Development 124, 4801-4810 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV5162

Post-transcriptional regulation of *gurken* by *encore* is required for axis determination in *Drosophila*

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SUMMARY

Establishment of anterior-posterior and dorsal-ventral polarity within the *Drosophila* egg chamber requires signaling between the germline and the somatic cells of the ovary. The gene *gurken (grk)* encodes a TGFα-like protein that is localized within the developing oocyte and is thought to locally activate *torpedo/Egfr* (*top*/*Egfr*), the *Drosophila* homolog of the EGF receptor, which is expressed throughout the follicular epithelium surrounding the oocyte. *grk-Egfr* signaling is required early in oogenesis for specification of posterior follicle cell fate and later in oogenesis for dorsal follicle cell fate determination, thus establishing the axes of the egg shell and embryo.

Previous studies have shown that these patterning processes are highly sensitive to changes in the levels and localization of *grk* mRNA. Here we show that post-transcriptional regulation of Grk protein levels is required for correct pattern formation. *encore (enc)*, a gene that functions in the regulation of germline mitosis and maintenance of oocyte identity, is also required for the accumulation of Grk protein during oogenesis. We present evidence that *enc* regulates Grk post-transcriptionally to ensure adequate levels of signaling for establishment of the anterior-posterior and dorsal-ventral axes.

Key words: axis formation, *Drosophila*, *gurken*, oogenesis, post-transcriptional regulation

INTRODUCTION

In *Drosophila*, dorsal-ventral (D/V) asymmetry of the egg is established during oogenesis and is most apparent in the structure of the egg shell. The dorsal surface of the egg is flat, the ventral surface is slightly curved and a pair of chorionic appendages are located on the dorsal anterior side of the egg shell. The polarity of the embryo that develops inside the egg is in exact alignment with the overlying egg shell. This alignment is produced during oogenesis and requires communication between the soma and germline, which establishes the axes of both the egg shell and embryo (Schüpbach, 1987).

In the ovary, each developing egg chamber is composed of two major cell types, those originating from the germline and those derived from the soma. The germline component consists of 15 nurse cells and an oocyte. These 16 cells are derived from a precise pattern of mitotic divisions of a stem cell daughter and are surrounded by a layer of somatically derived follicle cells. In later stages of oogenesis, the follicle cells secrete the egg shell, composed of the inner vitelline membrane and the outer chorion. The first sign of dorsal-ventral asymmetry occurs in midoogenesis at stage 8 when the oocyte nucleus moves from the posterior pole of the oocyte to the dorsal anterior corner (stages of oogenesis are reviewed in Spradling, 1993).

Female sterile alleles of the genes *gurken* (*grk)* and *torpedo* (*top*; also designated DER, or *Egfr*) cause a ventralization of both the egg shell and embryo. This phenotype is characterized by an expansion of ventral cell fates and a corresponding reduction in dorsal cell fates. Mosaic analysis revealed that *grk* is required in the germline and *top* (*Egfr*) is required in the soma, and demonstrated that a signaling process between the germ line and the overlying follicle cells is necessary for the establishment of dorsal-ventral polarity (Schüpbach, 1987).

Molecular analysis has supported the model that *Egfr* and *grk* participate in a signaling pathway. *Egfr* encodes the *Drosophila* homolog of the vertebrate epidermal growth factor receptor, a receptor tyrosine kinase (Price et al., 1989; Schejter and Shilo, 1989) while *grk* encodes a protein with similarity to the TGFα family of secreted growth factors. In vertebrate systems, TGFα is one of the ligands for the EGF receptor (Carpenter and Wahl, 1990). Thus, *grk* is a potential ligand for *Egfr* in the ovary. As expected from the mosaic analysis, *Egfr* is expressed in the follicle cells (Kammermeyer and Wadsworth, 1987). *grk* is expressed in the germline and accumulates in the developing oocyte (Neuman-Silberberg and Schüpbach, 1993). When the oocyte nucleus moves to the dorsal anterior corner of the oocyte, the *grk* mRNA becomes spatially restricted to a region overlying the oocyte nucleus. Throughout most of oogenesis, the distribution of Grk protein is similar to the localization pattern of the transcript. It is localized to the oocyte of early stage egg chambers (Roth et

al., 1995; Neuman-Silberberg and Schüpbach, 1996). At midoogenesis when the oocyte nucleus has moved to the dorsal anterior corner, the protein becomes restricted to the plasma membrane above the nucleus. By stage 10, the protein forms a narrow stripe that extends more posteriorly than the mRNA. This localization pattern is consistent with the model that *grk* acts as a spatially restricted dorsalizing signal which asymmetrically activates *Egfr* in the dorsal follicle cells thus distinguishing dorsal follicle cell fates from ventral follicle cell fates.

Recent work has shown that the *grk-Egfr* signaling pathway is also required for establishment of the anterior-posterior (A/P) axis during oogenesis (Gonzales-Reyes et al., 1995; Roth et al., 1995). The A/P axis is first evident early in oogenesis when the oocyte becomes positioned at the posterior pole of the egg chamber. A signal from the oocyte is necessary to induce the adjacent follicle cells to adopt a posterior follicle cell fate. This signaling process is mediated by the *grk-Egfr* pathway (Gonzales-Reyes and St Johnston, 1995; Roth et al., 1995). In egg chambers mutant for strong alleles of *grk*, *Egfr*, or *cornichon*, a gene required for *grk-Egfr* signaling, there is a duplication of anterior follicle cell fates at the posterior pole. This indicates that in the absence of a signal from the oocyte, the terminal follicle cells adopt a default anterior fate. As a result of this duplication, the eggs that are produced possess an anterior-specific structure, the micropyle, at both ends. The lack of posterior follicle cell fates in these mutants disrupts the anterior-posterior polarization of the oocyte, resulting in a symmetrical organization of the microtubule cytoskeleton within the oocyte. As a consequence, *bicoid* (*bcd*) is found at both the anterior and posterior poles of the oocyte, while *oskar* (*osk*) and a kinesin β-galactosidase fusion protein are localized to the center of the oocyte (Gonzales-Reyes et al., 1995; Roth et al., 1995).

Previously, we described a new gene, *encore* (*enc*), which is required for the regulation of germline mitosis and for maintenance of oocyte identity during oogenesis (Hawkins et al., 1996). Here we show that *enc* is also a member of the *grk-Egfr* signaling pathway during oogenesis and is required for D/V patterning. An analysis of eggs produced by females mutant for *enc* revealed D/V patterning defects similar to those produced by mutants in *grk* and *Egfr*. Our results indicate that these patterning defects are due to a requirement for *enc* in the post-transcriptional regulation of Grk. In addition, we show that *enc*, like *grk* and *Egfr*, has a role in A/P patterning during oogenesis.

MATERIALS AND METHODS

Drosophila strains

The isolation of the ethyl methanesulfonate-induced alleles enc^{D6}, enc^{DD7}, enc^{KK7}, enc^{L32}, enc^{M7}, enc^{N8}, enc^{OO6}, enc^{Q4}, enc^{R1}, enc^{T2}, enc^{UU3}, enc^{WW1}, enc^{XX1}, enc^{Z3} and the P-element-induced allele enc^{BB} was described previously (Hawkins et al., 1996). A second P-element allele of *enc*, enc^{A309}, was isolated during a P-element enhancer trap screen and was originally designated A309.1M3 (Grossniklaus et al., 1989) and enc^{R17} was generated by imprecise excision of this P-element. *Df(3L)A466* was described by Kulkarni et al. (1994). The enhancer trap lines BB127 and BB142 were generated by mobilization of the P[w^+lacZ] element of Bier et al. (1989). Descriptions of marker mutations and balancer chromosomes can be found in Lindsley and Zimm (1992).

Chorion and cuticle preparations

For microscopic analysis of chorions and embryonic cuticular phenotypes, eggs were processed as previously described (Wieschaus and Nusslein-Volhard, 1986).

In situ hybridization and antibody staining

For in situ hybridization, ovaries were dissected in Ringer's and the ovarioles teased apart with tungsten needles. The tissue was fixed in 4% paraformaldehyde in PBS + 0.1% Tween, 10% DMSO and 3 volumes of heptane for 20 minutes. For in situ hybridization to embryos, staged egg collections were covered with paraffin oil and embryos were hand selected to enrich for cellular blastoderms prior to fixation. All subsequent steps were carried out according to Tautz and Pfeifle (1989) using a hybridization temperature of 55˚C. Digoxigenin-labelled antisense RNA probes were synthesized using the RNA Genius kit (Boehringer Mannheim) according to manufacturer's protocol. The *grk* probe was prepared from a 1.7 kb cDNA (Neuman-Silberberg and Schüpbach, 1993), the *dpp* probe from a complete 3.5 kb cDNA (St Johnston and Gelbart, 1987), and the *rho* probe from a complete 2.5 kb cDNA (Bier et al., 1990).

For grk antibody staining, ovaries were fixed in 4% paraformaldehyde/0.5% NP-40 and 3 volumes of heptane for 20 minutes. To eliminate endogenous peroxidase activity, ovaries were treated with 0.3% H_2O_2 in methanol for 30 minutes then rehydrated through a methanol/PBS series. Ovaries were blocked for 1 hour in PBST + 1% BSA, rinsed with PBST + 0.1% BSA and incubated overnight at 4˚C with rat polyclonal anti-grk antiserum at a final dilution of 1:500. Prior to use, the grk antibody was purified by several rounds of preabsorption to fixed ovaries from grk^{HK} homozygotes. Ovaries were incubated with a 1:1000 dilution of biotinylated anti-rat (goat IgG) secondary antibody (Jackson labs), followed by coupling to HRP-avidin (Vector Laboratories, Peroxidase ABC system). Immunological staining was carried out according to Patel (1994). Antibody staining of embryos with anti-twist was performed as described by Roth et al. (1989).

Northern blot analysis

Ovarian poly(A)$^+$ RNA was isolated as described in Sambrook et al. (1989). Approximately 3 μg of poly(A)$^+$ RNA per lane was loaded on a 2% agarose formaldehyde gel and subsequently transferred in alkalai to Hybond-N nylon membrane (Amersham). The loading control used, *c550*, is a 549nt cDNA from the 88B region of chromosome III that is abundantly expressed in ovaries (R. Ray, personal communication). Band intensity was quantitated using Imagequant software (Molecular Dynamics).

Immunoblot analysis

For preparation of ovarian extracts, 50 ovaries were dissected on ice in Ringer's. The Ringer's was removed and replaced with 200 μl of lysis buffer (50 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl, 2 mM DTT, 1% SDS, 1% Triton X-100) containing protease inhibitors: 2 mg/ml aprotinin, 1 mg/ml pepstatin, 0.5 mg/ml leupeptin and 0.5 mg/ml Pefabloc SC (Boehringer Mannheim). Ovaries were homogenized using a pestle and cordless motor (Fisher) for 4× 15 seconds, cooling on ice in between, then centrifuged briefly to pellet debris. A small aliquot was removed and the protein concentration determined using the Bio-Rad DC protein assay kit as described by the manufacturer. A equal volume of 2× loading buffer (100 mM Tris pH 6.8, 200 mM DTT, 4% SDS, 20% glycerol, 2% bromophenol blue) was added and the sample was boiled for 5 minutes. 60 μg of protein sample was loaded per lane, fractionated by SDS-PAGE and electroblotted in transfer buffer (20 mM Tris, 150 mM glycine, 20% methanol) to nitrocellulose (Schleicher and Schuell). Immunoblots were blocked in 5% non-fat milk in TBST (150 mM NaCl, 10 mM Tris pH 8, 0.05% Tween-20) for 1 hour and incubated overnight with primary antibody in TBST + 0.1%BSA. Blots were washed 4× 15 minutes and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham) diluted to 1:2000 in TBST +

0.1%BSA. Blots were washed in TBST and bands visualized by ECL (Amersham) as described by the manufacturer. For grk westerns, the preabsorbed antibody was used at a final concentration of 1:500. The α-tubulin monclonal antibody (Sigma) was used at a final dilution of 1:5000.

RESULTS

enc mutations cause ventralization of the egg shell

Mutations in *encore* (*enc*) result in one extra round of mitosis in the germline. As a consequence, egg chambers are produced containing double the number of germline cells. Analysis of multiple *enc* alleles revealed that they were all temperature sensitive for this phenotype. In addition, a subset of *enc* alleles also displayed a cold-sensitive defect in oocyte differentiation (Hawkins et al., 1996). However, when eggs were analyzed under conditions where the defects in mitosis and oocyte differentiation are rare, it was discovered that *enc* also affects the D/V patterning of the egg shell. Eggs laid by *enc* mutant females exhibit varying degrees of ventralization. The wild-type egg shell is characterized by a pair of respiratory appendages on the dorsal anterior side of the egg (Fig. 1A). In weakly ventralized *enc* eggs, the dorsal appendages are closer together and fused at the base. More severely ventralized eggs are characterized by a complete fusion of the dorsal appendages, resulting in a single appendage on the dorsal midline (Fig. 1B). In the most extreme cases, the dorsal appendage material is absent (Fig. 1C). The eggs are slightly longer than wild type and assume a more pointed shape at the posterior pole.

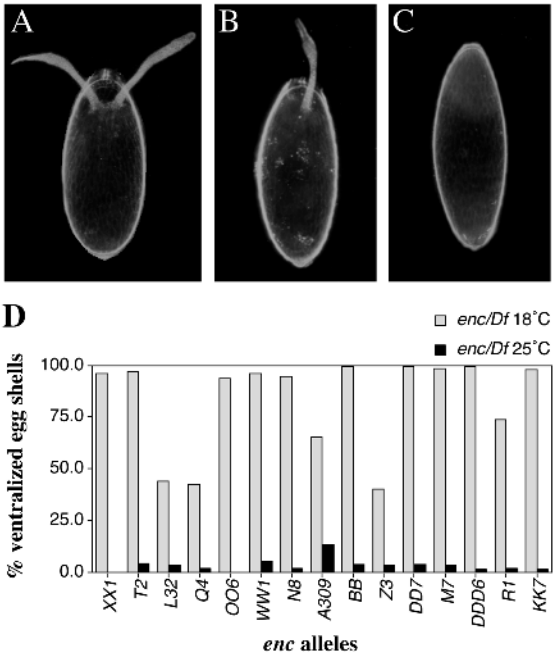

Fig. 1. Mutations in *enc* cause a ventralization of the egg shell. (A) A wild-type egg shell. (B,C) Egg shells derived from enc^{BB}/enc^{BB} females at room temperature. (D) The ventralized egg shell phenotype is cold-sensitive for all the *enc* alleles. The production of ventralized eggs was scored for each *enc* allele in trans to the deficiency *Df(3L)A466* (*Df*) at both 18˚C and 25˚C. For all the alleles at 18˚C, the vast majority of ventralized eggs (>95%) had a single dorsal appendage, while the production of the egg shells with no dorsal appendage material was infrequent. A total of 200-300 eggs were scored for each genotype.

enc mutants are cold sensitive for the D/V patterning defect

The allele exhibiting the strongest egg shell patterning defects was enc^{R17}. Less than 1% of the egg chambers produced by this mutant showed either a defect in germline mitosis or oocyte differentiation. The degree of ventralization of enc^{R17} was scored at both 18˚C and 25˚C. This analysis revealed that enc^{R17} was extremely cold sensitive (Table 1). At 18˚C, the majority of eggs produced by hemizygous mutant females have a single dorsal appendage (79%) and 20% of the egg shells have a further reduction in dorsal appendage material. None of the eggs produced at this temperature hatched. In contrast, at 25˚C, 97% of the eggs produced by enc^{R17}/Df females have a wild-type egg shell, while only 3% possess a single dorsal appendage. No egg shells showed a more severe loss of dorsal appendage material. At this temperature, 29% of the eggs hatched. The enc^{R17} allele behaves as a strong loss-of-function allele for the ventralized phenotype, since the egg shell phenotype produced by hemizygous females at 18˚C was similar to that produced by homozygous females at 18˚C (Table 1).

The egg shell phenotype produced by the remaining *enc* alleles was scored at both 18˚C and 25˚C to determine if the cold sensitivity exhibited by enc^{R17} was unique or a general property of all the *enc* alleles (Fig. 1D). This comparison showed that all the alleles were cold sensitive for ventralization. At 18˚C, the majority of the alleles produced 95-100% ventralized egg shells, except for *L32*, *Z3*, *Q4* and *A309* which produced a weaker phenotype. At 25˚C, all the alleles produced primarily wild-type egg shells. Any ventralized eggs that were produced at 25˚C had a very weak phenotype with the dorsal appendages fused only at the base.

The degree of ventralization observed at the nonpermissive temperature is similar to that caused by weak mutations in *grk* and *Egfr*. Of the eggs produced by females homozygous for grk^{ED}, 98% have a single dorsal appendage, similar to the weak alleles of *Egfr*, $Egfr^{QY1}$ and $Egfr^{CJ}$. In addition, it had been previously observed that mutations in both *grk* and *Egfr* are cold sensitive (Clifford and Schüpbach, 1989; Roth and Schüpbach, 1994).

enc mutants also produce ventralized embryos

An examination of the embryonic cuticle pattern revealed that

Table 1. Egg shell phenotypes of enc^{R17}

Genotype	°C	% eggs with two dorsal appendages	% eggs with one dorsal appendage	% eggs with no dorsal appendage
enc^{R17}/enc^{R17} *n*=317	18	5	70	24
$enc^{R17}/Df(3L)A466$ *n*=395	18	0	79	20
$enc^{R17}/Df(3L)A466$ *n*=350	25	97	3	0

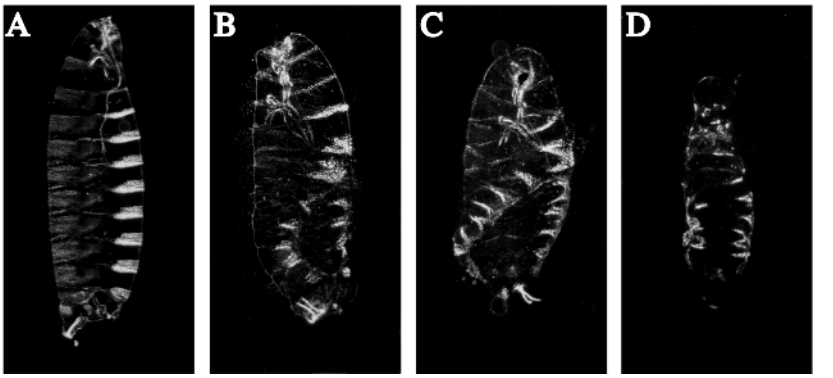

Fig. 2. *enc* mutants produce embryos with ventralized cuticles. Dark field micrographs of cuticle preparations. (A) Wild type; (B-D) cuticles produced by $enc^{R17}/Df(3L)A466$ at room temperature.

the embryos developing inside the ventralized egg shells were also ventralized. The wild-type cuticle is characterized by prominent denticle bands on the ventral side and fine hairs on the dorsal side. At the posterior pole are specialized tracheal structures, the Filzkörper (Fk), which are derived from the dorsal lateral region of the fate map (Fig. 2A). The cuticles derived from *enc* mutants fall into a phenotypic series. The weakest defect observed is frequently a ventral hole centered around abdominal segments A4-A6 (Fig. 2B). In moderately affected embryos, a ventral hole extends along the entire abdominal region, though in many of these embryos the head skeleton is still completely intact (Fig. 2C). The strongest phenotype produced is a completely ventralized cuticle reminiscent of mutations in *grk* and *Egfr*. All structures posterior to A8 are absent including posterior spiracles, Fk and anal pads (Fig. 2D). The range of cuticle phenotypes observed suggests that the degree of ventralization differs along the anterior-posterior axis with the extent of ventralization becoming progressively more severe towards the posterior end of the embryo.

To further characterize shifts in the D/V fate map, we examined the expression of three zygotic genes whose pattern of expression is restricted along the D/V axis. In wild-type embryos, Twist protein, a marker for the presumptive mesoderm, is expressed in a domain that comprises the ventralmost 20% of the embryo and extends from the anterior to the posterior end (Fig. 3A,D; Thisse et al., 1988). *decapentaplegic* (*dpp*) is expressed in 40% of the egg circumference on the dorsal side and expression extends over both poles (Fig. 3F; St Johnston and Gelbart, 1987). *rhomboid* (*rho*) is expressed in two ventrolateral stripes 8 cells wide adjacent to the Twist domain. In the late cellular blastoderm stage, a dorsal domain of expression is also observed (Fig. 4A; Bier et al., 1990).

In *enc* mutant embryos, the domain of Twist expression is expanded to encompass approximately 60% of the egg circumference (Fig. 3B,E). This expansion of mesodermal cell fates is similar to that observed in weak *Egfr* and *grk* mutants. However, unlike *grk* and *Egfr*, the expansion of the Twist domain progressively increases towards the posterior end of the embryo (Fig. 3B,C).

The expansion in ventral cell fates is accompanied by a corresponding reduction in dorsal cell fates. In *enc* mutant embryos, the domain of *dpp* expression is reduced. Although embryos are seen with only a narrow stripe of *dpp* expression remaining, the extent of reduction usually increases towards the posterior pole (Fig. 3G,H). Although *dpp* expression is

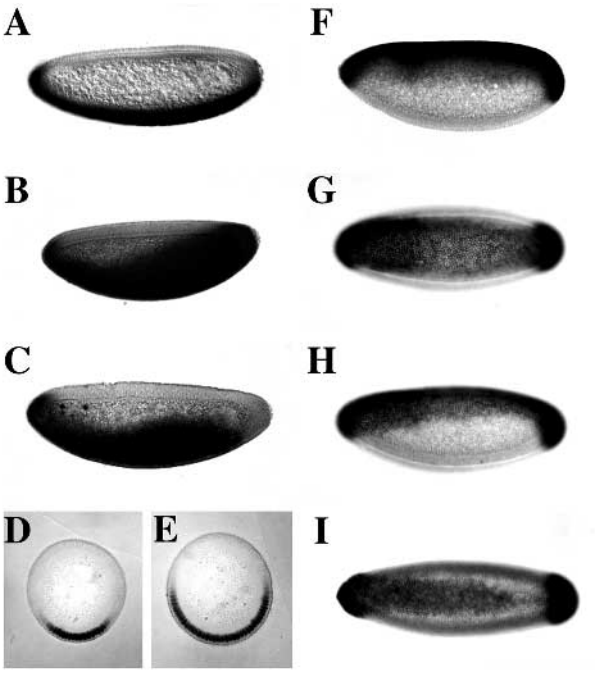

Fig. 3. *enc* mutant embryos have an expansion of ventral cell fates and a reduction in dorsal cell fates. (A-E) Twist protein expression. (A-C) Lateral views of whole mount embryos at the cellular blastoderm stage oriented with anterior to the left and dorsal up. (D,E) Cross sections of embryos at approximately 50% egg length at the cellular blastoderm stage. (A) A wild-type embryo with Twist expression on the ventral side. (B) An *enc* mutant embryo derived from enc^{KK7}/enc^{R17} at 18˚C showing an increased domain of Twist expression with a greater degree of expansion observed towards the posterior pole. (C) $enc^{R17}/Df(3L)A466$ embryo from 18˚C in which the Twist-expressing domain is expanded but expression is absent at the posterior end. (D) A wild-type embryo. Twist is expressed on the ventral side in a region encompassing 20% of the egg circumference. (E) An *enc* mutant embryo derived from $enc^{R17}/Df(3L)A466$ at 18˚C in which the Twist-expressing domain encompasses approximately 50% of the egg circumference. (F-I) *dpp* RNA distribution in cellular blastoderm stage embryos. (F) Lateral view of the *dpp* expression pattern in a wild-type embryo. (G) Dorsal view of *dpp* expression in a moderately ventralized embryo derived from $enc^{R17}/Df(3L)A466$ at 18˚C. (H) A dorsal lateral view of the embryo in (G) to emphasize the difference in the reduction of *dpp* expression along the anterior-posterior axis. (I) A dorsal view of a more severely ventralized embryo derived from $enc^{R17}/Df(3L)A466$. The domain of *dpp* expression narrows down to only a few cells in width towards the posterior end of the embryo.

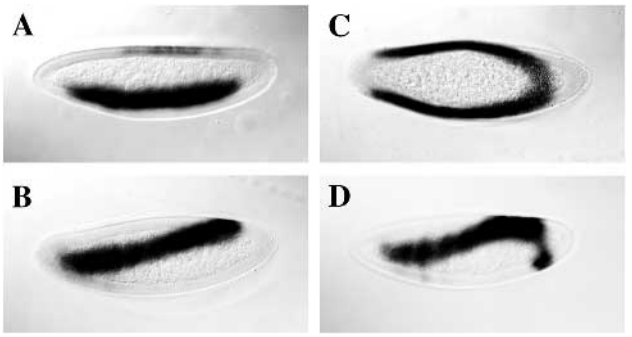

Fig. 4. *rho* RNA expression in *enc* mutant embryos. (A) Lateral view of the *rho* expression pattern in a wild-type embryo at the cellular blastoderm stage. (B) A lateral view of the *rho* expression in an $enc^{R17}/Df(3L)A466$ from 18˚C. (C) Dorsal view of the embryo pictured in B. The *rho* stripes fuse on the dorsal side near the posterior end of the embryo. (D) A more severely ventralized *enc* embryo derived from $enc^{R17}/Df(3L)A466$ in which a new domain of *rho* expression is induced on the ventral midline near the posterior pole.

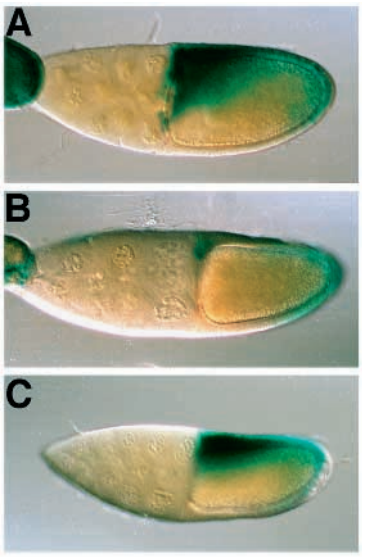

Fig. 5. Expression of the BB142 enhancer trap line in *enc* mutant egg chambers visualized by staining for β-galactosidase activity. (A) A wild-type egg chamber. Expression of the enhancer trap is observed in a large patch of dorsal anterior follicle cells. (B) Expression of the enhancer trap in a $enc^{R17}/Df(3L)A466$ stage 10 egg chamber raised at 18˚C. Very little staining is detectable in the dorsal anterior corner. (C) Expression of the enhancer trap line in a $enc^{R17}/Df(3L)A466$ stage 10 egg chamber raised at 25˚C.

never completely absent in the embryo, expression can be seen to disappear towards the posterior end (Fig. 3I). Expression at the poles, which is under control of the terminal system (Ray et al., 1991), appears unaffected.

The dorsal domain of *rho* expression is completely absent except for an occasional patch of expression at the anterior end. The lateral domains of *rho* expression are of normal width but are shifted more dorsally, indicating an expansion of ventral cell fates. Again, there is a pronounced difference along the anterior-posterior axis in the degree of ventralization (Fig. 4B). The ventrolateral stripes are frequently shifted completely onto the dorsal side at the posterior end of the embryo (Fig. 4C) and a new domain of *rho* expression is occasionally observed on the ventral side (Fig. 4D). The appearance of *rho* expression on the ventral midline, combined with a loss of Twist expression in this region, is indicative of a partial duplication of the dorsal-ventral pattern similar to the duplication that has been described previously for *grk* and *Egfr* (Roth and Schüpbach, 1994). In strong *grk* mutants, the Twist-expressing region is split into two domains and the region that lies in between on the ventral midline expresses ventrolateral markers such as *rho* and *Delta*. In *enc*, this pattern duplication is restricted to the posterior end of the embryo, the region that exhibits the greatest degree of ventralization.

enc mutants affect dorsal follicle cell fates

To examine the effect of *enc* on dorsal follicle cell fates, we monitored the expression of a reporter gene that marks dorsal follicle cells. For this purpose, we utilized the enhancer trap line BB142. In wild-type stage10 egg chambers, β-galactosidase expression from this insertion line is detected in the dorsal anterior follicle cells, forming a large triangular patch overlying the oocyte nucleus (Fig. 5A). Egg chambers derived from enc^{R17}/Df females raised at 18˚C containing the enhancer trap exhibited a significant reduction in the size of the β-gal-expressing region (Fig. 5B). Frequently, only a few stained cells were detected, indicating a loss of dorsal follicle cell fates. At 25˚C, the staining pattern was similar to wild type (Fig. 5C), consistent with the production of wild-type egg shells at this temperature. The wild-type staining pattern is also observed in egg chambers at 25˚C that have undergone an extra round of mitosis (data not shown). This confirms that an extra round of mitosis is not the underlying cause of the ventralized phenotype.

grk RNA expression

Since *enc* is required in the germline (Hawkins et al., 1996), we wanted to determine if the ventralization seen in *enc* mutants is the result of an alteration in *grk* RNA expression. Therefore, we examined the expression of *grk* transcript in *enc* mutant ovaries by in situ hybridization. In wild-type ovaries, *grk* mRNA is detected in the germarium and accumulates in the developing oocyte at the posterior pole. At stage 8, the RNA is briefly observed along the anterior margin of the oocyte, then becomes tightly localized to the dorsal anterior corner of the oocyte (Fig. 6A,B). In *enc* mutants, the early expression pattern is normal (Fig. 6C). However, a weak variable mislocalization is frequently observed in later stage egg chambers. In wild-type ovaries at stage 9 of oogenesis, a trail of *grk* RNA is seen along the anterior end of 37% of the egg chambers, whereas at stage 10, the *grk* RNA is strictly localized to the dorsal anterior corner. In enc^{R17}/Df at 18˚C, we observed 86% of stage 9 egg chambers with some mislocalized *grk* RNA and 47% of stage 10 egg chambers with mislocalized *grk* RNA (Fig. 6D). However, in most cases there is still a high point of RNA above the oocyte nucleus.

This pattern of mislocalization is similar, though not quite as severe, as that observed in mutants for *K10*, *spir*, *capu* and *sqd* (Neuman-Silberberg and Schüpbach, 1993; Roth and Schüpbach, 1994). However, in these mutants, the mislocalized *grk* RNA results in dorsalization, indicating that active Grk protein is produced from the mislocalized RNA, and induces dorsal follicle cell fates around the circumference of the egg. In contrast, *enc* produces exclusively ventralized eggs. We have never detected dorsalized eggs from any allelic combination analyzed.

We also examined the level of *grk* RNA in wild-type and mutant ovaries by northern analysis (Fig. 6E). In addition to wild type, ovaries from females heterozygous for a strong *gurken*

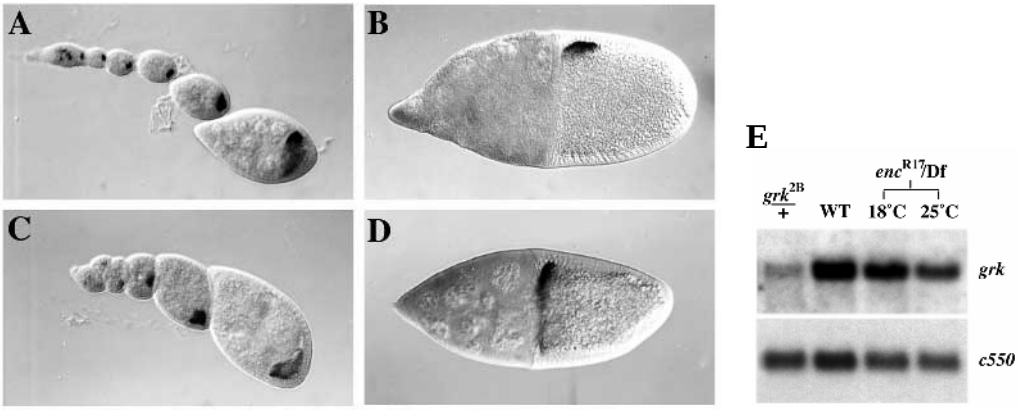

Fig. 6. Expression of *grk* transcript in *enc* mutant ovaries. (A) Early stage wild-type egg chambers. The transcript accumulates in the developing oocyte. (B) A wild-type stage 10 egg chamber. *grk* transcript is localized to the dorsal anterior corner of the oocyte. (C) Early stage egg chambers derived from $enc^{DD7}/Df(3L)A466$ at 18˚C. The early localization pattern of *grk* transcript in *enc* appears normal. (D) A stage 10 $enc^{DD7}/Df(3L)A466$ egg chamber from 18˚C. The *grk* mRNA is mislocalized along the anterior margin of the oocyte. (E) Northern blot analysis of grk RNA levels. The levels of *grk* RNA in $enc^{R17}/Df(3L)A466$ mutant ovaries at 18˚C and 25˚C were similar to wild type when normalized against a loading control*c550*, (see Materials and Methods) and significantly higher than the *grk* RNA levels in $grk^{2B}/+$ ovaries.

mutation that produces no RNA (grk^{2B}; Neuman-Silberberg and Schüpbach, 1994) were used as control. When the levels of *grk* RNA were normalized against the *c550* control RNA, it was found that ovaries from enc^{R17}/Df mutant females at both 18˚C and 25˚C have significantly more *grk* RNA than the $grk^{2B}/+$ control, which produces a considerably weaker phenotype than enc^{R17}/Df at 18˚C. In particular, the mutant ovaries at 18˚C had wild-type levels of *grk* RNA (relative levels of *grk* RNA/*c550* RNA were 0.29 for wild type and 0.32 for enc^{R17}/Df at 18˚C). Therefore, the ventralization produced by *enc* cannot be explained by a reduction in the level of *grk* RNA.

Grk protein expression is altered in *enc* mutants

Since the effect on *grk* RNA expression could not account for the *enc* ventralized phenotype, we examined Grk protein expression in *enc* ovaries to determine if *enc* affected Grk protein levels or localization. In wild-type ovaries, Grk protein expression is first detected in the germarium and is localized to the oocyte of early stage egg chambers. When the oocyte nucleus has moved to the dorsal anterior corner, the protein is localized to the cytoplasm and membrane above the nucleus. At later stages, the protein forms a narrow stripe that extends more posteriorly (Fig. 7A) (Serano et al., 1995; Neuman-Silberberg and Schüpbach, 1996).

There was a dramatic difference in the Grk expression pattern in *enc* ovaries. In enc^{R17}/Df at 18˚C, the level of Grk protein was extremely reduced and barely detectable. A lack of detectable Grk expression was also observed for other alleles examined (enc^{XX1}/Df, enc^{BB}/Df, enc^{L32}/Df, enc^{Z3}/Df). This extreme reduction in Grk protein expression included both the accumulation of the protein in the oocyte of early stage egg chambers and the asymmetric localization to the dorsal anterior corner in later stage egg chambers (Fig. 7B). At 25˚C, the permissive temperature, the staining pattern was similar to wild type (Fig. 7C). The levels of Grk protein in ovarian extracts prepared from enc^{R17}/Df mutants raised at 18˚C and 25˚C was assayed by western blotting (Fig. 7D). In wild-type extracts, the grk antibody recognizes a 47 kDa band that is absent in ovarian extracts prepared from grk^{HK} homozygotes (Neuman-Silberberg and Schüpbach, 1994). At 18˚C in enc^{R17}/Df, there is a significant reduction in the level of Grk protein. A similar reduction at 18˚C was observed in the other *enc* alleles examined (*Z3*, *XX1* and *L32* ; data not shown). At 25˚C, Grk protein levels were significantly increased compared to 18˚C. This increase in Grk protein levels at 25˚C was also observed in extracts prepared from *Z3*/Df, *XX1*/Df and *L32* (data not shown). These data show a clear correlation between the severity of the ventralization phenotype and the reduction in Grk protein. Therefore, the ventralization seen in *enc* mutants appears to be due to a reduction in Gurken protein, through an effect either on translation or protein stability.

enc is also involved in anterior-posterior patterning

Since it has been previously shown that *grk-Egfr* signaling pathway is also necessary to establish A/P polarity during oogenesis (Gonzalez-Reyes et al., 1995; Roth et al., 1995), we wanted to determine if *enc* was also involved in this process. The most sensitive indicator of a defect in A/P polarity is a duplication of anterior follicle cell fates. To visualize these cells, we used the enhancer trap line BB127 that expresses β-galactosidase in the nuclei of the nurse cells and centripetal follicle cells, a specialized group of anterior follicle cells that migrate between the nurse cells and oocyte in later stage egg chambers (Fig. 8A). In *enc* mutant egg chambers, there is frequently a duplication of these cells at the posterior end of the egg chamber (Fig. 8B).

In strong *grk*, *Egfr* and *cni* mutants, the defects in A/P patterning are apparent at later stages of oogenesis with the localization of *oskar* (*osk*) to the center of the developing oocyte (Gonzalez-Reyes et al., 1995; Roth et al., 1995). Therefore, we examined the localization of *osk* RNA in *enc* mutant egg chambers. In 40% of enc^{R17}/Df egg chambers, *osk* accumulates in the center of the oocyte instead of at its normal position at the posterior pole (Fig. 8C,D). Thus, *enc* also acts in the *grk-Egfr* signaling pathway for the establishment of anterior-posterior polarity. It has been previously shown that the posterior induction process is less sensitive to a reduction in *grk-Egfr* signaling than the establishment of dorsal follicle cell

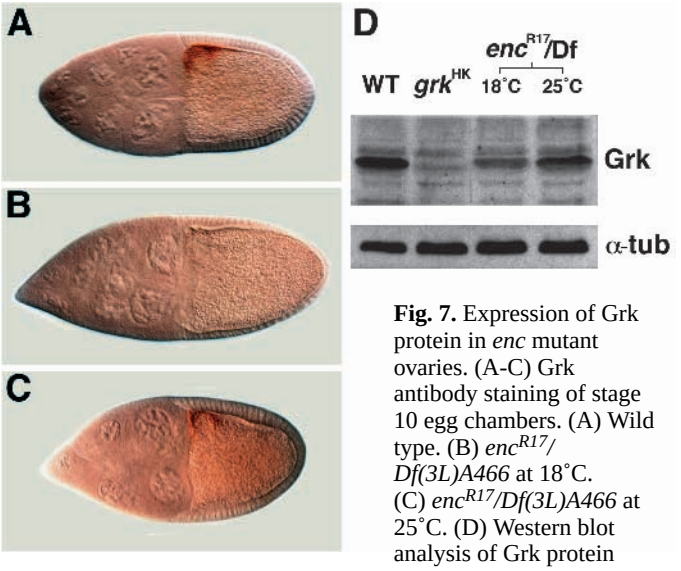

Fig. 7. Expression of Grk protein in *enc* mutant ovaries. (A-C) Grk antibody staining of stage 10 egg chambers. (A) Wild type. (B) enc^{R17}/*Df(3L)A466* at 18˚C. (C) enc^{R17}/*Df(3L)A466* at 25˚C. (D) Western blot analysis of Grk protein from ovarian extracts prepared from *enc* alleles at 18˚C and 25˚C. The Grk antibody recognizes a 47 kD band that is absent from ovaries of grk^{HK} homozygous females and strongly reduced in ovaries from enc^{R17}/*Df(3L)A466* females raised at 18˚C.

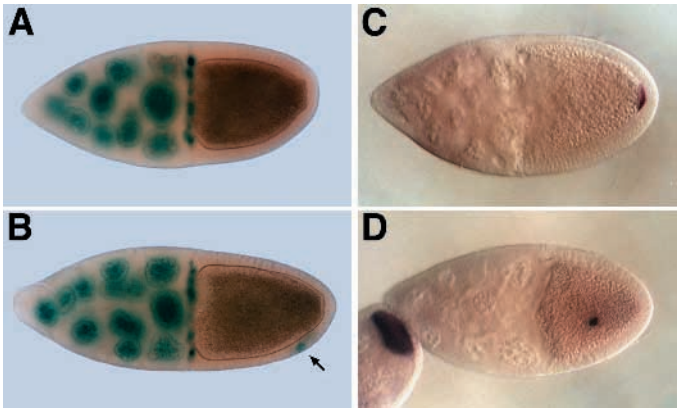

Fig. 8. *enc* mutations affect the anterior-posterior pattern of the egg chamber. (A,B) Expression of the BB127 enhancer trap line in stage 10 egg chambers. (A) Wild type; (B) enc^{R17}/*Df(3L)A466* at 18˚C. A cluster of posterior follicle cells show β-galactosidase expression (arrow) indicating a duplication of the anterior (centripetal) follicle cell fates at the posterior. (C,D) *oskar* RNA expression in midoogenesis. (C) Wild type; (D) enc^{R17}/*Df(3L)A466* at 18˚C. *oskar* RNA accumulates in the center of the oocyte.

fates (Gonzalez-Reyes et al., 1995; Roth et al., 1995). Similarly, a reduction in *enc* activity has a more severe effect on D/V patterning than A/P patterning.

DISCUSSION

enc is a novel member of the *grk-Egfr* signaling pathway during oogenesis

Striking similarities in the ventralized phenotype produced by *enc* mutants with those produced by mutations in *Egfr*, the *Drosophila* EGF receptor, and *grk*, a putative ligand for *Egfr*, have led us to conclude that *enc* is a novel member of this signaling pathway during oogenesis. Like mutations in *grk* and *Egfr*, mutations in *enc* cause a ventralization of both the egg shell and the embryo. An examination of the *enc* ventralized phenotype revealed that the extent of ventralization was comparable to that produced by weak *grk* and *Egfr* mutants. In strong *Egfr* and *grk* mutants, a partial pattern duplication along the dorsal-ventral axis has been observed. The ventral Twist-expressing domain is split into two stripes and is separated by a neurogenic region. A similar pattern duplication was observed in the most strongly ventralized *enc* embryos though, unlike *grk* and *Egfr*, this duplication was restricted to the posterior end of the embryo. Finally, like *grk* and *Egfr*, *enc* is also involved in anterior-posterior patterning during oogenesis. We observed both a duplication of anterior follicle cell fates and a mislocalization of *osk*. Taken together, these data show that *enc* activity is required for *grk-Egfr* signaling.

It is presently not known whether any of our *enc* alleles are complete null alleles. However, the ventralized phenotype of the strongest alleles is not enhanced in *trans* to a deficiency and thus they behave as strong loss-of-function alleles with respect to this defect. The phenotypes produced by these *enc* mutants are not as severe as those produced by the strongest alleles of *grk* and, therefore, it appears that, even in the absence of *enc* function, a low level of signaling occurs and *enc* functions to enhance this signal. However, we can presently not rule out the possiblity that a null allele of *enc* might produce a phenotype of equivalent strength to a null mutation in *grk*.

enc is required for the post-transcriptional regulation of *grk*

A more specific role for *enc* in the *grk/Egfr* signaling pathway was revealed by examination of Grk protein in *enc* mutant ovaries. This analysis showed that *enc* is involved in the post-transcriptional regulation of *grk*. By northern analysis, *grk* RNA levels are similar to wild type while, by western blot analysis, there was a signficant reduction in the amount of Grk protein at 18˚C. However, even at 18˚C there was not a complete absence of Grk protein. Since the strength of the *enc* ventralization is weaker than that produced by a strong *grk* mutant, the presence of a low level of Grk protein was expected. By antibody staining of *enc* mutant ovaries raised at 18˚C, little or no Grk protein was detected at any stage of oogenesis. Thus, *enc* is required during all stages of oogenesis for maintaining wild-type Grk protein levels. At the permissive temperature of 25˚C, in which >95% of the *enc* mutant egg shells are phenotypically wild type, Grk protein is correctly localized and, by western blot analysis, the levels of protein are similar to wild type.

The reduction in Grk protein is unlikely to be an indirect effect of weak mislocalization of the *grk* RNA observed in *enc* mutant ovaries. Previous analysis of genes required for *grk* RNA localization has suggested that correct localization is not necessary for translation. In *K10* and *sqd* mutants in which *grk* RNA is mislocalized along the anterior margin of the oocyte, wild-type levels of protein are observed (Neuman-Silberberg and Schüpbach, 1996). In addition, the mislocalization of the *grk* RNA in *enc* mutant ovaries is considerably less pronounced, with only a fraction of the egg chambers exhibiting detectable mislocalization, whereas the reduction in Grk protein levels is observed in all egg chambers.

There are two explanations to account for the reduction in Grk protein levels in *enc* mutants. *enc* could be required for the synthesis of the Grk protein by functioning to enhance translation or, alternatively, *enc* could be necessary to stabilize the Grk protein. Regardless of the exact mechanism, our results demonstrate that regulation at the post-transcriptional level provides one mechanism by which to precisely control the level of Grk protein. Previous studies have shown that D/V patterning is very sensitive to changes in the level of *grk* activity. *grk* exhibits weak haploinsufficiency such that lowering the gene dosage by half results in the production of a low percentage of eggs with a weakly ventralized egg shell (Christerson and McKearin, 1994). Conversely, increasing the gene dosage results in the production of dorsalized eggs (Neuman-Silberberg and Schüpbach, 1994). Therefore, *enc's* role in the post-transcriptional regulation of Grk is to ensure optimal levels of Grk protein necessary for correct dorsal-ventral patterning.

Another gene that has been implicated in the post-transcriptional regulation of *grk* is *aubergine* (*aub*). Mutations in *aub* were orginally identified that caused the production of eggs with either a fusion or an absence of dorsal appendages, characteristic of a ventralized phenotype (Schüpbach and Wieschaus, 1991). Subsequently, isolation of additional alleles revealed a role for *aub* in posterior body patterning, in that embryos derived from these mutant alleles lacked abdominal segments and pole cells (Wilson et al., 1996). An analysis of *aub's* role in posterior body patterning revealed that the level of Osk protein was substantially reduced and that *aub* was involved in the synthesis of the Osk protein. The level of Grk protein was also reduced, accounting for the egg shell defects associated with *aub* mutants and suggesting a role for *aub* in enhancing Grk translation.

The *Drosophila* gene *orb*, which encodes a homolog of the vertebrate translational control factor CPEB (Hake and Richter, 1994), also affects Grk protein accumulation (Neuman-Silberberg and Schupbach, 1996; Jacqueline Chang and Paul Schedl, personal communication). *orb* has been shown to be involved in localizing mRNAs to specific regions of the developing oocyte (Christerson and McKearin, 1994; Lantz et al., 1994). It has also been suggested that Orb may act as a translational control factor in oogenesis involved in the translation of localized RNAs, perhaps through association with the cytoskeleton of the oocyte (Christerson et al., 1995). It is therefore possible that a translational control complex has to form in oogenesis that activates translation of *gurken* RNA. Encore could be a member of such a complex and serve to relieve the *grk* RNA from negative factors that would initially inhibit its translation. One potential factor that may inhibit Grk translation is Bruno. Bruno was orginally identified as an ovarian RNA binding protein that bound to Bruno response elements (BREs) in the 3′UTR of *osk* and serves to repress *osk* mRNA translation prior to its localization to the posterior pole of the oocyte (Kim-Ha et al., 1995). Interestingly, there are two BREs in the 3′UTR of the *grk* RNA, raising the possibiltiy that the translational repressor Bruno could directly bind to the *grk* RNA. However, it is presently not known whether the putative BRE elements are indeed required for translational control of *grk* RNA.

Shift in the dorsal-ventral axis

Although the phenotype of *enc* is similar to that produced by weak alleles of *grk* and *Egfr*, one major difference was observed. In *grk* and *Egfr* mutant embryos, the extent of ventralization is roughly equivalent along the anterior-posterior axis while, in *enc* mutant embryos, there is a significant difference in the degree of ventralization along the anterior-posterior axis. There is a shift in the D/V axis such that *enc* embryos become progressively more ventralized towards the posterior end. This shift is not observed in weak alleles of *grk*, thus a reduction in Grk protein levels cannot alone explain the gradient of ventralization seen in *enc* mutants.

Female sterile mutations in three other genes, *K10*, *sqd* and *orb* also differentially affect D/V patterning along the A/P axis (Roth and Schüpbach, 1994; Haenlin et al., 1995). An examination of embryonic cuticle patterns revealed that the embryos were dorsalized anteriorly and ventralized posteriorly, and an analysis of the embryonic fate map using molecular markers revealed that the orientation of the D/V axis had shifted and, in the most extreme cases, was almost parallel with the A/P axis. This shift in the D/V axis is presumably due to the mislocalization of *grk* transcript to the anterior margin of the oocyte where it produces a functional dorsalizing signal that is received by follicle cells around the circumference of the egg chamber at the anterior end. The shift in the D/V axis observed in *enc* mutant embryos may therefore be explained by the weak mislocalization of *grk* RNA. Unlike *K10* and *sqd*, the mislocalization of *grk* RNA in *enc* is coupled with a significant reduction in the level of Grk protein. As a result, low levels of mislocalized protein at the anterior margin of the oocyte may provide sufficient dorsalizing signal to obtain a wild-type or only weakly ventralized phenotype at the anterior end of the embryo and a more pronounced ventralization towards the posterior end.

Dorsal-ventral patterning defines a third independent requirement for *enc* during oogenesis

As described previously, there are two other requirements for *enc* during oogenesis. It is involved both in regulating the number of germline mitoses and in the process of oocyte differentiation (Hawkins et al., 1996). Although it is conceivable that either a doubling in nurse cell number or abnormal oocyte differentiation could affect the production of Grk, our analysis demonstrates that the ventralized phenotype observed in *enc* mutants is not a secondary consequence of either defect. The mitotic defect is temperature sensitive, in contrast to the D/V patterning defect, which is cold sensitive. At 18˚C, the non-permissive temperature for the D/V defect, most egg chambers contain the normal number of nurse cells. In addition, at 25˚C, egg chambers were observed which contained extra nurse cells but there was no visible ventralization of the follicle cells as assayed with the BB142 enhancer trap line. The ventralized phenotype is also unlikely to be an indirect result of a defect in oocyte differentiation. Although this phenotype is also cold sensitive, it was only observed in a subset of the *enc* alleles while ventralized eggs are produced by all the alleles. In addition, if egg chambers containing a polyploid oocyte nucleus developed into mature eggs, presumably they would never produce embryos upon fertilization. However, mutations in *enc*, in addition to producing eggs with ventralized egg shells, also produced ventralized embryos. These embryos must have been derived from eggs containing a

normal haploid oocyte nucleus. Finally, the allele that produces the strongest ventralized phenotype, enc^{R17}, does not exhibit other oogenesis defects. Thus, a requirement for *enc* in the *grk/Egfr* signaling pathway represents a third independent requirement for *enc* during oogenesis.

Two other genes, *egl* and *Bic-D*, share phenotypes with *enc* in that they are required for oocyte differentiation and have also been shown to affect *grk-Egfr* signaling during oogenesis (Swan and Suter, 1996; Mach and Lehmann, 1997). However, unlike mutations in *enc*, the effect of mutations in *egl* and *Bic-D* on *grk-Egfr* signaling do not appear to arise from a direct affect on Grk protein. A reduction in *Bic-D* activity during midoogenesis results in the production of eggs with ventralized egg shells. This defect in D/V patterning appears to be a secondary consequence of reduced oocyte growth resulting in a misalignment of the follicle cells and the underlying germ line. In the case of *egl*, defects in D/V polarity may be due to a reduction in *grk* RNA levels or localization.

The extra round of germline mitosis in *enc* mutants is most likely due to an overproduction of *bag-of-marbles* (*bam*) RNA early in oogenesis (Hawkins et al., 1996). In contrast, the ventralized phenotype appears to result from a lack of Gurken protein. Given that, in wild-type ovaries, *bam* RNA shows a very restricted distribution (McKearin and Spradling, 1990), it is possible that its turn-over is highly regulated. Encore could be a protein that regulates RNA function and stability in oogenesis, and thus may be involved in the turn-over of *bam* RNA and the translational control of *grk* RNA.

In conclusion, we have identified a novel member of the *grk-Egfr* signaling pathway during oogenesis. Several genes are known to be required for the proper localization of the *grk* transcript. It now appears that post-transcriptional regulation of Grk levels is also required to ensure correct A/P and D/V patterning of the egg and embryo.

We are grateful to Christopher Beh and Elizabeth Gavis for critical reading of the manuscript and members of the Schüpbach lab, especially Robert Ray, for stimulating discussions. We thank W. J. Gehring, in whose lab enc^{A309} and enc^{R17} were isolated. We thank Robert Ray for help with the northern analysis and Gordon Gray for the preparation of fly food. This work was supported by the Howard Hughes Medical Institute and by the Public Health Service Grant PO1 CA41086 to T. S.

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(Accepted 18 September 1997)