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Dorsoventral axis formation in *Drosophila* depends on the correct dosage of the gene *gurken*

F. S. Neuman-Silberberg and T. Schupbach

Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA

SUMMARY

The *Drosophila* gene *gurken* participates in a signaling process that occurs between the germ line and the somatic cells (follicle cells) of the ovary. This process is required for correct patterning of the dorsoventral axis of both the egg and the embryo. *gurken* produces a spatially localized transcript which encodes a TGF-α-like molecule (Neuman-Silberberg and Schupbach, *Cell* 75, 165-174,1993). Mutations in *gurken* cause a ventralized phenotype in egg and embryo. To determine whether the *gurken* gene product plays an instructive role in dorsoventral patterning, we constructed females containing extra copies of a *gurken* transgene. Such females produce dorsalized eggs and embryos, which is expected if *gurken* acts as a limiting factor in the dorsoventral patterning process. In addition, the expression pattern of the gene *rhomboid* in the follicle cells is altered in ovaries of females containing extra copies of *gurken*. Our results indicate that changing *gurken* dosage in otherwise wild-type ovaries is sufficient to alter the number of somatic follicle cells directed to the dorsal fate. Therefore the *gurken-torpedo* signaling process plays an instructive role in oogenesis. It induces dorsal cell fates in the follicle cell epithelium and it controls the production of maternal components that will direct the embryonic dorsoventral pattern after fertilization.

Key words: *Drosophila*, oogenesis, dorsoventral pattern, signal transduction

INTRODUCTION

The two *Drosophila* genes *gurken (grk)* and *torpedo* (*top*)/DER (*Drosophila* EGF receptor homolog, Schejter and Shilo, 1989; Price et al., 1989), are involved in dorsoventral patterning during oogenesis. Females homozygous for mutations in either *top*/DER or *grk* produce ventralized eggs and embryos, indicating that loss of either *grk* or *top* function leads to a shift in cell fates, both in the somatically derived follicle cell epithelium surrounding the oocyte and in the embryo that develops inside the mutant egg. Analysis of the mutant phenotypes, as well as studies involving mosaic egg chambers, have shown that *grk* and *top* are components of a signaling process between the germ line and the somatic follicle cells, and form a putative ligand-receptor pair. The *grk-top*/DER signal is required for dorsal follicle cell differentiation and controls embryonic dorsoventral patterning (Schupbach, 1987; Price et al., 1989; Neuman-Silberberg and Schupbach, 1993). Dorsoventral asymmetry in the embryo is achieved through asymmetric activation of the *Toll* receptor present on the embryonic membrane. The *grk-top* signaling process inhibits the production of an active ligand for Toll on the dorsal side of the egg and thus restricts activation of the *Toll* receptor to the ventral side (Schupbach, 1987; Hashimoto et al., 1988; 1991; Manseau and Schupbach, 1989; Roth et al., 1989; Steward, 1989; Stein et al., 1991; Stein and Nusslein-Volhard, 1992; Chasan and Anderson, 1994).

It has never been shown, however, that the *grk-top* signal induces dorsal cell fates in a follicle cell epithelium that does not yet possess any dorsoventral polarity. It is possible, and in fact has been proposed, that initial patterning of the follicle cells depends on other spatially regulated factors in the egg chamber (see e.g. Ruohola-Baker et al., 1993). Although the *grk* RNA is localized to the dorsal anterior corner of the oocyte, the *grk-top* signal might only trigger a differentiation response in a prepatterned follicle cell epithelium. In this case, the restricted accumulation of the *grk* RNA would be fortuitous, or at least not an essential aspect of the *grk* function. Mislocalization of the *grk* RNA was observed in egg chambers from females mutant for *fs(1)K10*, *squid*, *cappuccino* and *spire* which produce dorsalized eggs and embryos (Neuman-Silberberg and Schupbach, 1993). This observation does not, however, prove that *grk* is the limiting factor in dorsoventral patterning, since the effects of the dorsalizing mutations might not be restricted to *grk*. If other unknown localized factors establish dorsoventral polarity in the egg chamber, they might also be aberrantly localized in the dorsalizing mutants. That maternal mutants might affect the localization of more than one factor in the egg chamber is not without precedent. Mutations in at least two of these genes, *cappuccino* and *spire*, are known to affect the localization of a number of maternal components (for review see Lehmann, 1992).

Identificaton of the key regulated components that establish dorsoventral polarity in the egg chamber requires a procedure whose initial effects are restricted to a single component or gene product. In the experiments described in this paper, we

have constructed females with extra copies of the *gurken* gene. We show that an increase in the amount of *grk* transcripts is sufficent to dorsalize the follicle epithelium. These results argue that *gurken* plays a crucial instructive role in dorsoventral patterning.

MATERIALS AND METHODS

Females carrying extra copies of the *gurken* transgene were obtained from stocks carrying two different P-element-mediated insertions of the genomic *gurken* region over balancer chromosomes (Neuman-Silberberg and Schupbach, 1993). Transgenic females carrying more than three copies of the *gurken* transgene are sterile. We were unable to obtain flies with more than four copies of the transgene. The reason for this lethality is unknown.

For characterization of the egg shell phenotypes, freshly laid eggs were mounted in Hoyer's medium. For inspection of the embryonic cuticle, fully developed embryos were dechorionated in bleach, manually removed from the vitelline membrane, and mounted in Hoyer's/lactic acid (7:3) (Wieschaus and Nusslein-Volhard, 1986).

Immunological staining of embryos was performed as previously described (Macdonald and Struhl (1988) using the Avidin/biotin ABC system (Vector). Anti-twist antibody was kindly provided by S. Roth. Stained embryos were mounted in 50% methyl salicylate/50% Canada Balsam.

For in situ hybridization, whole-mount ovaries were hybridized with digoxigenin-labeled DNA (*grk*) or RNA (*rho*) probes prepared with the Boehringer Mannheim kit**.** *rho* probe was provided by S. Roth. Fixation and hybridization were done according to the procedure described by Tautz and Pfeifle (1989) with modifications (Suter and Steward, 1991), hybridizing at 45°C for DNA and 55°C for RNA probes.

RESULTS

To determine the effects of grk dosage on dorsoventral patterning, we analyzed the eggs and embryos produced by females that carried multiple copies of a *grk* transgene, inserted at different places in the genome.

The transgenes are, most likely, not transcribed at the level of the endogenous wild-type genes, since a single copy of a particular transgene restored wild-type morphology to about 50-70% of the eggs produced by a mutant female homozygous for the strong allele grk^{HK36}. With two copies of this transgene crossed into a grk^{HK36} background, we found that approximately 90% of the eggs showed wild-type morphology. This is similar to females heterozygous for grk^{HK36}, which typically produce 80-90% wild-type eggs (*grk* does show a slight haploinsufficiency, which can be enhanced in some genetic backgrounds). We therefore estimate that this particular transgene may produce somewhat more than half as much *grk* product as one endogenous gene. Females carrying four extra copies of the *gurken* transgene were chosen for the analysis, since we were unable to obtain flies with higher copy numbers of the *gurken* transgene. We would predict that these females contain about two- to three-fold higher levels of *grk* RNA than wild-type females.

Females that carry two copies of the *gurken* transgene, in addition to the two endogenous copies, produce a small fraction of partially dorsalized eggs. Females with four copies of the transgene lay a significant fraction of partially dorsal-

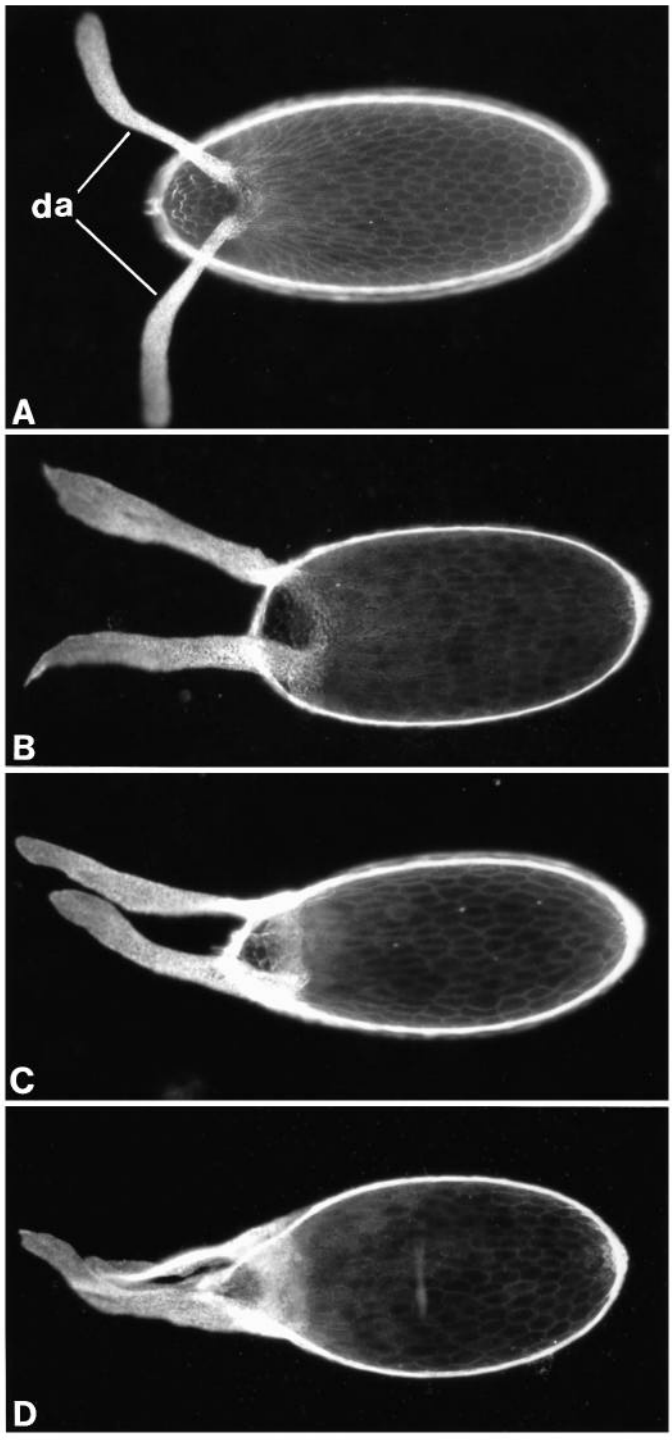

Fig. 1. Egg shells from wild-type females and from females carrying extra copies of the *grk* gene. (A) Wild-type egg with two dorsal appendages (da); (B-D) dorsalized eggs produced by females with two endogenous copies and four transgenic copies of the *grk* gene. The dorsalized eggs are somewhat smaller and rounder than the wild-type eggs. (B) Weakly dorsalized egg. The dorsal appendages are spaced considerably further apart than in wild type. (C) Intermediate phenotype with excess of dorsal appendage material over the dorsal side of the egg. (D) Strongly dorsalized phenotype with dorsal appendage material being secreted around the entire egg circumference. In all photographs, eggs are shown in a dorsal view and anterior is to the left.

ized eggs, and a few severely dorsalized eggs (Fig. 1). There is a variability in the amount of dorsalization observed in eggs produced by genotypically identical transgenic females (or even by a single female). On average, in 33% of the eggs, the dorsal appendages are spaced considerably further apart than in wild type (Fig. 1B). In 51% of the eggs, there is an excess of dorsal appendage material which usually results in a broad fusion of appendage material over the dorsal side (Fig 1C). 4% of the eggs have a strongly dorsalized phenotype with dorsal appendage material being secreted around the entire egg circumference (Fig. 1D). Few embryos hatch from the eggs laid by the females with four extra copies of the *gurken* transgene, and the majority of the unhatched embryos show a partial dorsalization. This dorsalization appears more extreme in the head and thoracic region, whereas the abdomen and telson often remain relatively normal (Fig. 2). In general, it appeared that there was a good correspondence between the degree of dorsalization of the embryonic cuticle and of the egg shell. In order to assess the dorsalization phenotype of the embryos more carefully, we stained a collection of such embryos with antibodies to the twist protein, which in wild type is expressed along the entire ventral region of the cellular blastoderm embryo. In the embryos produced by the transgenic flies, we observed a narrowing or deletion of the ventral twist domain particularly in the anterior part of the embryo (Fig. 3). There is, however, a considerable variability in the reduction of the twist domain ranging from embryos in which twist-expressing cells are missing only in an anterior domain, or in anterior and middle regions of the embryo, to the most severe cases in which only the poles still express the twist protein. Since expression of twist at the poles is regulated by the terminal genes (Ray et al., 1991), this extreme phenotype represents a complete loss of ventral mesoderm. The progressive loss of twist expression from anterior to posterior correlates well with the anterior-to-posterior dorsalization observed in the embryonic cuticle preparations.

To test how the addition of extra *grk* gene copies would affect the RNA localization, we performed in situ hybridization experiments on ovaries from females with four copies of the transgene (Fig. 4). These ovaries stain more intensely than the wild-type control at all stages of oogenesis. In early stages (stages 1-7), the *grk* RNA accumulates normally in the posteriorly situated oocyte. In wild-type oocytes, the *grk* RNA briefly accumulates along the anterior border of the oocyte during stage 8 of oogenesis, before assuming a dorsally restricted accumulation pattern in early stage 9. In ovaries from transgenic females, *grk* RNA requires longer to assume the tightly localized perinuclear pattern since in the majority of stage 9 oocytes the *grk* RNA is still found in a ring around the anterior margin of the oocyte (Fig. 4). During stage 10 of oogenesis, the *grk* RNA appears normally localized. Extra copies of the *grk* gene lead to an increase in the amount of *grk* RNA present in the oocyte, which presumably causes the

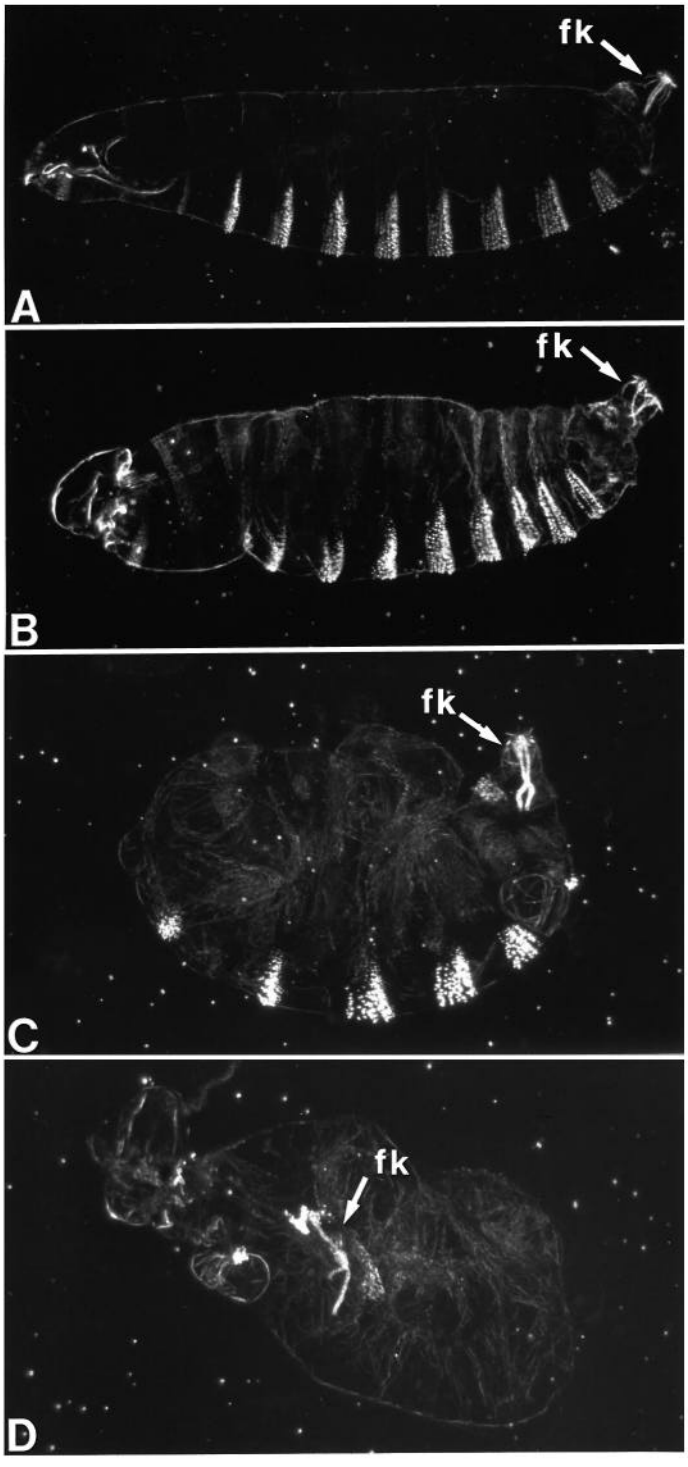

Fig. 2. Embryonic cuticles. (A) Wild-type embryo, with normal head structures, three thoracic segments, eight abdominal segments carrying ventral denticle belts and telson (filzkörper, fk). (B-D) Progressively dorsalized embryos produced by transgenic mothers with four extra copies of the *grk* gene. The majority (up to 70%) of the eggs produced by females carrying four extra copies of *grk* are not fertilized. Among the fertilized eggs, 15% of the embryos appeared normal, and often hatched. (B) Weakly dorsalized embryo. In 40% of the embryos defects consistent with a dorsalization are apparent in head and thoracic structures, whereas all eight abdominal denticle belts are present and the posterior structures appear normal. (C) In 34% of the embryos in addition to head and thoracic structures, a variable range of abdominal defects are apparent. The posterior end appears normal. (D) Strongly dorsalized embryo. In 11% of the embryos, head and ventral denticle belts are completely missing and defects are seen at the posterior end, although the embryo is not completely dorsalized at the posterior end. In all photographs, anterior is to the left and ventral is down.

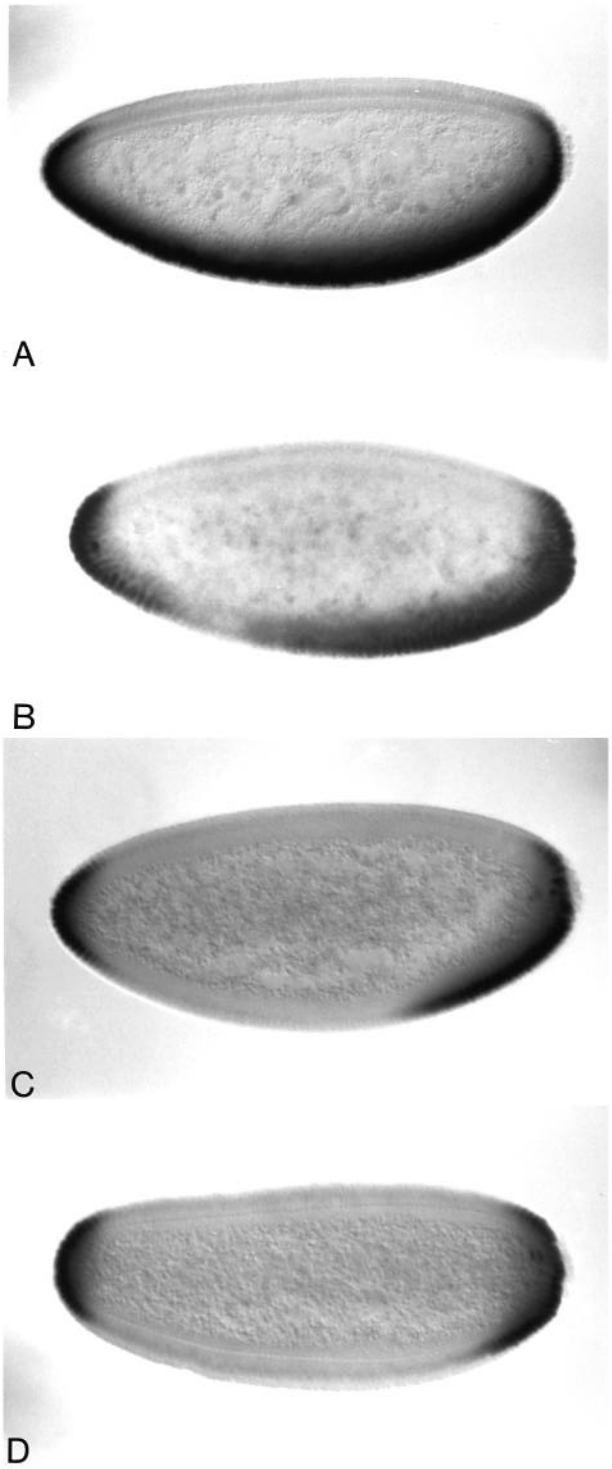

Fig. 3. Anti-twist antibody staining of cellular blastoderm embryos from wild-type and transgenic mothers. (A) Wild-type embryo. The twist protein is expressed in the ventral mesoderm and at both poles. (B-D) Progressively dorsalized embryos produced by mothers carrying four extra copies of the *grk* transgene. In all embryos, the twist-expressing domain is reduced. (B) In a weakly dorsalized embryo, the Twist domain is lost only in an anterior domain. (C) Moderately dorsalized embryo. Loss of the twist domain occurs in anterior and middle regions of the embryo. (D) In the most severely dorsalized embryos, only the poles still express the twist protein.

observed lag in the localization of *grk* RNA to the dorsal side of the oocyte.

The gene *rhomboid* (*rho*), which is expressed in the dorsal follicle cells during oogenesis, is required for dorsoventral patterning (Ruohola-Baker et al., 1993) and can be used as a molecular marker for dorsal follicle cell fate. We tested whether *rho* expression changes in ovaries containing an increased level of *grk*. The wild-type expression pattern of *rho* undergoes dynamic changes during development (Ruohola-Baker et al., 1993). In our experiments, *rho* expression in wild-type ovaries was first detected in early stage 10 of oogenesis. The RNA is expressed on the dorsal side of the egg chamber in a patch of follicle cells forming an apron-like shape overlying the oocyte nucleus (Fig. 5A). In addition, the RNA seems to be expressed in a single row of follicle cells forming a lateral to ventral semicircle along the anterior margin of the oocyte. In mid-stage 10 egg chambers, the initial broad staining on the dorsal anterior side is resolved into a pattern of two dorsolateral stripes (Fig. 5B). This pattern persists till later stages of oogenesis. In females carrying four extra copies of *grk* both the early and the late expression patterns of *rho* are altered on the dorsal side (Fig. 5C,D,E). The initial patch of staining on the dorsal side becomes broader expanding more ventrally and, in extreme cases, encircling the entire egg chamber. When the broad pattern resolves, the two dorsolateral stripes are further apart with a larger dorsal gap between them than in wild type (Fig. 5E). This result indicates that a higher dosage of *grk* leads to an expansion in the dorsal population of follicle cells that initially express *rho* and are later bordered by the lateral *rho* stripes.

DISCUSSION

The pattern alterations observed in eggs and embryos from females carrying multiple copies of the *gurken* gene indicate that *gurken* is a limiting factor in the dorsoventral patterning of follicle cells. The dorsalization of the egg shell and the changes in *rho* expression pattern demonstrate that an increased level of *grk* product induces dorsal fates in a larger group of follicle cells than in wild type. The size of the follicle cell population that assumes a dorsal fate depends on the local amount of *grk* product. Therefore, activation of the *top*/DER receptor by *gurken* produces instructive information for dorsal follicle cell determination.

In egg chambers from females with four copies of the *grk* transgene, the localization of *grk* RNA to the anterior-dorsal corner of the oocyte appears to be delayed as compared to wild type, and *grk* RNA can still be found along the anterior margin of the oocyte during stage 9 of oogenesis. This suggests that the *grk* RNA localization process is saturable by a two- to three-fold increase in the amount of *grk* RNA. The increased amount of *grk* RNA, possibly in conjunction with some unlocalized RNA, leads to a variable degree of dorsalization of egg shell and embryo. The variability of the observed dorsalization most likely indicates that the dosage level of *grk* in our experiments just barely reached a threshold of dorsalization, where small physiological differences between different egg chambers lead to a big range of egg shell and embryonic phenotypes. Unfortunately we were unable to obtain females with even higher copy numbers of the *grk* transgene. Such females

would have allowed us to test at which level of extra *grk* RNA the dorsalization of egg shell and embryo would have become completely penetrant.

The aberrantly localized *grk* RNA at the anterior end of the stage 9 egg chambers from transgenic females resembles the alteration of *grk* RNA localization seen in egg chambers homozygous for mutations in *fs(1)K10*, *squid*, *cappuccino* and *spire* (Neuman-Silberberg and Schupbach, 1993). Similarly, the dorsalization pattern of the embryos derived from transgenic females also resembles the dorsalization of embryos produced by females mutant for *fs(1)K10.* A stronger dorsalization of these embryos was observed at the anterior end (Wieschaus, 1979). The direct biochemical targets of *fs(1)K10*, *squid*, *spire* or *cappuccino* in oogenesis are presently unknown. At least in the case of *cappuccino* and *spire* it has been shown that these mutations affect the localization of several gene products in oogenesis (for review see St. Johnston and Nusslein-Volhard, 1992). Here we show that increasing the dosage of *grk* alone is sufficient to produce dorsalization suggesting that *grk* is the major factor whose mislocalization causes the observed dorsalization in these mutant genotypes.

Rhomboid is expressed in a population of follicle cells overlying the anterior dorsal side of the egg chamber, in proximity to the oocyte nucleus (Ruohola-Baker et al., 1993). Loss of *rho* expression results in ventralized eggs and embryos, and *rho* is therefore necessary for dorsoventral pattern formation during oogenesis. Ectopic expression of *rho* in the follicle cells was shown to dorsalize the egg and the embryo. *Rho* appears to act as a limiting factor of dorsal signal reception in the follicle cells (Ruohola-Baker et al., 1993). Our data indicate that expression of *rho* on the dorsal side of the egg chamber is most likely induced in the follicle cells by the *grk-top/*DER signaling process. *Rho*, in turn, may be necessary to facilitate the ligand-receptor interactions (see discussions in Ruohola-Baker et al., 1993, and Sturtevant et al., 1993). The dorsoventral signaling process therefore utilizes a limiting factor both in the production as well as in the reception of the signal. We also observed expression of *rho* in a single row of cells along the ventral and lateral sides of the egg chamber which was not affected by increasing the *grk* gene dosage. This ring of follicle cells are situated precisely at the border between the nurse cells and the oocyte, and they have been previously shown to specifically express a number of *lacZ* enhancer insertions, indicating that they constitute a distinct subpopulation of follicle cells (Grossniklaus et al., 1989; Schupbach and Wieschaus, 1991, Spradling, 1994**)**. This ring of *rho* expression is present in wild type and in *grk* mutant ovaries (data not shown, but visible in

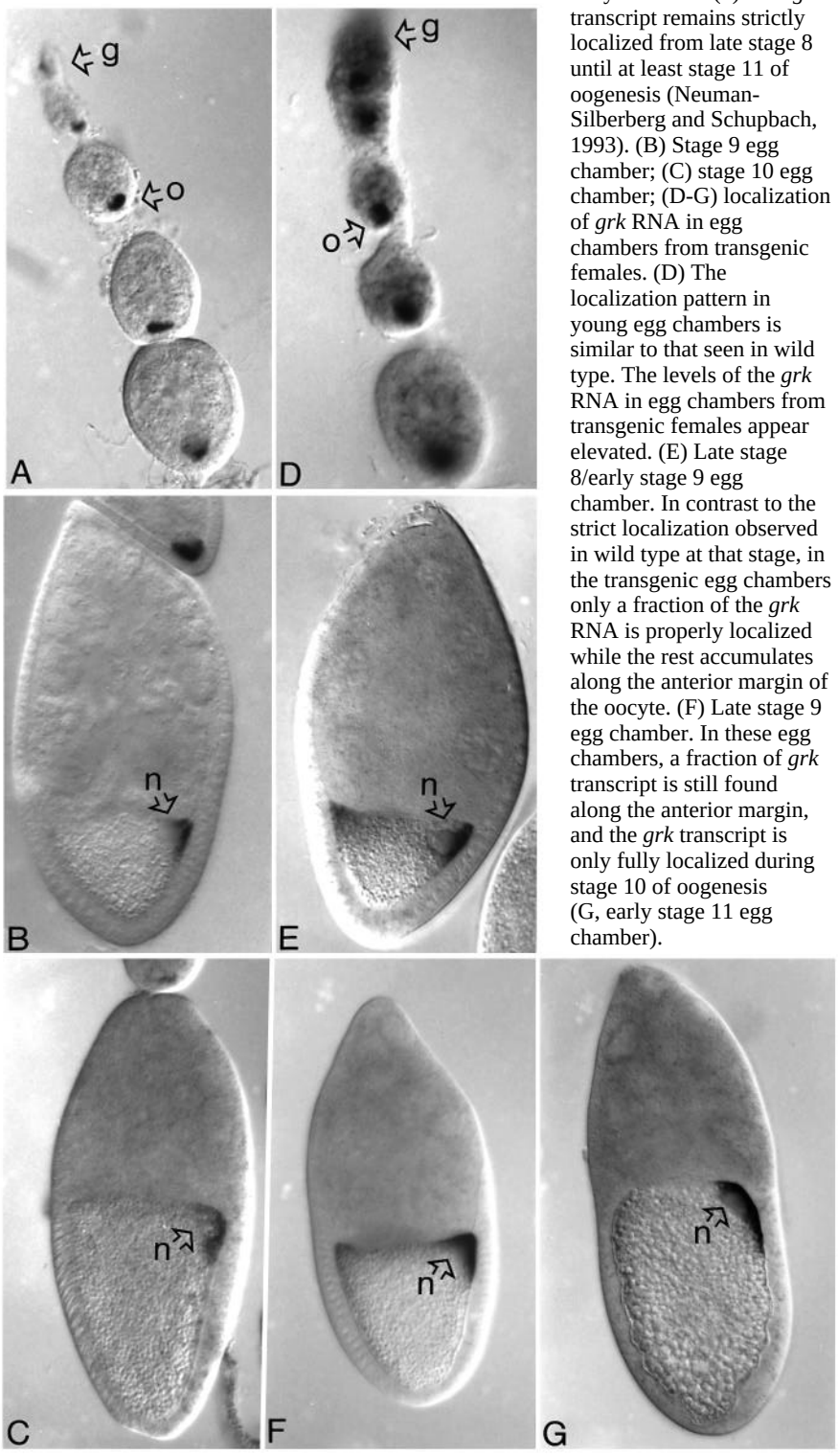

Fig. 4. Expression and localization pattern of *grk* RNA in egg chambers from wild-type and from transgenic females. (A-C) Egg chambers from wild-type females. (A) The *grk* transcript is first detected in the germarium (g). In young egg chambers, it is localized to the oocyte (o). During stage 8 of oogenesis, the *grk* transcript becomes strictly localized to the anterior side of the oocyte in close association with the asymmetrically positioned oocyte nucleus (n). The *grk* transcript remains strictly localized from late stage 8 until at least stage 11 of oogenesis (Neuman-Silberberg and Schupbach, 1993). (B) Stage 9 egg chamber; (C) stage 10 egg chamber; (D-G) localization of *grk* RNA in egg chambers from transgenic females. (D) The localization pattern in young egg chambers is similar to that seen in wild type. The levels of the *grk* RNA in egg chambers from transgenic females appear elevated. (E) Late stage 8/early stage 9 egg chamber. In contrast to the strict localization observed in wild type at that stage, in the transgenic egg chambers only a fraction of the *grk* RNA is properly localized while the rest accumulates along the anterior margin of the oocyte. (F) Late stage 9 egg chamber. In these egg chambers, a fraction of *grk* transcript is still found along the anterior margin, and the *grk* transcript is only fully localized during stage 10 of oogenesis (G, early stage 11 egg chamber).

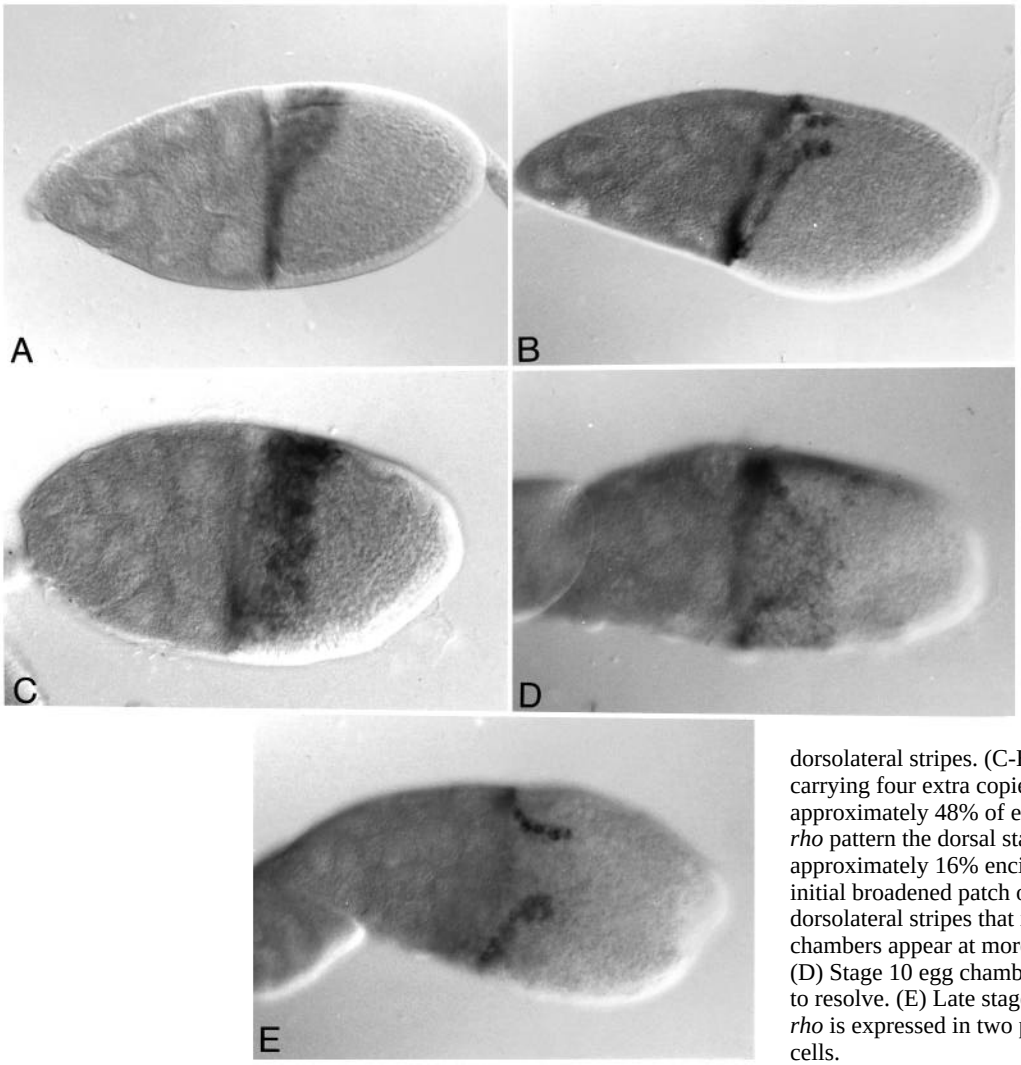

Fig. 5. *rho* expression in ovaries from wild-type and from transgenic females. (A,B) Egg chambers from wild-type females. (A) *rho* expression is first detected at early stage 10 of oogenesis in a spatially limited population of follicle cells. On the dorsal side, *rho* is expressed in a patch of follicle cells forming an apron-like shape overlying the oocyte nucleus. In addition, on the ventral and lateral sides, *rho* is expressed in a single row of follicle cells surrounding the anterior margin of the oocyte. (B) In slightly older stage 10 egg chambers, the initial broad pattern on the dorsal side is resolved into two dorsolateral stripes. (C-E) Egg chambers from females carrying four extra copies of the *grk* transgene. (C) In approximately 48% of egg chambers that display the early *rho* pattern the dorsal staining expands ventrally and in approximately 16% encircles the egg chamber. (D,E) The initial broadened patch of *rho* staining resolves into two dorsolateral stripes that in approximately 80% of egg chambers appear at more lateral positions than in wild type. (D) Stage 10 egg chamber; the initial dorsal *rho* pattern starts to resolve. (E) Late stage 10 to early stage 11 egg chamber; *rho* is expressed in two populations of dorsolateral follicle cells.

Fig. 6F of Ruohola-Baker et al., 1993), indicating that this part of the *rho* expression pattern is independent of *grk*. We believe that this anterior subpopulation of follicle cells is determined by an anterior-posterior patterning system operating in the follicle cell epithelium, and is independent of the dorsoventral system.

The effects of *gurken* dosage are similar to the effects of *bicoid* and *oskar*, two other localized and limiting maternal factors in *Drosophila* development, which cause pattern abnormalities when present in extra maternal gene copies (Driever et al., 1988; Smith et al., 1992) *bicoid* acts as a morphogen whose concentration specifies several different cell fates in the target tissue (Struhl et al., 1989). *oskar*, in contrast, is not thought to act as a morphogen, but serves to localize the morphogen *nanos* into a translation competent complex (Gavis and Lehmann, 1992; Lehmann, 1992). Extra copies of *oskar* lead to ectopic sources of nanos protein, and this causes embryonic abnormalities. Our studies demonstrate that extra gene doses of *gurken* affect follicle cell patterning and expand the population of follicle cells assuming a dorsal cell fate. The experiments do not determine whether specific follicle cell fates are directly controlled by different Grk protein concentrations. It may well be that *gurken* induces a relatively unpatterned dorsal primordium in the follicle cells, and that subsequently secondary patterning mechanisms operating within and between the dorsal and ventral follicle cell primordia specify the exact follicle cell fates. In this view, the initial expression of *rho* in a dorsal domain might be a relatively direct response to the *grk-top* signaling process, whereas the later lateral stripes of *rho* expression would be regulated by secondary patterning mechanisms possibly requiring interactions between dorsal and ventral follicle cells.

In summary our data show that *grk* is the major regulated factor in oogenesis whose concentration and spatial distribution are regulated by the germ line to establish the dorsoventral fate of the follicle cells, and subsequently, of the embryo.

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