S. and T.B. Kornberg. 1990. Repression of *ci^P* in posterior compartments of *Drosophila* by *engrailed*. *Genes & Dev.* **4**: 1068–1077.
- Figdor, M.C. and C.D. Stern. 1993. Segmental organization of embryonic diencephalon. *Nature* **363**: 630–634.
- Frasch, M. and M. Levine. 1987. Complementary patterns of *even-skipped* and *fushi tarazu* expression involve their differential regulation by a common set of segmentation genes in *Drosophila*. *Genes & Dev.* **1**: 981–995.
- Frasch, M., T. Hoey, C. Rushlow, H. Doyle, and M. Levine. 1987. Characterization and localization of the *even-skipped* protein of *Drosophila*. *EMBO J.* **6**: 749–759.
- Frischauf, A.-M., H. Lehrach, A. Poustka, and N. Murray. 1983. Lambda replacement vectors carrying polylinker sequences. *J. Mol. Biol.* **170**: 827–842.
- Garcia-Bellido, A., P. Ripoll, and A.G. Morata. 1973. Developmental compartmentalization of the wing disc of *Drosophila*. *Nature New Biol.* **245**: 251–253.
- . 1976. Developmental compartmentalization of the dorsal mesothoracic disc of *Drosophila*. *Dev. Biol.* **48**: 132–147.
- Gergen, J.P. and A.B.A. Butler. 1988. Isolation of the *Drosophila* segmentation gene *runt* and analysis of its expression during embryogenesis. *Genes & Dev.* **2**: 1179–1193.
- Goto, T., P. Macdonald, and A.T. Maniatis. 1989. Early and late periodic patterns of *even-skipped* expression are controlled by distinct regulatory elements that respond to different spatial cues. *Cell* **57**: 413–422.
- Grossniklaus, U., R.H. Pearson, and W.J. Gehring. 1992. The *Drosophila sloppy-paired* locus encodes two proteins involved in segmentation that show homology to mammalian transcription factors. *Genes & Dev.* **6**: 1030–1051.
- Hafen, E., A. Kuroiwa, and A.W.J. Gehring. 1984. Spatial distribution of transcripts from the segmentation gene *fushi tarazu* during *Drosophila* embryonic development. *Cell* **37**: 833–841.
- Howard, K. and A.P. Ingham. 1986. Regulatory interactions between the segmentation genes *fushi tarazu*, *hairy* and *engrailed* in the *Drosophila* blastoderm. *Cell* **44**: 949–957.
- Ingham, P.W. 1988. The molecular genetics of embryonic pattern formation in *Drosophila*. *Nature* **335**: 25–34.
- . 1991. Segment polarity genes and cell patterning within the *Drosophila* body segment. *Curr. Opin. Genet. Dev.* **1**: 261–267.
- Ingham, P.W. and A.A. Martinez-Arias. 1986. The correct activation of *Antennapedia* and *bithorax complex* genes requires the *fushi tarazu* gene. *Nature* **324**: 592–597.
- Ingham, P.W., K.R. Howard, and D. Ish-Horowicz. 1985. Transcription pattern of the *Drosophila* segmentation gene *hairy*. *Nature* **318**: 439–445.
- Ingham, P.W., N.E. Baker, and A. Martinez-Arias. 1988. Regulation of segment polarity genes in the *Drosophila* blastoderm by *fushi tarazu* and *even-skipped*. *Nature* **331**: 73–75.
- Jürgens, G., E. Wieschaus, C. Nüsslein-Volhard, and A.M. Kluding. 1984. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. II. Zygotic loci on the third chromosome. *Wilhelm Roux's Arch. Dev. Biol.* **193**: 283–295.
- Kakkis, E., K.J. Riggs, W. Gillespie, and K. Calame. 1989. A transcriptional repressor of *c-myc*. *Nature* **339**: 718–721.
- Kania, M.A., A.S. Bonner, J.B. Duffy, and A.J.P. Gergen. 1990. The *Drosophila* segmentation gene *runt* encodes a novel nuclear regulatory protein that is also expressed in the developing nervous system. *Genes & Dev.* **4**: 1701–1713.
- Karens, R.E. and G.M. Rubin. 1984. Analysis of P transposable element functions in *Drosophila*. *Cell* **38**: 135–146.
- Kellerman, K.A., D.M. Mattson, and A.I. Duncan. 1990. Mutations affecting the stability of the *fushi tarazu* protein of *Drosophila*. *Genes & Dev.* **4**: 1936–1950.
- Kilchherr, F., S. Baumgartner, D. Bopp, E. Frei, and A.M. Noll. 1986. Isolation of the *paired* gene of *Drosophila* and its spatial expression during early embryogenesis. *Nature* **321**: 493–499.
- Kornberg, T. 1981. *Engrailed*: A gene controlling compartment and segment formation in *Drosophila*. *Proc. Natl. Acad. Sci.* **78**: 1095–1099.
- Kornberg, T., I. Siden, P. O'Farrell, and A.M. Simon. 1985. The *engrailed* locus of *Drosophila*: *In situ* localization of transcripts reveals compartment-specific expression. *Cell* **40**: 45–53.
- Krause, H.M., R. Klemenz, and A.W.J. Gehring. 1988. Expression modification and localization of the *Fushi Tarazu* protein in *Drosophila* embryos. *Genes & Dev.* **2**: 1021–1036.
- Lawrence, P.A., P. Johnston, P. Macdonald, and G. Struhl. 1987. Borders of parasegments in *Drosophila* embryos are delimited by the *fushi tarazu* and *even-skipped* genes. *Nature* **328**: 440–442.
- Letsou, A., S. Alexander, K. Orth, and S.A. Wasserman. 1991. Genetic and molecular characterization of *tube*, a *Drosophila* gene maternally required for embryonic dorsoventral polarity. *Proc. Natl. Acad. Sci.* **88**: 810–814.
- Lewis, E. 1978. A gene complex controlling segmentation in *Drosophila*. *Nature* **276**: 565–570.

- Licht, J.D., M.J. Gossel, J. Figge, and U.M. Hansen. 1990. *Drosophila* Kruppel protein is a transcriptional repressor. *Nature* **346**: 76–79.
- Lumsden, A. and R. Keynes. 1989. Segmental patterns of neuronal development in the chick hindbrain. *Nature* **337**: 424–428.
- MacDonald, P.M., P. Ingham, and G. Struhl. 1986. Isolation, structure and expression of *even-skipped*: A second pair-rule gene of *Drosophila* containing a homeobox. *Cell* **47**: 721–734.
- Manoukian, A.S. and H.M. Krause. 1992. Concentration-dependent activities of the *even-skipped* protein in *Drosophila* embryos. *Genes & Dev.* **6**: 1740–1751.
- . 1993. Control of segmental asymmetry in *Drosophila* embryos. *Development* **118**: 785–796.
- Martinez-Arias, A. and P.A. Lawrence. 1985. Parasegments and compartments in the *Drosophila* embryo. *Nature* **313**: 639–642.
- Martinez-Arias, A., N.E. Baker, and P.W. Ingham. 1988. Role of segment polarity genes in the definition and maintenance of cell states in the *Drosophila* embryo. *Development* **103**: 157–170.
- Meer, S.V.D. 1977. Optical clean and permanent whole mount preparation for phase contrast microscopy of cuticular structures of insect larvae. *Dros. Inf. Serv.* **52**: 160–161.
- Mohler, J., E.D. Eldon, and V. Pirrotta. 1989. A novel spatial transcription pattern associated with the segmentation gene *giant* of *Drosophila*. *EMBO J.* **8**: 1539–1548.
- Morata, G. and P.A. Lawrence. 1975. Control of compartment development by the *engrailed* gene in *Drosophila*. *Nature* **255**: 608–617.
- Morrissey, D., D. Askew, L. Raj, and M. Weir. 1991. Functional dissection of the *paired* segmentation gene in *Drosophila* embryos. *Genes & Dev.* **5**: 1684–1696.
- Noordermeer, J., P. Johnston, F. Rijsewicz, R. Nusse, and P.A. Lawrence. 1992. The consequences of ubiquitous expression of the *wingless* gene in the *Drosophila* embryo. *Development* **116**: 711–719.
- Nüsslein-Volhard, C. and E. Wieschaus. 1980. Mutations affecting segment number and polarity in *Drosophila*. *Nature* **287**: 795–801.
- Nüsslein-Volhard, C., H. Kluding, G. Jürgens. 1985. Genes affecting the segmental subdivision of the *Drosophila* embryo. *Cold Spring Harbor Symp. Quant. Biol.* **50**: 145–154.
- Orenic, T.V., D.C. Shusarski, K.L. Kroll, and R.A. Holmgren. 1990. Cloning and characterization of the segment polarity gene *cubitus interruptus* Dominant of *Drosophila*. *Genes & Dev.* **4**: 1053–1067.
- Pankratz, M.J., E. Seifert, N. Gerwin, B. Billi, U. Nauber, and H. Jäckle. 1990. Gradients of *krüppel* and *knirps* gene products direct pair-rule gene stripe patterning in the posterior region of the *Drosophila* embryo. *Cell* **61**: 309–317.
- Park, K. and M.L. Atchison. 1991. Isolation of a candidate repressor/activator, NF-E1 (*yy-1*, *delta*), that binds to the immunoglobulin kappa 3' enhancer and the immunoglobulin heavy chain muE1 site. *Proc. Natl. Acad. Sci.* **88**: 9804–9808.
- Perrimon, N. and A.P. Mahowald. 1986. *l(1)hopscotch*, a larval-pupal zygotic lethal with a specific maternal effect on segmentation in *Drosophila*. *Dev. Biol.* **118**: 28–41.
- Poole, S.J. and T.B. Kornberg. 1988. Modifying expression of the *engrailed* gene of *Drosophila melanogaster*. *Development (Suppl.)* **104**: 85–93.
- Preiss, A., U.B. Rosenberg, A. Kienlin, E. Seifert, H. Jäckle. 1985. Molecular genetics of *Kruppel* a gene required for segmentation of the *Drosophila* embryo. *Nature* **313**: 27–32.
- Rauskolb, C., M. Peifer, and E. Wieschaus. 1993. *extradenticle*, a regulator of homeotic gene activity, is a homolog of the homeobox-containing human proto-oncogene *pbx1*. *Cell* **74**: 1101–1112.
- Rosenberg, A.H., B.N. Lade, D. Chui, S. Lin, J.J. Dunn, and F.W. Studier. 1987. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* **56**: 125–135.
- Ruppert, J.M., B. Vogelstein, K. Arheden, and K.W. Kinzler. 1990. *GLI3* encodes a 190-kilodalton protein with multiple regions of GLI similarity. *Mol. Cell. Biol.* **10**: 5408–5415.
- Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* **74**: 5463–5467.
- Sauer, F. and H. Jäckle. 1991. Concentration-dependent transcriptional activation or repression by *Krüppel* from a single binding site. *Nature* **353**: 563–566.
- Shi, Y., E. Seto, L.-S. Chang, and T. Shenk. 1991. Transcriptional repression by YY-1, a human GLI-Kruppel-related protein, and relief of repression by adenovirus E1A protein. *Cell* **67**: 377–388.
- Stanojevic, D., T. Hoey, and M. Levine. 1989. Sequence-specific DNA-binding activities of the gap proteins encoded by *hunchback* and *Kruppel* in *Drosophila*. *Nature* **341**: 331–335.
- Zarkower, D. and J. Hodgkin. 1992. Molecular analysis of the *C. elegans* sex determining gene *tra-1*: A gene encoding two zinc finger proteins. *Cell* **70**: 237–249.
- Zuo, P., D. Stanojevic, J. Colgan, K. Han, M. Levine, and J.L. Manley. 1991. Activation and repression of transcription by the gap proteins *hunchback* and *Krüppel* in cultured *Drosophila* cells. *Genes & Dev.* **5**: 254–264.

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

M J Benedyk, J R Mullen and S DiNardo

Genes Dev. 1994, **8**:

Access the most recent version at doi:[10.1101/gad.8.1.105](https://doi.org/10.1101/gad.8.1.105)

References

This article cites 77 articles, 25 of which can be accessed free at:
<http://genesdev.cshlp.org/content/8/1/105.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).