orb is required for anteroposterior and dorsoventral patterning during *Drosophila* oogenesis

Lori B. Christerson and Dennis M. McKearin

Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9038 USA

We describe mutations in the *orb* gene, identified previously as an ovarian-specific member of a large family of RNA-binding proteins. Strong *orb* alleles arrest oogenesis prior to egg chamber formation, an early step of oogenesis, whereas females mutant for a maternal-effect lethal *orb* allele lay eggs with ventralized eggshell structures. Embryos that develop within these mutant eggs display posterior patterning defects and abnormal dorsoventral axis formation. Consistent with such embryonic phenotypes, *orb* is required for the asymmetric distribution of *oskar* and *gurken* mRNAs within the oocyte during the later stages of oogenesis. In addition, double heterozygous combinations of *orb* and *grk* or *orb* and *top/DER* alleles reveal that mutations in these genes interact genetically, suggesting that they participate in a common pathway. Orb protein, which is localized within the oocyte in wild-type females, is distributed ubiquitously in stage 8–10 *orb* mutant oocytes. These data will be discussed in the context of a model proposing that Orb is a component of the cellular machinery that delivers mRNA molecules to specific locations within the oocyte and that this function contributes to both D/V and A/P axis specification during oogenesis.

[Key Words: RNA binding; oogenesis; dorsoventral; posterior group; polarity; RNA localization]

Received October 5, 1993; accepted in revised form January 20, 1994.

One of the earliest events in embryonic development is the establishment of anteroposterior (A/P) and dorsoventral (D/V) axes, patterning processes that are particularly well characterized in *Drosophila* (for review, see St Johnston and Nüsslein-Volhard 1992). The *Drosophila* embryo organizes its body plan by using maternally inherited positional information. The organization of the anterior and posterior patterns in the embryo is dependent on the asymmetric distribution of specific mRNAs and proteins within the egg during oogenesis (for review, see Lasko 1992; Lehmann 1992; St Johnston and Nüsslein-Volhard 1992). Transcripts for the anterior determinant *bicoid* (*bcd*) are localized in the oocyte cytoplasm at the anterior pole of the egg and are translated after fertilization to produce an anterior-to-posterior concentration gradient of Bicoid protein (Frigerio et al. 1986; Berleth et al. 1988; Driever and Nüsslein-Volhard 1988; St Johnston et al. 1989). Mutations that disrupt *bcd* mRNA localization result in distortion of pattern elements along the A/P axis (Schüpbach and Wieschaus 1986; Frohnhofer and Nüsslein-Volhard 1987; Stephenson and Mahowald 1987). In the posterior patterning system, transcripts for the posterior group gene *oskar* (*osk*) are among the first that must be localized to the oocyte posterior pole for assembly of a specialized cytoplasm, the polar plasm (Lehmann and Nüsslein-Volhard 1986; Ephrussi et al. 1991; Kim-Ha et al. 1991). The polar plasm contains two localized signals: the posterior de-

terminant *nanos*, which is required for normal abdominal segmentation in the embryo (Wang and Lehmann 1991; Gavis and Lehmann 1992), and a second signal that directs the formation of the posterior pole cells, the germ cell precursors (Ephrussi and Lehmann 1992).

The D/V pattern in the embryo is thought to form largely independently of the A/P pattern (St Johnston and Nüsslein-Volhard 1992) and probably is determined by positional information present in the vitelline membrane of the egg (Stein et al. 1991; Stein and Nüsslein-Volhard 1992). The vitelline membrane, along with the eggshell, is secreted during oogenesis by the follicle cells that surround the oocyte (Mahowald and Kambyzellis 1980). The determination of follicle cell states along the D/V axis requires signaling between the oocyte and follicle cells (Manseau and Schüpbach 1989; Schüpbach et al. 1991). Distribution of the signal to a limited number of follicle cells produces a distinctly patterned dorsal eggshell surface. Signal production may be associated with the oocyte nucleus, because only the follicle cells overlying the oocyte nucleus adopt dorsal fates and laser ablation of the oocyte nucleus blocks any follicle cells from becoming dorsal (Montell et al. 1991).

Genetic analysis of maternal-effect mutations has identified a number of genes required to establish the D/V axis of both the eggshell and embryo. Females mutant for the genes *fs(1)K10* and *squid* (*sqd*) produce eggshells and embryos that are dorsalized and deficient in

ventral structures (Wieschaus et al. 1978; Kelley 1993). Since this phenotype suggests that too many follicle cells receive a dorsalizing signal, these genes may act to restrict the expression of the signal temporally and/or spatially. The opposite phenotype is produced by *gurken* (*grk*), *torpedo* (*top/DER*), *cornichon* (*cni*), *rhomboid* (*rho*), and *brainiac* (*brn*) mutations, which cause cells that normally develop as dorsal to adopt a ventral fate on the eggshell (Schüpbach 1987; Ashburner et al. 1990; Schüpbach et al. 1991; Goode et al. 1992; Ruohola-Baker et al. 1993). The embryos within *grk*, *top/DER*, *cni*, and *rho* eggs are ventralized as well. *top/DER* and *rho* activities are required in the follicle cells during oogenesis for dorsal follicle cell determination (Schüpbach 1987; Ruohola-Baker et al. 1993), whereas *grk*, *cni*, and *brn* activities are required in the germ line for proper dorsal follicle cell fate (Schüpbach 1987; Schüpbach et al. 1991; Goode et al. 1992). *top/DER* is an oogenesis-specific allele of the *Drosophila* epidermal growth factor receptor (DER; Schejter and Shilo 1989; Price et al. 1989) and is hypothesized to be a receptor for the dorsalizing signal (Schüpbach 1987; Price et al. 1989). Recently, Neuman-Silberberg and Schüpbach (1993) have determined that *grk* encodes a transforming growth factor- α (TGF- α)-like protein and have shown that *grk* transcripts are asymmetrically localized to the dorsal corner of the oocyte. They have postulated that the dorsal localization of *grk* mRNA may result in a spatially restricted ligand that asymmetrically activates the *top/DER* receptor.

Mutations in *cappuccino* (*capu*) and *spire* (*spir*) produce both ventralized and dorsalized eggshells and embryos (Manseau and Schüpbach 1989; Neuman-Silberberg and Schüpbach 1993; L. Manseau, pers. comm.) and also block abdominal segmentation in the embryo (Manseau and Schüpbach 1989). Thus, these genes are required for the formation of both the A/P and D/V axes (Manseau and Schüpbach 1989), suggesting that a common process may be involved in the establishment of both polarities. In this report we describe a third gene, *orb*, that is necessary for proper A/P and D/V patterning in the oocyte and embryo. We find that *orb* is required for the formation of the dorsal surfaces of the eggshell and interacts genetically with *grk* and *top/DER*. We also show that mutation of the *orb* locus results in both expansion of ventral fates and posterior patterning defects in the embryo. In addition, we describe a strong *orb* mutation that disrupts oogenesis by arresting cyst development prior to egg chamber formation.

The *orb* gene product contains domains that match the consensus sequences of the RNA recognition motif (RRM) family of RNA-binding proteins (Lantz et al. 1992). Members of this family bind a wide variety of RNA molecules (Kenan et al. 1991; Kim and Baker 1993). We show that *orb* is required for the distribution of *grk* and *osk* mRNAs within the oocyte during the later stages of oogenesis. These results suggest that the RRM sequence similarity may be of functional significance. On the basis of these data, we postulate that Orb is a component of cellular machinery in the egg chamber

that asymmetrically distributes mRNA molecules to specific regions of the oocyte.

Results

Identification of a mutation that arrests cyst development

Each *Drosophila* ovary is composed of ~15–20 ovarioles, each containing egg chambers at different stages of development (for review, see King 1970; Mahowald and Kambyzellis 1980; Spradling 1993b). At the anterior end of each ovariole is a region called the germarium (Fig. 1A). At the onset of oogenesis, cystoblasts at the anterior end of the germarium undergo four mitotic divisions with incomplete cytokinesis, producing syncytial clusters (cysts; Spradling 1993a) of 16 cystocytes interconnected by cytoplasmic bridges (ring canals; Brown and King 1964; Xue and Cooley 1993). The presumptive oocyte resides at the posterior end of the cluster, whereas the more anterior cells develop as nurse cells and synthesize products that will be transported into the oocyte. Somatic cells derived from follicle cells surround the cyst in a uniform monolayer to form the egg chamber.

The *orb^{dec}* allele was identified in a collection of enhancer-trap female sterile mutants and contains a transposon insertion (referred to as *P[lacZ-ry⁺]/94E*) in 94E on the right arm of the third chromosome (see Table 1A for *orb* alleles used in this study). Ovaries of *orb^{dec}* females are tiny, perhaps 1–5% of wild-type volume. Each *orb^{dec}* ovariole consists of a germarium-like region and occasionally one or two egg chambers. These egg chambers are often empty, indicating that the germ cells have degenerated. Comparing *orb^{dec}* germaria to wild type using differential interference contrast (DIC) microscopy (Fig. 1B,C), we found that cystocytes at the anterior end (region 1) appear normal. These cells are expected to be two-, four-, and eight-cell clusters that arise from the mitotic divisions of the cystoblasts. However, beginning in the germarial mid-region when cystocytes have completed their divisions, cellular morphology begins to appear aberrant. In this region, blebs appear on the surfaces of the *orb^{dec}* cystocytes (Fig. 1C), and these cells do not grow in volume or undergo the extensive genomic polyploidization that characterizes their wild-type counterparts (data not shown). By the time that follicle cells have surrounded the germ-line cells to form the egg chamber, the cystocytes appear to have partially or completely degenerated.

The disruption of cystocyte differentiation in *orb^{dec}* ovaries can be seen more clearly by using rhodamine-labeled phalloidin to stain F-actin in the cortical cytoskeleton of cystocytes and follicle cells as well as the ring canals in the cystocytes. Figure 1, D and E, shows confocal images of rhodamine-phalloidin-stained wild-type and mutant germaria, respectively. Consistent with the data obtained from visible light microscopy (Fig. 1, B and C), the cells in the anterior region of mutant germaria appeared similar to those seen in wild type, whereas

Figure 1. The *orb^{dec}* transposon insertion produces arrest of early oogenesis. (A) Schematic diagram of the *Drosophila* germarium (adapted from Mahowald and Strasshiem 1970). At the anterior end of the germarium (left), stem cell daughters (cystoblasts) divide mitotically with incomplete cytokinesis to produce 2-, 4-, 8-, and 16-cell syncytial clusters. Germarial region 1 contains the stem cells and the mitotic cystoblasts. Germarial region 2a contains the newly formed 16-cell clusters. Follicle cells surround the 16-cell clusters to produce lens-shaped egg chambers in region 2b. Egg chambers, composed of cystocytes (c) and follicle cells (fc) assume a spherical shape in germarial region 3. (B) Wild-type ovariole. Germarial regions 1–3 are indicated. A more mature egg chamber is present at the posterior end (right) of the germarium. Bar, 10 μ m (applies to B and C). (C) *orb^{dec}* ovariole. Cells at the anterior end (approximately region 1) appear normal, followed by cellular blebs (arrows) in the mid-region that are presumed to be evidence of degenerating cells and cellular debris. The disruption of germarial morphology that is caused by the mutation allows only an approximate designation of the germarial regions. (D) Confocal image of a wild-type ovariole stained with rhodamine-phalloidin. Large germ-line cells are outlined by the reagent in the anterior half of the germarium. Immediately posterior to the midpoint (arrow at top) and spanning the width of the germarium can be a lens-shaped cluster of cystocytes being surrounded by migrating follicle cells. Two more mature egg chambers are visible in this micrograph; one in region 3 and a larger, more spherical chamber that has been released from the germarium. Note also the easily detectable ring canals (rc) in egg chambers at the lens-shaped stage and beyond. Bar, 10 μ m (applies to D and E). (E) *orb^{dec}* ovariole stained with rhodamine-phalloidin. The cortical pattern of F-actin in cells at the anterior end of the germarium appears similar to wild type. Cyst assembly appears aberrant in the mid-region and completed cysts appear devoid of cystocytes (see text). The open arrow marks a cyst containing a ring canal but apparently without cystocytes. Note that D and E do not represent confocal images of B and C. (F) Schematic representation of the 5' portion of the *orb* gene, indicating boundaries between exons (open boxes) and introns (solid lines). The *orb^{dec}* mutagenic transposon insertion site (between nucleotides +499 and +500) in exon 2 is indicated. PCR and sequence analysis of the *orb* gene has identified a previously unrecognized intron at nucleotide position +301 (Lantz et al. 1992; see Materials and methods).

the mid-region of mutant germaria contained only poorly formed cystocyte clusters and little evidence of enclosure by follicle cells. This region corresponds to the area of cellular blebbing seen in the DIC micrographs such as Figure 1C. When enclosed clusters were detect-

able, the central cavity, which should be occupied by cystocytes, appeared empty. Often, ring canals appeared to be free floating and not embedded in membrane (Fig. 1E, e.g., see cyst marked by open arrow). From these observations, we conclude that the *orb^{dec}* mutation dis-

Table 1. Mutations used in this study

A. orb mutations		
Allele	Phenotype	Source/reference
<i>orb^{dec}</i>	degenerating cysts	A.C. Spradling; this work
<i>orb^{F343}</i>	degenerating cysts	Lantz et al. (1994)
<i>orb^{mel}</i>	maternal-effect lethal	this work
<i>Df(3R)MSu244</i>	strong Minute and recessive lethal	Reuter et al. (1986)
B. Mutations used to test genetic interactions		
Gene	Phenotype of eggshells and embryos	Source/reference
<i>grk^{HK36}</i>	intermediate and strong ventralization	Schüpbach (1987)
<i>grk^{HC21}</i>	weak and intermediate ventralization	Schüpbach (1987)
<i>top^{CO}</i>	embryonic lethality	Clifford and Schüpbach (1989); Price et al. (1989)
<i>top¹</i>	weak and intermediate ventralization	Schüpbach (1987)
<i>cni^{AA12}</i>	strong ventralization	Ashburner et al. (1990)
<i>fs(1)K10^{LM00}</i>	strong dorsalization	T. Schüpbach

rupts cyst formation near the time that the 16-cell cluster is formed and causes cystocytes to degenerate.

Rhodamine-phalloidin staining of *orb^{dec}* ovaries revealed that follicle cell migrations (to surround the cystocyte clusters) appeared retarded, raising the possibility that *orb⁺* activity might be required in the follicle cells. However, molecular evidence supports a role that is restricted to the germ-line cells. In females that are heterozygous for the *orb^{dec}* allele, LacZ expression (from the mutagenic *P[lacZ-ry⁺]*94E transposon) is detected only in the germ-line cells of the egg chamber throughout oogenesis (data not shown). In addition, the absence of the *orb* transcript in germ-line-less flies (Lantz et al. 1992) and the restriction of *orb* mRNA (Lantz et al. 1992) and protein expression (Lantz et al. 1994; this paper) to the germ line strongly suggests that *orb⁺* activity is required only in germ-line cells. Therefore, we suspect that egg chamber formation is retarded in *orb^{dec}* ovaries because clusters of degenerating cystocytes are poor substrates for follicle cell migrations.

We confirmed that *P[lacZ-ry⁺]*94E was the cause of the mutant phenotype by two methods. First, a chromosome deficient for the 94E region, *Df(3R)MSu244* (Table 1A), failed to complement the *orb^{dec}* phenotype. Second, precise excision of the transposon resulted in simultaneous reversion of the mutant phenotype to wild type. *orb^{dec}* was judged to be a strong loss-of-function allele because *orb^{dec}/Df(3R)MSu244* flies displayed the same phenotype as *orb^{dec}* homozygotes.

Molecular analysis of the *orb^{dec}* allele

The *orb* locus was cloned from the *orb^{dec}* allele using transposon sequences to recover genomic DNA flanking *P[lacZ-ry⁺]*94E. By comparing the *orb* cDNA sequence (Lantz et al. 1992) with that of the *orb^{dec}* allele, we determined that *P[lacZ-ry⁺]*94E was located in the 5'-untranslated region (UTR) of the female *orb* transcript between positions +499 nucleotides and +500 nucleotides (Fig. 1F). Consistent with genetic results, *orb^{dec}*

behaves as a strong loss-of-function allele, since Northern analysis and in situ hybridization to ovaries revealed that homozygous *orb^{dec}* females produced little or no *orb* mRNA (data not shown), and immunoblotting showed that Orb protein was undetectable in *orb^{dec}* ovaries (Lantz et al., this issue).

Generation of a weak *orb* allele, *orb^{mel}*

Transposase-induced mobilization (Cooley et al. 1988) was used to produce additional *orb* alleles from the *orb^{dec}* allele. Most of these produced phenotypes that were similar to the *orb^{dec}* phenotype or slightly weaker, like that described by Lantz et al. (1994) for the *orb^{F303}* allele. However one allele, *orb^{mel}*, which produces a weaker phenotype than *orb^{dec}*, was particularly informative with regard to the function of *orb* and is the focus of the remainder of this paper.

orb^{mel} fails to complement strong *orb* alleles, producing ovarian morphologies that are intermediate in phenotype between the strong phenotype (see *orb^{dec}*) and a weak maternal-effect phenotype (see below). Therefore, we conclude that *orb^{mel}* acts as a recessive partial loss-of-function allele.

Southern blot analysis of *orb^{mel}* showed that the excision event removed the entire transposon as well as ~500 bp of *orb* genomic DNA. We sequenced the *orb^{mel}* 5' UTR and determined that the 5' breakpoint of the deletion was located at the precise site of transposon insertion in the *orb^{dec}* allele and that the deletion extended into intron 2, removing the 5' splice donor site (Fig. 2A). Comparison of cDNA sequence data from *orb^{mel}* mutant flies to the wild-type *orb* cDNA sequence (Lantz et al. 1992) showed that the *orb^{mel}* deletion resulted in a complete loss of exon 2 (+302 to +800 nucleotides) from the *orb^{mel}* mRNA; thus, exon 1 is spliced directly to exon 3. Such exon skipping is consistent with the exon definition model for RNA splicing proposed by Berget and colleagues, in which the intact exon (with its 3' and 5' splice sites) is the unit of assembly for splicing factors (Robberson et al. 1990; Talerico and Berget 1990).

Figure 2. Molecular analysis of the *orb^{mel}* allele. (A) Schematic representation of 5' portion of the *orb* gene. The *orb^{mel}* deletion (solid rectangle) removes 300 bp of 5' UTR in exon 2 and ~200 bp of adjacent intron sequence. This deletion interferes with normal splicing (broken lines) of *orb* transcript, producing *orb^{mel}* mRNA that is missing exon 2 altogether (*orb^{mel}* splicing is depicted by solid lines). Open boxes represent exons. (B) Northern blot analysis of *orb^{mel}* mRNA. The autoradiograph shows that *orb^{mel}* transcript migrates at a position ~500 nucleotides shorter than wild-type (WT) *orb* transcript. Both lanes contain ~2 μ g of ovarian poly(A)⁺ RNA hybridized with a portion of the *orb* cDNA. Hybridization to *Adh* (Goldberg 1980) is shown as a control for RNA loading.

Northern analysis revealed that the transcript produced by *orb^{mel}* is smaller than wild-type *orb* mRNA by ~500 nucleotides, as predicted by the sequence data, and is present at approximately wild-type levels (Fig. 2B).

orb^{mel} eggs display D/V defects

In females that are homozygous for the *orb^{mel}* mutation, the morphology of structures on the dorsal side of the eggshell is aberrant. The most striking aspect of these defects is the fusion or absence of the two respiratory appendages, called dorsal appendages, that normally

arise from the dorsolateral anterior surface of the egg (Fig. 3). Similar eggshell defects have been described for *grk*, *top/DER*, *cni*, *rho*, and *brn* mutations, which block dorsal follicle cell fate determination and produce eggshells referred to as ventralized (Schüpbach 1987; Ashburner et al. 1990; Schüpbach et al. 1991; Goode et al. 1992; Ruohola-Baker et al. 1993). As shown in Table 2, 23% of the eggs produced by *orb^{mel}/orb^{mel}* females displayed fused dorsal appendages and 6% lacked both dorsal appendages. These effects were enhanced when the amount of *orb* gene product was reduced by placing *orb^{mel}* in *trans* to *orb^{dec}* [data not shown]. However, because *orb^{mel}* was derived from the *orb^{dec}* chromosome, we were concerned that noncomplementing background mutations might contribute to these effects. Therefore, in the following experiments we have used a different strong loss-of-function allele, *orb^{F343}*, an ethylmethane sulfonate-induced mutation identified as an *orb* allele by its failure to complement *orb^{dec}* (Lantz et al., this issue).

Figure 3. *orb^{mel}* eggshells display a reduction of dorsal structures. (A) Wild-type eggshell. Two respiratory appendages extend from the dorsolateral anterior surface. Anterior is left. Bar, 100 μ m (applies to all panels). (B,C) *orb^{mel}* eggshells. Dorsal appendage material is fused and originates at the dorsal midline (B) or is missing (C).

Table 2. Maternal-effect lethal eggshell phenotypes of *orb^{mel}* females

Genotype	Number ^a	Percentage		
		fused dorsal appendages	lacking dorsal appendages	two dorsal appendages
Controls				
<i>orb^{F343}/TM3</i>	237	<1	0	>99
<i>orb^{mel}/TM3</i>	263	<1	<1	>99
<i>orb</i> mutants				
<i>orb^{mel}/orb^{mel}</i>	556	23	6	71
<i>orb^{mel}/orb^{F343}</i>	1437	21	78	<1

Fused dorsal appendage phenotype ranges from fusion at base of appendages to fusion along entire length of appendage.

^aNumber of eggs scored.

Of the eggs produced by *orb^{mel}/orb^{F343}* females, 21% displayed fused dorsal appendages and 78% were missing both dorsal appendages (Table 2). These data indicate that dorsal appendage formation is sensitive to the dose of *orb⁺* activity.

A significant fraction of *orb^{mel}/orb^{F343}* eggs are approximately one-half to three-fourths of the normal egg length (data not shown). Consistent with this observation, we frequently see smaller-than-normal late-stage *orb^{mel}/orb^{F343}* oocytes, the apparent result of the incomplete transfer of nurse cell contents into the oocyte; this phenotype is similar to that described for *kelch* and *chickadee* mutants (Schüpbach and Wieschaus 1991; Cooley et al. 1992; Xue and Cooley 1993). In addition, we have observed that the follicle cells surrounding these smaller-than-normal *orb^{mel}/orb^{F343}* oocytes appear more columnar in shape than wild type (e.g., see Fig. 6D, below). Although the cause of this altered follicle cell morphology is presently unknown, we suspect that it may be a consequence of the reduced surface area that these smaller oocytes provide.

orb interacts genetically with *grk* and *top/DER*

Because eggshells of *orb^{mel}* females showed defects similar in many respects to those produced by *grk*, *top/DER*, and *cni* mutations, we tested for genetic synergism between these mutations and *orb* by constructing double heterozygous females (Table 3). The alleles used for these genetic complementation tests are described in Table 1B. Examination of eggs produced by *grk^{HK36}/+* females showed that dorsal follicle cell determination was sensitive to the dose of *grk* alone, as a reproducible percentage of eggshells carried fused dorsal appendages (Table 3). Evidence for this incompletely penetrant dominant effect of *grk* mutations has been obtained independently (Forlani et al. 1993; T. Schüpbach, pers. comm.). Remarkably, 82% of the eggs laid by females doubly heterozygous for *orb^{F343}* and *grk^{HK36}* carried fused dorsal appendages, whereas 69% of the eggs laid by females doubly heterozygous for *orb^{F343}* and a weaker *grk* allele, *grk^{HG21}*, carried fused dorsal appendages. Similar results were obtained with double heterozygous combinations of *orb^{dec}* and *grk* alleles. Thus, mutations in *orb* act as

dominant enhancers of the partially dominant effects of *grk* alleles.

We also tested for genetic synergism with *top/DER* alleles; these mutations behave as recessive loss-of-function alleles (Schüpbach 1987; Clifford and Schüpbach 1989). *orb^{F343}* fully complemented *top¹*, a weak allele; eggshells with single dorsal appendages occurred at background levels (<1%) in *top¹/+*; *orb^{F343}/+* ovaries. However, the *orb^{F343}* allele displayed dramatic second-site noncomplementation of *top^{CO}*, a strong allele, such that 71% of eggs laid by *top^{CO}/+*; *orb^{F343}/+* females carried single dorsal appendages (Table 3). Similar effects on dorsal appendage formation were observed using *orb^{dec}* in place of *orb^{F343}*, although the percentage of eggshells with fused dorsal appendages was less than that pro-

Table 3. Percentages of fused dorsal appendages produced by double heterozygotes

Genotype	Number ^a	Fused dorsal appendages (%)
Controls		
<i>orb^{F343}/TM3</i>	237	<1
<i>orb^{dec}/TM3</i>	414	<1
<i>grk^{HK36}/+</i> ; <i>TM3/+</i>	265	13
<i>grk^{HG21}/+</i> ; <i>TM3/+</i>	414	2
<i>top^{CO}/+</i> ; <i>TM3/+</i>	144	1
<i>cni^{AA12}/+</i> ; <i>TM3/+</i>	320	0
<i>K10^{LM00}/+</i> ; <i>TM3/+</i>	167	0
Double heterozygotes		
<i>grk^{HK36}/+</i> ; <i>orb^{F343}/+</i>	262	82
<i>grk^{HK36}/+</i> ; <i>orb^{dec}/+</i>	230	57
<i>grk^{HG21}/+</i> ; <i>orb^{F343}/+</i>	240	69
<i>grk^{HG21}/+</i> ; <i>orb^{dec}/+</i>	283	11
<i>top^{CO}/+</i> ; <i>orb^{F343}/+</i>	218	71
<i>top^{CO}/+</i> ; <i>orb^{dec}/+</i>	227	6
<i>cni^{AA12}/+</i> ; <i>orb^{F343}/+</i>	340	2
<i>K10^{LM00}/+</i> ; <i>orb^{F343}/+</i>	280	6
<i>grk^{HK36}/+</i> ; <i>top^{CO}/+</i>	424	90

Fused dorsal appendage phenotype ranges from fusion at base to fusion along entire length of appendage. Eggshells with fused dorsal appendages represent the most severe phenotype produced by these double heterozygotes.

^aNumber of eggs scored.

duced with *orb*^{F343}. In contrast, eggshells produced by females doubly heterozygous for a strong *cni* allele, *cni*^{AA12}, and *orb*^{F343} showed very weak effects on dorsal appendage formation.

Finally, as predicted by the synergisms described above, *grk* and *top/DER* alleles interact genetically as *trans*-heterozygotes: 90% of the eggshells produced by females doubly heterozygous for *grk*^{HK36} and *top*^{CO} displayed fused dorsal appendages. Similar genetic interactions between *grk* and *top/DER* mutations have been observed by T. Schüpbach (pers. comm.).

To test *orb* for potential genetic interactions with genes of the dorsalizing class of eggshell mutations, we constructed females that were doubly heterozygous for *K10*^{LM00} (Table 1B) and *orb*^{F343}. A small but reproducible percentage of these eggshells carried fused dorsal appendages, suggesting at best only weak genetic interaction (Table 3).

The distribution of grk mRNA is disrupted in orb mutant oocytes

Phenotypic interactions between mutations at genetically unlinked loci have successfully identified genes that function in biochemically related pathways (Cline 1988; Regan and Fuller 1990; Hoffmann 1991; Rubin 1991). The genetic interactions between *orb*, *grk*, and *top/DER* suggested that *orb* might function in the pathway of dorsalizing signal production. Recently, Neumann-Silberberg and Schüpbach (1993) have shown that *grk* mRNA is localized within the oocyte and that this localization is disrupted by mutations known to affect the D/V pattern of the eggshell and embryo. They postulate that *grk* mRNA localization is important for spatially restricting the dorsalizing ligand that asymmetrically activates the *top/DER* receptor in follicle cells. The cytoplasmic distribution of Orb protein (Lantz et al., this issue; see below) and the potential RNA-binding domains in the Orb protein sequence (Lantz et al. 1992) made it a candidate for involvement in mRNA localizations. Therefore, we examined *grk* transcript distribution in egg chambers from *orb*^{mel}/*orb*^{F343} females.

Localization of the *grk* mRNA in mutant egg chambers from the germarium to stage 7 was normal (data not shown). However, beginning at stages 8–9, the localization pattern of *grk* mRNA was altered. Rather than being tightly restricted to the dorsal side of the oocyte nucleus as in wild type (Fig. 4A), the *grk* transcript was distributed across the entire anterior end of the mutant oocyte (Fig. 4B). Interestingly, this pattern of *grk* mRNA distribution is similar to that observed for *fs(1)K10* and *sqd* mutations, which dorsalize the eggshell, as well as *capu* and *spir* mutations, which produce both dorsalized and ventralized eggshells (Manseau and Schüpbach 1989; L. Manseau, pers. comm.). The potential significance of these results for the production of the dorsalizing signal will be discussed later.

D/V polarity defects extend to orb mutant embryos

Most mutations that affect the polarity of the eggshell

Figure 4. *grk* mRNA is mislocalized in late-stage *orb*^{mel}/*orb*^{F343} oocytes. (A) Detection of *grk* mRNA distribution by in situ hybridization in stages 6, 8, and 9 wild-type egg chambers (lateral view). In the late stages of oogenesis, *grk* transcript is detected in the dorsal corner of the oocyte (see also Neumann-Silberberg and Schüpbach 1993). Bar, 25 μ m (applies to both panels). (B) *orb*^{mel}/*orb*^{F343}, lateral view of stages 6, 8, and 10 egg chambers. In late-stage mutant oocytes, *grk* mRNA is not restricted to the dorsal corner of the oocyte nucleus (arrowhead) but is distributed along the entire margin of the oocyte anterior end.

also affect the polarity of the embryo (Ashburner et al. 1990; Schüpbach et al. 1991; Kelly 1993; Ruohola-Baker et al. 1993). Because we expected that the reduced dorsal eggshell structures of *orb* mutant eggs might indicate a reduction of dorsal cell fates (ventralized phenotype) in embryos, we examined the cuticles produced by embryos of both *orb*^{mel}/*orb*^{mel} and *orb*^{mel}/*orb*^{F343} females. We were surprised to find that none of the *orb* mutant embryonic cuticles displayed any recognizable D/V patterning defects (Table 4).

To check the possibility that the classes of embryos with incomplete or no cuticle (Table 4, i.e., scraps of cuticle and undeveloped classes) might have D/V polarity defects, we analyzed a much earlier indicator of the D/V pattern, the ventral-to-dorsal nuclear translocation gradient of the dorsal protein (Steward et al. 1988; Roth et al. 1989; Rushlow et al. 1989; Steward 1989). In wild-type embryos, the dorsal protein is localized in nuclei on the ventral surface, whereas the protein is excluded from nuclei on the dorsal surface (Fig. 5A). Mutations in ma-

Table 4. Phenotypes of embryos produced by *orb^{mel}/orb^{mel}* and *orb^{mel}/orb^{F343}* females

Embryonic phenotype	Percentage ^a	
	<i>orb^{mel}/orb^{mel}</i> (n = 201)	<i>orb^{mel}/orb^{F343}</i> (n = 322)
Undeveloped	46	67
Scraps of cuticle	7	32
Abdominal segmentation defects		
no abdominal segments	25	1
few abdominal segments	18	<1
fusion of abdominal segments	5	0
Wild type	2	<1
unhatched	22	0
hatched	4	—
hatched	18	—

Embryos that had arrested prior to cuticle deposition were classified as undeveloped. By this assay, we could not distinguish undeveloped embryos from eggs that were unfertilized (see text).

^a[n] Number scored.

ternally active genes that are essential for embryonic D/V polarity produce shifts in the dorsal protein gradient in a ventral-to-dorsal manner (Roth et al. 1989; Stewart 1989). In completely ventralized embryos, dorsal protein is present exclusively in the nuclei around the circumference of the embryo. Partially ventralized embryos display an expansion of dorsal protein nuclear localization toward the dorsal surface.

Because *orb^{mel}/orb^{F343}* eggs showed the highest penetrance of ventralized eggshell defects, we tested these embryos by immunohistochemical detection of nuclear dorsal distribution. In most of the blastoderm-stage *orb^{mel}/orb^{F343}* embryos (>90%), the domain of nuclear dorsal was expanded considerably, indicating an increase in ventral cell fates in the embryo (Fig. 5, cf. A and B). However, in at least half the embryos examined, the pattern of dorsal-positive nuclei was different from that described for embryos ventralized by mutations in other maternal dorsoventral genes such as *Toll* and *top/DER* (Roth et al. 1989; Stewart 1989). Figure 5C shows an example of this novel dorsal protein distribution. Note that dorsal protein was excluded from nuclei on the ventral surface at the embryo's anterior end and included in nuclei on the dorsal surface at the posterior end. The basis for this pattern of dorsal protein translocation is unknown at this time. Nevertheless, the patterns of dorsal-positive nuclei presented in Figure 5, B and C, both indicate that the active domain of embryonic ventralizing signal is expanded in *orb* mutant embryos.

Embryos produced by *orb^{mel}* females display posterior group defects

Our analysis of embryonic cuticles (see Table 4) and of D/V polarity in embryos produced by *orb^{mel}/orb^{F343}* females indicated early developmental disruptions. However, embryos produced by *orb^{mel}/orb^{mel}* females (hereafter referred to as *orb^{mel}* embryos), which can complete

more stages of embryogenesis, revealed another aspect of the *orb* mutant phenotype and suggested that *orb* was required for A/P axis formation in addition to the requirement described previously for D/V patterning. *orb^{mel}* embryos displayed abdominal segment deletions and lacked pole cells, defects typical of the posterior class of maternal-effect mutants (for review, see by Lasko 1992; Lehmann 1992; St Johnston and Nüsslein-Volhard 1992). Twenty-five percent of *orb^{mel}* embryonic

Figure 5. Confocal optical sections of dorsal protein distribution in embryos produced by wild-type and *orb^{mel}/orb^{F343}* females. (A) The dorsal-to-ventral gradient of dorsal protein distribution in a wild-type blastoderm-stage embryo. Nuclei on the ventral surface (lower region of embryo) contain the highest levels of dorsal protein, whereas the protein is excluded from nuclei and present in the cytoplasm on the dorsal surface (upper region of embryo). In the lateral region of the embryo, dorsal protein is distributed equally in the nuclei and cytoplasm, which appears as a diffuse stripe across the length of the embryo. Anterior is left. Bar, 25 μ m (applies to all panels). (B,C) Pattern of dorsal distribution in blastoderm-stage embryos from *orb^{mel}/orb^{F343}* females. The region of nuclear dorsal distribution is expanded (B), indicating an expansion of ventral fates in the embryo. Also frequently observed is a pattern (C) in which the region of cytoplasmic dorsal distribution is shifted toward the anterior portion of the embryo and the region of nuclear dorsal distribution is shifted toward the posterior portion of the embryo. The arrow marks the embryo's anterior end.

cuticles manifested abdominal segmentation defects (Table 4; Fig. 6, cf. A and B). In a related study, in which developing embryos were observed directly, we found that pole cells were absent in 27 of 33 *orb^{mel}* embryos that completed cellularization.

Even though 22% of *orb^{mel}* embryos produced wild-type cuticles, severe defects were observed as well (Table 4). Approximately half of *orb^{mel}* embryos failed to form cuticle (undeveloped), and a small fraction appeared severely aberrant (resembling scraps of cuticle). By comparison, examination of *orb^{mel}/orb^{F343}* embryos showed that almost none can make wild-type cuticle. Instead, a majority of these embryos failed to deposit cuticle and the remainder formed mostly scraps of cuticle. Thus, both allelic combinations produced classes of arrested embryos that could not be distinguished from eggs that were unfertilized. Therefore, we determined the percentages of unfertilized eggs produced by *orb* mutant females by performing immunocytochemistry with antibodies that recognized a sperm tail antigen (Karr 1991). We found that 23% of *orb^{mel}/orb^{mel}* eggs ($n = 198$) and 29% of *orb^{mel}/orb^{F343}* eggs ($n = 307$) failed to become fertilized. Because the numbers of unfertilized eggs can account for only half of the undeveloped embryos observed (see Table 4), we conclude that some of the *orb* mutant embryos arrest early in embryogenesis. We suspect that these undeveloped embryos and those that produce scraps of cuticle result from the simultaneous disruption of several developmental processes that are sensitive to the amount of functional *orb* gene product.

orb mutants fail to localize *osk* mRNA at the posterior pole of the oocyte

Because proper pole cell formation and abdominal segmentation require the localization of *osk* mRNA to the posterior pole of the oocyte (Ephrussi et al. 1991; Kim-Ha et al. 1991), we examined the distribution of *osk* mRNA in the egg chambers of *orb^{mel}/orb^{F343}* females. In wild-type egg chambers, *osk* mRNA is present in the nurse cells and is localized at the posterior pole of the oocyte during late oogenesis (Ephrussi et al. 1991; Kim-Ha et al. 1991). Figure 6C shows an example of *osk* mRNA distribution in a wild-type stage 10 egg chamber. In contrast, posterior *osk* mRNA localization was never observed in *orb^{mel}/orb^{F343}* oocytes. Rather, *osk* mRNA was detected throughout the oocyte cytoplasm, with some enrichment in the peripheral regions of the oocyte (Fig. 6D). Localization of *osk* mRNA to the oocyte appeared normal for egg chambers from germarium through stage 7 in *orb^{mel}/orb^{F343}* ovaries (Fig. 6, cf. E and F).

bcd and *fs(1)K10* mRNA localization in the *orb* mutant

To this point, the phenotypic consequences of the *orb* mutation had predicted successfully that *grk* and *osk* mRNA localizations were *orb* dependent. To determine whether *orb* was required for general mRNA localization in the oocyte, we examined the pattern of two other well-characterized transcript localizations, *bcd* and

Figure 6. Embryos of *orb* mutant females display abdominal segment deletions and fail to localize *osk* mRNA to the posterior pole of the oocyte. (A) Wild-type embryonic cuticle, showing distinct denticle bands over each abdominal segment. Anterior is left and dorsal is up. Bar, 100 μ m (applies to A and B). (B) Representative posterior group phenotype of embryonic cuticles produced by *orb^{mel}* mutant mothers. Abdominal segments are absent. (C) Detection of *osk* mRNA by in situ hybridization in a wild-type stage 10 egg chamber. *osk* mRNA is detected in the nurse cells (left) and is localized within the oocyte to the posterior pole as a tight band (arrow; see also Ephrussi et al. 1991; Kim-Ha et al. 1991). Anterior is left. Bar, 25 μ m (applies to C and D). (D) *orb^{mel}/orb^{F343}* egg chamber (stage 10). *osk* mRNA is detected in the nurse cells and throughout the oocyte and never becomes concentrated at the oocyte posterior pole. Some enrichment of *osk* mRNA is detected in the cytoplasm that underlies the oocyte cortex. No consistent difference in *osk* mRNA abundance in the nurse cells has been observed between wild-type and *orb* mutant egg chambers. (E) Wild-type ovariole (germarium through stage 7). *osk* mRNA localizes to the oocyte in each egg chamber. Anterior is left. Bar, 50 μ m (applies to E and F). (F) *orb^{mel}/orb^{F343}* ovariole (germarium through stage 7). *osk* mRNA localization to the oocyte in each egg chamber appears identical to wild type. Less intense staining observed in F is a consequence of shorter reaction development time than E.

fs(1)K10 (Haenlin et al. 1987; Berleth et al. 1988; Cheung et al. 1992), in *orb^{mel}/orb^{F343}* egg chambers. The distribution of *bcd* and *fs(1)K10* transcripts within *orb* mutant oocytes appeared normal throughout oogenesis. This included the later stages when both *bcd* and *fs(1)K10* transcripts were correctly localized to the anterior end of *orb* mutant oocytes (Fig. 7, A–D). However, in some cases, *fs(1)K10* mRNA localization in *orb* mutant oocytes appeared less tightly restricted to the anterior margin of the oocyte (Fig. 7, cf. C and D).

orb^{mel} mutants produce an aberrantly localized protein

In wild-type ovaries, Orb protein is distributed in a dynamic and specific pattern during oogenesis (Lantz et al., this issue). Especially significant for the phenotypes produced by the *orb^{mel}* allele is the observation that Orb protein is associated with the cortex of the oocyte and is enriched at the anterior corners and the posterior pole of the oocyte during stage 8–10 (Fig. 8A; see also Lantz et al., this issue). In light of the *orb*-dependent localization of transcripts to the oocyte dorsal–anterior and posterior ends, we investigated the distribution of Orb protein in *orb^{mel}/orb^{F343}* egg chambers.

Before stage 8, the accumulation of Orb protein in

Figure 7. *bcd* and *K10* mRNA anterior localization in late-stage *orb^{mel}/orb^{F343}* egg chambers. (A) Detection of wild-type *bcd* mRNA by in situ hybridization in a wild-type stage 10 egg chamber. *bcd* mRNA is detected along the anterior margin of the oocyte (see also Berleth et al. 1988). Bar, 25 μ m [applies to all panels]. Anterior is left. (B) *orb^{mel}/orb^{F343}* egg chamber (stage 10). *bcd* mRNA is correctly localized to the anterior margin of the oocyte. (C) Detection of *fs(1)K10* mRNA by in situ hybridization in a wild-type stage 9 egg chamber. *fs(1)K10* mRNA is detected at the anterior end of the oocyte as a tight band (see also Haenlin et al. 1987; Cheung et al. 1992). (D) *orb^{mel}/orb^{F343}* egg chamber (stage 9). *fs(1)K10* mRNA correctly localizes along the anterior margin of the oocyte. This micrograph shows an example of the lower efficiency of *fs(1)K10* mRNA localization observed in some mutant oocytes (see text).

Figure 8. Confocal optical sections of Orb protein distribution in wild-type and *orb^{mel}/orb^{F343}* stage 9 egg chambers. (A) Immunolocalization of Orb protein in a wild-type stage 9 egg chamber. Orb protein is enriched in the cortical region that underlies the oocyte plasma membrane and especially in the anterior corners and at the posterior pole of the oocyte. The cytoplasm of the nurse cells closest to the oocyte contains higher levels of Orb protein than the cytoplasm of the remaining nurse cells (see also Lantz et al. 1994). Bar, 25 μ m [applies to both panels]. (B) *orb^{mel}/orb^{F343}* egg chamber (stage 9). Orb protein is distributed throughout the cytoplasm of the oocyte, with regions in which the protein appears punctate. The levels of Orb protein in the nurse cells closest to the oocyte appear reduced.

oocytes of *orb^{mel}/orb^{F343}* egg chambers appeared indistinguishable from wild type (data not shown). However, beginning at stage 8, and especially evident during stage 9, Orb protein distribution in these oocytes was aberrant. In stage 9 *orb^{mel}/orb^{F343}* oocytes, Orb was distributed in a diffuse pattern across the oocyte and never became concentrated at the anterior, posterior, or cortical regions of the oocyte (Fig. 8B). In addition, in some areas, the protein appeared punctate instead of homogeneous. These punctate areas may represent regions of protein aggregation, but the nature of such aggregation sites is unknown. Finally, the level of Orb in the nurse cells of stages 8–10 *orb^{mel}/orb^{F343}* egg chambers appeared lower than that of wild type.

Discussion

orb function during oocyte polarization

In this paper we present evidence that *orb* is required for both A/P and D/V patterning in the eggshell and embryo. We also show that the distribution of at least two transcripts, namely *osk* and *grk*, whose localization is apparently critical for formation of these axes and *orb*

dependent during stage 8–10 of oogenesis. During these stages, the oocyte cytoplasm becomes considerably reorganized (Theurkauf et al. 1992) and shows signs of polarization (e.g., the asymmetric distribution of mRNAs) into D/V and A/P axes (St Johnston and Nüsslein-Volhard 1992; Neuman-Silberberg and Schüpbach 1993). The similarity of Orb protein to RNA-binding proteins (Lantz et al. 1992) and the enrichment of Orb in the anterior and posterior regions of the oocyte cytoplasm during the mid- and late stages of oogenesis (Lantz et al., this issue; see Fig. 8A) are consistent with a role for *orb* in localizing asymmetrically distributed mRNAs during stage 8–10 of oogenesis.

Because *orb* clearly has a role during early oogenesis, could the apparent mislocalization of transcripts during stage 8–10 be a consequence of earlier requirements for *orb* function? For example, might the loss of *orb*-dependent activities during earlier oogenesis disrupt egg chamber polarity to such an extent that the subsequent oocyte axes do not form? Although our data do not exclude this possibility, a considerable amount of evidence supports a function for *orb* beyond the early egg chamber. First, the morphology of *orb^{me1}/orb^{F343}* egg chambers displays normal polarity (see Fig. 4B). The oocyte occupies its usual posterior position in the egg chamber, and the follicle cells execute their normal posterior-ward migrations to surround the oocyte. Second, no indication of early disruptions (prior to stage 8) in the distribution of oocyte-specific mRNAs [*osk*, *grk*, *bcd*, and *fs(1)K10*] can be detected in *orb^{me1}/orb^{F343}* egg chambers, suggesting that polarity in the egg chamber is intact. Third, the genetic synergism displayed between *orb* and *grk* (see Table 3) is most consistent with a role for *orb* during the time that the dorsalizing signal is passed from the oocyte to the overlying follicle cells. This signaling event is estimated to begin during stage 8 or 9, based on the timing of the movement of the oocyte nucleus to the future dorsal corner of the oocyte (Schüpbach et al. 1991), studies of the stages in which laser ablation of the oocyte nucleus can phenocopy the ventralized phenotype (Montell et al. 1991), and the redistribution of *grk* mRNA to the dorsal anterior corner during these stages (Neuman-Silberberg and Schüpbach 1993). Therefore, we postulate that *orb* is required specifically during stage 8–10 of oogenesis for the localization of transcripts.

Linkage between A/P and D/V polarity systems

Unlike most of the genes that can produce a reduction in the dorsal surfaces of the eggshell and embryo, mutation of *orb* also disrupts A/P axis formation (see Figs. 3 and 6; Tables 2 and 4). A functional oocyte nucleus (Montell et al. 1991) and the genes *capu* and *spir* are also required for the proper formation of both axes (Manseau and Schüpbach 1989). Mutations in *capu* and *spir* result in both ventralized and dorsalized eggshells as well as embryos that display D/V patterning and posterior group defects (Manseau and Schüpbach 1989; L. Manseau, pers. comm.). Manseau and Schüpbach (1989) addressed such effects on both polarity systems by speculating that *capu*

and *spir* function as cytoskeletal elements that organize cytoplasm or move molecules within cytoplasm. Our results suggest that the mechanisms of mRNA localization link A/P and D/V axes formation during oogenesis. Because *orb* mutations disrupt both axes, Orb is probably insufficient by itself to specify the address of a particular transcript. Rather, a role as an RNA-binding component of a larger complex that shuttles transcripts to specific sites seems more plausible.

Involvement in dorsal follicle cell determination

The genetic synergism displayed by double heterozygous combinations of *orb* and *grk* or *orb* and *top/DER* mutations (see Table 3) suggests that these genes might contribute to the same developmental pathway. Such tests for second-site noncomplementation or dominant enhancement have been useful for revealing interactions among genes mutable to similar phenotypes, as well as for the isolation of new mutations in genes acting in related pathways (Botas et al. 1982; Dambly-Chaudière et al. 1988; Torres and Sánchez 1989). We propose that *orb*, together with other maternal D/V genes including *grk* and *top/DER*, is required for proper communication between the germ line and follicle cells to signal dorsal follicle cell fates.

Recently, Neumann-Silberberg and Schüpbach (1993) provided an elegant model for how localization of the *grk* transcript might establish the spatially restricted dorsalizing signal. Our finding that *grk* mRNA localization is dependent on *orb*⁺ function provides an explanation for why eggshell polarity is disrupted in *orb* mutants and why dosage-sensitive genetic interactions exist between *orb* and *grk* or *top/DER*. Noting that these double heterozygotes produced eggshells that resembled those produced by loss-of-function *grk* or *top/DER* alleles, we hypothesize that a decrease in *orb* dosage in *orb*/+; *grk*/+ egg chambers produces a subthreshold level of *grk* mRNA in the dorsal anterior region of the oocyte, decreasing the amount of dorsalizing signal in that region and resulting in a reduction of dorsal follicle cell fates. Likewise, in *orb*/+; *top*/+ egg chambers, a decrease in *top/DER* expression in the follicle cells may reduce their sensitivity to the dorsalizing signal when combined with a subthreshold level of localized *grk* caused by the *orb* mutation. The fact that double heterozygous combinations of *grk* and *top/DER* loss-of-function alleles produce similar reductions in dorsal eggshell structures (see Table 3) is consistent with this explanation.

It is worth noting that the effects of *orb* (see Fig. 4B) and *fs(1)K10* mutations (Neuman-Silberberg and Schüpbach 1993) on *grk* transcripts may not be limited to localization alone, as *orb* and *fs(1)K10* mutants fail to localize *grk* mRNA in similar ways yet produce largely opposite phenotypes. Other factors, such as effects on abundance or translational efficiency of *grk* mRNA, might influence the final phenotypic consequences. In fact, Neumann-Silberberg and Schüpbach (1993) noted that the abundance of *grk* mRNA might be reduced in *capu* and *spir* mutants compared with the level of *grk*

transcript found in *fs(1)K10* mutant egg chambers. The production of both dorsalized and ventralized phenotypes in eggshells and embryos mutant for *capu* and *spir* (Manseau and Schüpbach 1989; L. Manseau, pers. comm.) suggests that the intensity of the dorsalizing signal might be influenced by several factors.

A model for orb function in osk mRNA localization

The fact that *osk* mRNA localization to the oocyte posterior end is *orb* dependent is sufficient to explain the posterior group defects observed in *orb^{mel}* embryos (see Fig. 6 B; Table 4). How might *orb* regulate *osk* mRNA distribution? One possibility is that Orb protein acts as a docking site for *osk* mRNA at the posterior pole of the oocyte, where it might maintain posterior *osk* mRNA localization. The posterior enrichment of Orb protein during the stages when *osk* mRNA is posteriorly localized is consistent with such a role. However, this explanation is weakened by the observation that *osk* mRNA does not become transiently localized at the posterior pole in *orb* mutant oocytes. In contrast, such transient localization at the posterior pole has been associated with some mutant alleles of *osk* (Ephrussi et al. 1991).

A second possibility is that Orb is involved in *osk* mRNA transport within the egg chamber. Evidence from several laboratories suggests that *osk* mRNA distribution can be divided into several phases (Ephrussi et al. 1991; Kim-Ha et al. 1991, 1993; St Johnston et al. 1991). First, *osk* mRNA is transported to the oocyte from its principal site of synthesis in the nurse cells. Second, *osk* mRNA is transported to the posterior pole of the oocyte. In fact, recently reported experiments have identified several elements in the *osk* 3' UTR that affect different steps of this process (Kim-Ha et al. 1993). Such stepwise localization may explain why *osk* mRNA distribution in the oocyte requires the activities of several genes [*Bicaudal-D* (*Bic-D*), *egalitarian*, *staufer*, *capu*, and *spir*; Ephrussi et al. 1991; Kim-Ha et al. 1991; St Johnston et al. 1991] as well as *orb*. St Johnston et al. (1991) have postulated that Staufen protein, which contains a double-stranded RNA-binding domain (St Johnston et al. 1992; Gagnon et al. 1993), and *osk* mRNA are localized to the posterior pole as part of a complex. The fact that *osk* mRNA localization is also *orb* dependent leads us to postulate that both Orb and Staufen might be part of a multiprotein complex that binds to *osk* mRNA and transports the mRNA to the oocyte posterior pole via the cytoskeleton. In this case, the distribution of Orb protein could reflect its relative abundance along the routes of transport. Experiments that test direct binding of Orb protein to *osk* mRNA are in progress.

An early requirement for orb in oogenesis

Phenotypic analysis of *orb^{dec}* indicates an early requirement for *orb* in oogenesis (Fig. 1C,E; Lantz et al. 1994). Because intact 16-cell clusters are seldom observed in *orb^{dec}* germaria, the cysts apparently begin to degenerate almost as soon as they form. Considerable evidence sug-

gests that several mRNAs accumulate in the presumptive oocyte of the young cysts (Suter et al. 1989; Ephrussi et al. 1991; Kim-Ha et al. 1991; Cheung et al. 1992; Dalby and Glover 1992; Lantz et al. 1992; Yue and Spradling 1992), either in response to oocyte determination or as part of the actual mechanism of oocyte determination. Thus, RNA-binding proteins are probably active during formation of the germ-line cyst. Analysis of oocyte-specific mRNA localization in *orb^{F303}* mutant ovaries, which display a slightly weaker phenotype than *orb^{dec}*, has shown that both *Bic-D* and *fs(1)K10* mRNAs fail to become localized (Lantz et al., this issue). Considering that *orb* activity is essential for mRNA localization late in oogenesis, we speculate that the loss of *orb*-dependent mRNA localization in the 16-cell cluster might be so catastrophic that degeneration ensues.

Materials and methods

Fly stocks

All fly stocks were maintained under standard culturing conditions. *y;ry⁵⁰⁶* and *w¹¹⁸* stocks were used as wild-type controls. *K10^{LM00}* is described by Cheung et al. (1992). The balancer chromosome *TM3* is described by Lindsley and Zimm (1992).

Generation of orb mutant alleles

The original P-allele of *orb* (*orb^{dec}*) was identified as a female sterile mutation by D. Stern and A. Spradling (pers. comm.) and was produced by standard procedures (Spradling 1993b). *orb^{dec}* carries a transposon insertion containing a *lacZ* gene (Hiromi et al. 1985). The source of transposase for the transposase-induced reversion of *orb^{dec}* was *P[ry⁺ 42-3](99B)* (Robertson et al. 1988).

Microscopy

Microscopy was performed on a Zeiss Axiophot microscope equipped with phase and differential interference contrast objectives. Confocal images were collected using the MRC 600 system (Bio-Rad Microsciences Division) attached to a Zeiss Axioplan microscope. Confocal images were photographed using a Polaroid FreezeFrame Video Recorder. To examine the cytology of ovaries, tissue was dissected in 1× PBS (130 mM sodium chloride, 3 mM monobasic sodium phosphate, 7 mM dibasic sodium phosphate) and fixed with 4% paraformaldehyde in 1× PBS; ovarioles were then teased apart to expose the egg chambers. Heterozygous *orb^{dec}* females from a balanced stock (*orb^{dec}/TM3*) were used for staining for LacZ activity in ovaries, which was carried out as described by Cooley et al. (1992). F-actin was stained using rhodamine-phalloidin as described by Xue and Cooley (1993).

Nucleic acid hybridization

Southern blotting procedures and labeling of DNAs by random hexamer priming were as described in Sambrook et al. (1989) and Feinberg and Vogelstein (1984). Hybridizations using DNA probes and subsequent washing protocols were performed at 65°C using the procedure of Church and Gilbert as described in Sambrook et al. (1989).

Cloning of the orb locus

A 4.5-kb fragment of genomic DNA contiguous with 7.0 kb of transposon DNA was isolated by plasmid rescue (Pirrotta 1986; Cooley et al. 1988) from *orb^{dec}* DNA. A 2.3-kb *EcoRI* fragment near the site of transposon insertion was used to screen 20,800 colonies of a genomic library supplied by J. Tamkun (Tamkun et al. 1992). Two overlapping genomic clones were recognized as derived from the *orb* locus by cross-hybridization to phage clones of *orb* genomic DNA (kindly provided by V. Lantz and P. Schedl, Princeton University, NJ). DNA was sequenced with Sequenase version 2.0 (U.S. Biochemical) according to the manufacturer's instructions. A PCR fragment (+17 to +797 nucleotides of *orb* cDNA) of wild-type genomic DNA was ~1 kb larger than expected from published *orb* cDNA sequence (Lantz et al. 1992). Sequencing this genomic fragment confirmed the presence of an intron at +301 nucleotides of the *orb* gene.

In situ hybridization and immunocytochemistry

Whole-mount *in situ* hybridization to ovaries using either digoxigenin-labeled DNA or RNA probes was performed according to Tautz and Pfeifle (1989) with modifications as follows: After hybridization with digoxigenin-labeled probes in hybridization solution (HS) at 48°C (described by McKearin and Spradling 1990), ovaries were washed three times with HS for 15 min each as follows: once in HS and once in HS/PBT (4:1), both at 48°C; once at room temperature (RT) in HS/PBT (3:2, 2:3, and 1:4). After staining, ovaries were washed twice at RT in PBT (1× PBS, 0.1% Tween 20) for 15 min each and then incubated overnight at 4°C in anti-digoxigenin antibody conjugated to alkaline phosphatase (AP) (Boehringer Mannheim), 1:2000 in PBT (anti-digoxigenin-AP antibodies were first diluted to 1:100 in PBT and preincubated with fixed ovaries for 2 hr at RT). Ovaries were mounted onto slides in 25% glycerol in 1×PBS. All digoxigenin-labeled probes were synthesized using the Boehringer Mannheim Genius kit by the following methods. RNA probes (for *osk* hybridization) were prepared as described by Cox et al. (1984). Single-stranded digoxigenin-labeled cDNA probes [for *fs(1)K10* hybridization] were prepared using a single primer cycling protocol provided by N. Patel (pers. comm.). Double-stranded digoxigenin-labeled cDNA probes (for *bcd* and *grk* hybridizations) were prepared as described by Yue and Spradling (1992). Immunocytochemistry of ovaries was performed using a 1:20 dilution of Orb monoclonal antibody (provided by V. Lantz and P. Schedl), as described by Xue and Cooley (1993), with the exception that the blocking and incubation buffer used consisted of 1× PBT, 1.5% bovine serum albumin, and 0.5% Triton X-100. Immunocytochemistry of embryos using either monoclonal antibodies to sperm (Karr 1991) or polyclonal antibodies to dorsal protein (S. Gillespie and S. Wasserman, pers. comm.) was performed as described by Karr et al. (1989) with modifications by S. Gillespie (pers. comm.) using fluorescently labeled secondary antibodies (Jackson ImmunoResearch).

Characterization of mutant phenotypes

Eggshells, embryonic cuticles, and living embryos were prepared and examined as described by Wieschaus and Nüsslein-Volhard (1986). The presence of pole cells was determined in living embryos between embryonic stages 4 and 6.

Acknowledgments

We are grateful to R. Lehmann, P. Macdonald, and R. Cohen for their gifts of plasmids; to T. Karr for providing sperm tail anti-

bodies; to S. Gillespie and S. Wasserman for dorsal antibodies; to T. Schüpbach, R. Cohen, S. Roth, V. Lantz, and P. Schedl for sending fly stocks; and to V. Lantz and P. Schedl for providing an *orb* cDNA clone and Orb monoclonal antibodies. We are especially grateful to V. Lantz, L. Manseau, F. S. Neuman-Silberberg, P. Schedl, and T. Schüpbach for generously sharing unpublished results and reagents. Many thanks go to S. Gillespie for advice on immunocytochemistry, and to S. Gillespie and S. Alexander for assistance in analyzing embryonic phenotypes. We thank C. Hilliard and M. Kuhn for excellent technical assistance. We thank L. Avery, M. Brown, G. Buckles, K. Clark, L. Cooley, F. Katz, B. Ohlstein, A. Spradling, S. Wasserman, and anonymous reviewers for comments on the various drafts of this manuscript. We especially thank L. Cooley, A. Spradling, and S. Wasserman for many helpful discussions. This work was supported by an National Institutes of Health grant GM-45820 and an American Cancer Society Institutional Grant IN-142I.

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L B Christerson and D M McKearin

Genes Dev. 1994, **8**:

Access the most recent version at doi:[10.1101/gad.8.5.614](https://doi.org/10.1101/gad.8.5.614)

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