THE ROLE OF THE GENE *EGALITARIAN* IN *DROSOPHILA* OOCYTE DETERMINATION AND POLARITY

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Abstract

Development can be described as the process by which cells become different from their siblings. The *Drosophila* oocyte forms from one of sixteen interconnected sister cells; the other fifteen cells form polyploid feeder cells known as nurse cells. In *egalitarian* (*egl*) mutants, the cell that should become the oocyte instead follows the same fate as its sister cells, forming a sixteenth nurse cell. The function of the *egl* gene is required in the oocyte and nurse cells of the ovary and *egl* mRNA and protein localize to the developing oocyte very early in oogenesis.

Three lines of evidence show that *egl* interacts with *BicaudalD* (*BicD*), another gene required for oocyte determination. First, the Egl and BicD proteins colocalize within the egg and this localization requires the function of both genes. Second, the penetrance of *BicD* dominant mutations can be altered by changing the *egl* gene dosage, showing a genetic interaction between the loci. Third, Egl and BicD proteins can be coimmunoprecipitated from ovary extracts, indicating that the two proteins form part of a protein complex.

The polarity of the oocyte forms in several steps, each of which requires the polarized microtubule cytoskeleton. Early in oogenesis, microtubules emanate from a microtubule organizing center (MTOC) at the posterior cortex of the oocyte. At this stage, microtubules are essential for oocyte determination; later in oogenesis, the location of the MTOC shifts to the anterior cortex of the oocyte and microtubules are essential for the dorso-ventral polarity of the oocyte. Egl protein is localized in a pattern reminiscent of the location of the minus ends of microtubules and Egl protein localization requires microtubules. Also, *egl* mutants expressing low amounts of *egl* produce eggs that lack dorsal structures, indicating that *egl* is also required for the dorso-ventral polarity of the oocyte. *egl* function may provide a link between microtubule function in oocyte determination and RNA localization in oocyte axis formation. Indeed, the elaboration of the oocyte/nurse cell axis may be the first step in the iterative elaboration of polarity in the *Drosophila* oocyte

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CHAPTER 1

Introduction

Summary

The *Drosophila* oocyte forms as one of a cluster of sixteen interconnected sister cells; the other fifteen cells form polyploid feeder cells known as nurse cells. Failure to specify the oocyte results in all sixteen cells developing as nurse cells. Oocyte determination requires the action of several genes, including *egalitarian* (*egl*) and *BicaudalD* (*BicD*), as well as function of the microtubule cytoskeleton (Schupbach and Wieschaus, 1991; Mohler and Wieschaus, 1986; Koch and Spitzer, 1983). The determination of the oocyte and its migration to the posterior of the oocyte-nurse cell cluster may be the first step in the specification of oocyte polarity, which is elaborated in two subsequent steps. First, *gurken* (*grk*) RNA is localized to the oocyte and Gurken protein signals to the surrounding somatic follicle cells, leading to anterior-posterior polarity in the oocyte (Gonzales-Reyes et al., 1995). Second, at a later stage of oogenesis, *grk* RNA is localized proximal to the oocyte nucleus and Grk protein signals to the overlying follicle cells, leading to dorso-ventral polarity in the oocyte (Neuman-Silberberg and Schupbach, 1993).

Cell polarity and asymmetric divisions in development

Development can be described as the process by which a cell becomes different from its sibling cells. I study this process in a system where one cell follows a strikingly different developmental program from that of its sister cells. By characterizing a mutant in which that one cell follows the same developmental program as its sisters, I can examine both the asymmetries that result in the specification of one cell as different from its sister cells and the asymmetries that result in the formation of polarity within that cell. The *Drosophila* oocyte forms from one of sixteen interconnected sister cells. This cell not only becomes different from its sisters, but it also becomes polarized to pattern the embryo that it will form. For example, localization of *bicoid* and *oskar* RNAs to the anterior and posterior poles of the oocyte pattern the anterior and posterior of

the embryo, respectively. Recent findings suggest that polarization of the oocyte cytoplasm depends on reciprocal signalling between the germ line derived cells, including the oocyte, and the surrounding somatic follicle cells (González-Reyes et al., 1995; reviewed in Ray and Schüpbach, 1996). Specification of the oocyte with respect to its sister cells is likely to be the first event required for the subsequent intracellular polarization of the oocyte.

Drubin and Nelson (Drubin and Nelson, 1996) define three stages in the establishment of cell polarity and examine these steps in bud or mating projection formation in *Saccharomyces cerevisiae* and in the formation of a polarized epithelium. The first stage involves the decoding of an internal or external cue in cell polarity. For example, in yeast a complex of actin and septin cytoskeletal proteins marks the previous bud site, which defines the next bud site. The formation of the yeast mating projection responds to an external gradient of pheromone. The basal side of an epithelial cell forms in contact with the extracellular matrix; this signal is transmitted through the cell membrane by integrins. In the next step, this cue is reinforced by the localization of proteins, including signalling molecules and components of the cytoskeleton that mark the site of polarity. In the third step, the signal for cell polarity is propagated or implemented by changes in the cellular architecture, including the cytoskeleton. In epithelial cells, this allows for the sorting of vesicles from the golgi and endosomes, allowing the targeted delivery of proteins to the apical or basolateral surfaces of the cell. Thus, the cell becomes polarized in response to a signal that indicates the location of a special region of the cell.

This polarity can provide the basis for asymmetric cell divisions, in which a cell follows a different cell fate from its sibling. For example, the polarity of the epithelium from which Drosophila neuroblasts delaminate defines the localization of the Inscuteable protein (Kraut et al., 1995; reviewed in Doe, 1996). Inscuteable localization, in an actin-dependent process, defines the localization of the Numb protein and the orientation of the subsequent cell division. Intriguingly, the localization pattern of Inscuteable, in an apical crescent, is the reciprocal of the localization pattern of Numb, which forms a basal crescent. Asymmetric segregation of Numb to one of the daughter cells causes it to adopt a different fate than its sibling (Rhyu et al., 1994; Spana et al., 1995).

Specification of the *Drosophila* oocyte from sixteen sister cells that share a common cytoplasm resembles the establishment of cell polarity described above in that spatial cues cause restructuring of the cytoskeletal architecture of the oocyte, thus allowing specialization of a region of the cytoplasm. Elaboration of oocyte anterior-posterior polarity follows from this specialization. Oocyte anterior-posterior polarity subsequently sets up the spatial cues that trigger a second cytoskeletal restructuring and elaboration of oocyte dorso-ventral polarity. Thus, polarity of the *Drosophila* oocyte is established by sequential iterations of a process analogous to the establishment of polarity in simpler cell types. In order to describe my study of the role of the gene

egalitarian in *Drosophila* oocyte determination and subsequent oocyte polarity, I will first describe the process of oocyte formation, including what is known about the genes that affect the process.

Oogenesis in *Drosophila*

Formation of the ovary and the oogenic cyst

The *Drosophila* ovary is composed of two different tissue types, germ line cells surrounded by mesodermal follicle cells (oogenesis is reviewed in King, 1970 and Spradling, 1993). The germ cells derive from the embryonic pole cells and the follicle cells derive from the somatic mesoderm. During embryogenesis, the pole cells migrate from their origin in the posterior midgut, through the gut to associate with the gonadal mesoderm. They remain as a loosely organized mass of cells, with large central germ cells enclosed by apical and basal populations of mesodermal cells, until the third larval instar. The first sign of incipient ovarian morphogenesis is the formation of the terminal filaments, stacks of 8-10 disc-shaped mesodermal cells. These stacks form in a regular array by intercalation of mesodermal cells in a layer and recruitment of additional mesodermal cells to the stack (Godt and Laski, 1995). The apical mesodermal cells then migrate around the terminal filament, dividing the population of germ cells into a series of tubes of developing cells that will become the ovarioles.

Mosaic analyses have shown that each ovariole contains two or three germ line stem cells. These must undergo and maintain germ line sex determination, a process very different from somatic sex determination. Both germ line and somatic sex determination act through the gene *Sex lethal* (*Sxl*) (Steinmann-Zwicky, 1994b), but the action of *Sxl* in the germ line is controlled by a different set of genes than in the soma (reviewed in Mahowald and Wei, 1994). These genes belong to a class of genes that, when mutant, cause an ovarian tumor phenotype. This class includes *ovo* and *ovarian tumor* (*otu*) (Oliver et al., 1987; Storto and King, 1988). Sxl^- germ cells also have an ovarian tumor phenotype when transplanted into a wild-type host to circumvent the XX lethality of *Sxl* due to a failure of dosage compensation (Steinmann-Zwicky et al., 1989). *ovo* and *otu* mutants can be suppressed by Sxl^{M1}, an allele that constitutively expresses *Sxl* (Pauli et al., 1993). Thus, although mutations that produce an ovarian tumor phenotype might be expected to control the pattern of cell divisions, many such mutations actually seem to affect germ line sex determination.

To both produce oocytes and renew the stem cell population, each germ line stem cell divides to produce a stem cell and a cell, called the cystoblast, that then differentiates. In this division, a vesicle-rich organelle called the spectrosome segregates asymmetrically, remaining only in the apical region of the stem cell (Lin and Spradling, 1995). A tumorous ovary gene, *bag-of-marbles* (*bam*) (McKearin and Ohlstein, 1995), affects the differentiation of cystoblasts from stem

cells and does not appear to be involved in germ line sex determination. *bam* mutants have an ovarian tumor phenotype, because in the absence of the ability to differentiate as cystoblasts, both daughters of the stem cell continue dividing as stem cells.

After the cystoblast forms, it then goes through four rounds of synchronous mitoses to produce a cluster of sixteen cells (diagrammed in the lower right corner of Figure 1-1). At each division, incomplete cytokinesis causes the formation of a cytoplasmic bridge between the two daughter cells. This bridge, or ring canal, becomes surrounded by a rim of filamentous actin and other proteins and allows for transport into the developing oocyte. The ring canals do not segregate randomly in each mitosis; rather, one cell inherits all previously made ring canal connections. After four rounds of mitosis, this produces a cluster of cells, two cells with four ring canals (grey in Figure 1-1, inset), two cells with three ring canals, four cells with two ring canals, and eight cells with one ring canal. This pattern of segregation indicates that the sixteen daughters of the cystoblast are not equivalent. Indeed, of the cluster of sixteen germ cells, one of the cells with four ring canals always becomes the oocyte; the other fifteen cells become polyploid feeder cells known as nurse cells.

Another example of asymmetric segregation in the cystoblast divisions is the behavior of the fusome (Storto and King, 1989). The fusome is a vesicle-rich organelle, very similar to the spectrosome found in the germ line stem cells. Both the fusome and the spectrosome contain membrane-skeleton proteins, such as spectrin, and have a similar appearance by electron microscopy (Lin and Spradling, 1995). The fusome forms in the cystoblast after the stem cell division and seems to orient the spindle in the cystoblast divisions. One of the centrosomes of the spindle associates with the fusome at each division (Lin et al., 1994). After the division, the fusome extends through the newly formed ring canal into the daughter cell where it can serve to orient the next division. In *hu-li tai shao* (*hts*) mutants, which lack the fusome, the pattern of cell divisions is perturbed, such that clusters form with fewer than sixteen germ cells (Yue and Spradling, 1992). The asymmetric segregation of the fusome may be important for oocyte determination because *hts* mutants rarely form oocytes.

Several mutations that affect the process of cystoblast divisions also affect the process of oocyte determination. In addition to being required for differentiation of the cystoblast in the stem cell division, *bam* also seems to act in the cystoblast divisions. Bam protein is expressed in the dividing cystoblast, where it localizes to the fusome as well as filling the cytoplasm of dividing cells. The divisions that form the sixteen cell cyst are also affected by the gene *encore* (*enc*). *enc* mutant germ cells undergo an extra round of mitosis, producing cysts with thirty-one nurse cells and one oocyte (Hawkins and Thorpe, 1996). This mutation also affects oocyte determination; the oocyte that forms becomes partially polyploid, even as it differentiates as an oocyte by accumulating yolk. *enc* seems to act through *bam*, because *enc* mutant females express more Bam

in the germarium. Also, *bam* mutations act as dominant suppressors of *enc*, such that *bam/+;enc/enc* females have fewer mutant egg chambers than *+/+;enc/enc* females. Thus, the control of the cystoblast divisions is linked to the control of oocyte determination, as shown by two different mutant phenotypes. *enc* mutants produce too many germ cells and make an oocyte with some nurse cell characteristics. *hts* mutants produce too few germ cells and rarely make an oocyte.

Figure 1-1 Introduction to oogenesis

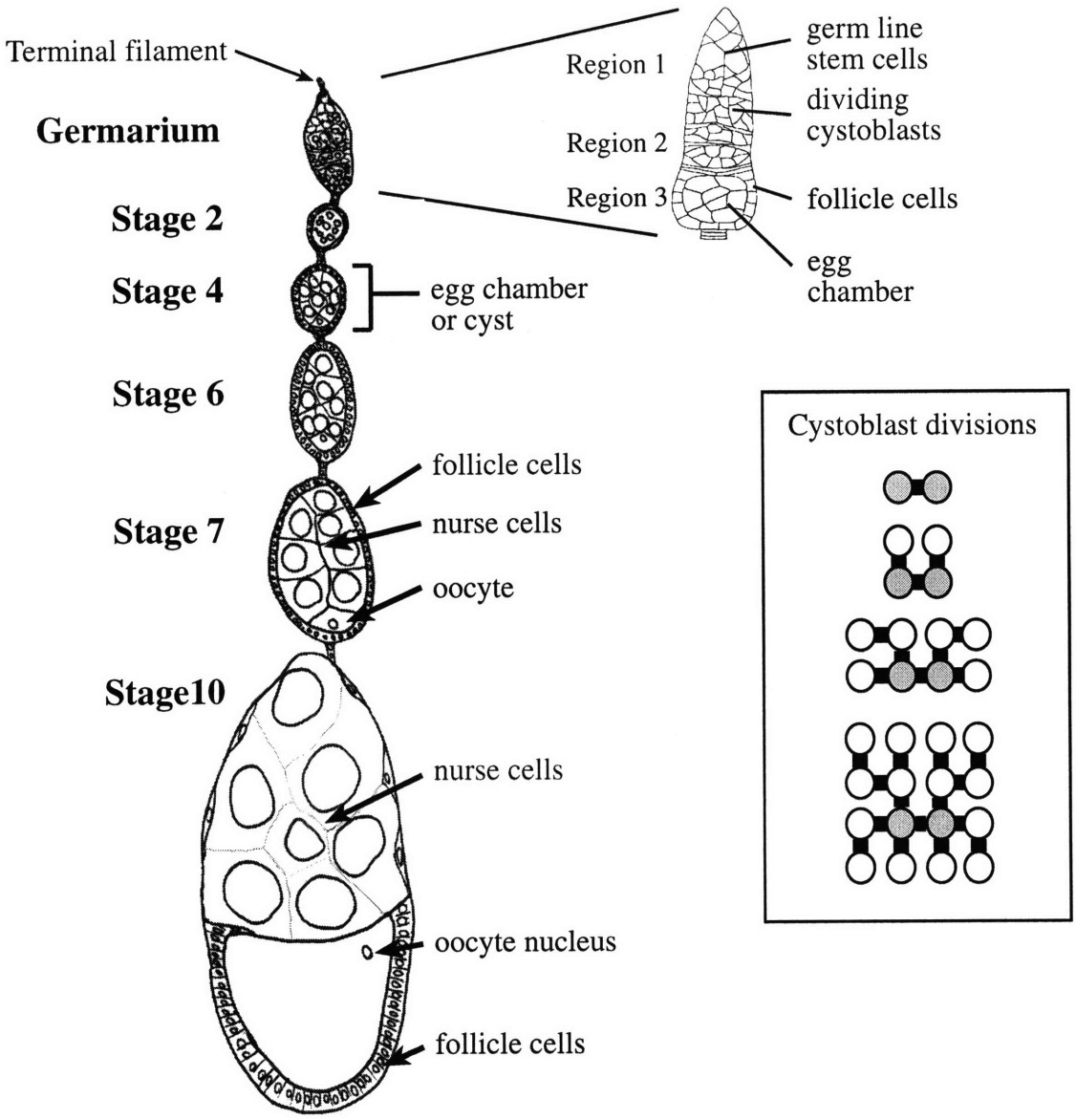
The Drosophila ovary is composed of strings of developing oocytes called ovarioles. A single ovariole is shown here. The stem cells reside at the anterior tip of the ovary, in the germarium; the cystoblast divisions, which form the cluster of sixteen cells, also occur in region one of the germarium. The cystoblast divisions are diagrammed in the inset at the lower right corner of the figure. At each division, incomplete cytokinesis leaves a cytoplasmic bridge, or ring canal, which is represented as a black bar. At the end of four rounds of mitosis, two cells (grey) have four ring canals; one of these becomes the oocyte. In region two of the germarium, the mesodermal follicle cells move in to surround the cysts and the two cells with four ring canals form synaptonemal complex. The cysts flatten and then round up with the oocyte at the posterior in region three, which is the same as stage one of oogenesis.

The sixteen-cell cyst, or egg chamber, is surrounded by follicle cells and buds off from the germarium at stage 1. As the stages of oogenesis progress, the cyst moves toward the posterior as it is displaced by younger cysts; thus, the ovariole forms a developmental progression from the youngest egg chambers at the anterior, to the oldest egg chambers at the posterior. From stage 2 to stage 7, the oocyte and nurse cells grow at approximately the same rate. The nurse cell nuclei have several distinguishable stages: at stage 4, the nurse cell nuclei appear polytene; in stages 5 and 6, they are no longer polytene, but continue to be polyploid; by stage 7, the nurse cell nuclei closer to the oocyte have a higher DNA content than those further from the oocyte.

At stage 8, the oocyte begins to accumulate yolk and begins to expand much faster than the nurse cells. The oocyte nucleus moves to the dorsal anterior corner of the oocyte. At stage 9, the follicle cells begin to migrate over the oocyte, which continues to expand. At stage 10, the follicle cells have completed their migrations and the oocyte is the same size as the entire cluster of nurse cells.

The stages after 10 are not shown. The second half of stage 10 is marked by cytoplasmic streaming from the nurse cells into the oocyte; the nurse cell nuclei remain at the anterior of the cluster and eventually degenerate. In these later stages, the follicle cells migrate to seal the anterior of the oocyte, secrete the egg coverings and form specialized egg shell structures such as the dorsal appendages. After stage 14, the egg is activated in the uterus, fertilized and laid.

Figure 1-1 Introduction to oogenesis



Structure of the ovary

All of the divisions that produce the sixteen-cell cyst occur in region 1 of the germarium at the anterior tip of the ovary (Figure 1-1). After the complete cyst forms, the two cells with four ring canals produce synaptonemal complex (Carpenter, 1975). Complete cysts in region 2 become flattened and follicle cells begin to migrate to surround the cluster of germ cells. As it is surrounded by follicle cells, the cluster rounds up such that one of the cells with four ring canals, the cell that will become the oocyte, is at the posterior. The localization of the oocyte to the posterior allows for the passage of two rounds of reciprocal signals between the oocyte and the follicle cells. These signals, which will be discussed below, polarize the oocyte first along its anterior-posterior axis, and then along its dorso-ventral axis. Thus, the determination of the oocyte is the first asymmetry that sets the stage for future cytoplasmic asymmetries that polarize the egg and control embryonic polarity.

The development of the oocyte after it leaves the germarium has been divided into fourteen stages, beginning with stage 1, which is the equivalent of germarial region 3, the cyst surrounded by follicle cells and budding off from the germarium. The stages of oogenesis are reviewed in King, 1970 and Spradling, 1993 and are diagrammed in Figure 1-1. At approximately stage 1, one of the two cells with four ring canals decondenses its chromosomes; the other cell with four ring canals remains in meiosis and becomes the oocyte. At about stage 3, the oocyte chromatin condenses to form the karyosome, a hollow ball with a dense core of chromatin in prophase. The nurse cell nuclei become polyploid and synthesize cytoplasmic components that are transported into the oocyte by a microtubule-dependent mechanism. The synaptonemal complex in the oocyte karyosome disappears by approximately stage 7. The oocyte expands relative to the follicle cells and begins to accumulate yolk at stage 8; by stage 10 the oocyte has expanded to make up half of the cluster. In stage 10b, the nurse cells contract and expel their contents into the oocyte, leaving only their degenerating nuclei at the anterior. At stage 14, the oocyte has passed from prophase into metaphase arrest, the egg coverings are complete and the egg is ready to be activated and fertilized in the uterus, then laid.

The next sections will consider in detail some of the processes in oocyte development, specifically, the development of the somatic follicle cells, the process of oocyte determination, the role of the microtubule cytoskeleton in oogenesis and the establishment of oocyte polarity.

Follicle cell morphogenesis

The mesodermal follicle cells contribute to both the structure of the ovary and the cell-cell interactions of oogenesis. During embryogenesis the germ cells migrate to make the gonad, but during oogenesis different populations of follicle cells migrate to surround the germ cell cyst,

separate it from the germarium, cover the oocyte and become positioned to make important structures in the eggshell. The follicle cells are also involved in several signalling processes that affect the germ cells in oogenesis. For example, ablation of the terminal filament causes an increase in egg chamber production from isolated transplanted germaria (Lin and Spradling, 1993), indicating an interaction between these cells and the germ line stem cells.

Mosaic analysis and BrdU labelling showed that there are approximately two stem cells for the follicle cells located in the germarium, in region 2b (Margolis and Spradling, 1995). These stem cells divide to form the approximately sixteen cells that migrate to surround the germ cell cluster in region 2 of the germarium and form several specialized populations of follicle cells. These cells stop dividing at stage 6 of oogenesis, when approximately one thousand cells cover the cyst. The divisions of the follicle cells are stimulated by *hedgehog* (*hh*) signalling from the terminal filament cells at the anterior of the germarium (Forbes et al., 1996). *hh* is an evolutionarily conserved secreted signalling molecule required for multiple patterning events during development, including segmentation and wing disc patterning. Reduction of the amount of *hh* activity during oogenesis causes the follicle cells to fail to encapsulate cysts; augmentation of the amount of *hh* activity during oogenesis causes the follicle cells to overproliferate, producing an excess of follicle cells between cysts.

The somatic follicle cells form a monolayer around the cyst and separate it from the germarium. They also form specialized stalk cells that connect the cysts to each other in the ovariole. The process of stalk cell specification involves many neurogenic genes, including *Notch* (*N*), *Delta* (*Dl*), and the multifunctional transcription regulator *daughterless* (*da*) (Cummings and Cronmiller, 1994; Ruohola et al., 1991). *N*, *Dl* and *da* are expressed in the follicle cell layer during early oogenesis. Reduction in the activity of any of these genes causes a failure of stalk formation and cyst encapsulation, resulting in follicles containing multiple sixteen-cell clusters.

N and *Dl* mutations also affect another specialized group of follicle cells, the polar cells. By staining with various markers, Ruohola *et al.* (Ruohola et al., 1991) found that *N* and *Dl* mutant ovaries have an excess of polar follicle cells. The polar follicle cells form at the anterior and posterior end of each cyst. They cease dividing long before the other follicle cells of the cyst (Margolis and Spradling, 1995) and express several different markers, including FasciclinIII and various lacZ enhancer traps. The polar cells at the posterior of the egg chamber respond to *gurken* signalling from the oocyte, as discussed below. In the absence of this signal, the polar cells develop as anterior cells, forming migratory cells known as border cells.

At stage 9 of oogenesis, as the oocyte expands with yolk, the follicle cells migrate off of the nurse cells to cover the oocyte. Only approximately fifty follicle cells remain over the nurse cells, where they lie in the crevices between the large nurse cells. At this stage, the border cells detach from the anterior of the follicle cell epithelium and migrate between the nurse cells to the

anterior of the oocyte. The border cells are joined at the anterior of the oocyte by centripetally migrating follicle cells from around the oocyte, which serve to close off the anterior of the egg. After the nurse cells expel their cytoplasm into the oocyte, the follicle cells migrate to close off the anterior of the egg. The follicle cells secrete the vitelline membrane and the chorion, the two layers covering the egg. The follicle cells also construct several specialized structures, including the micropyle, which provides an entry point for the sperm at the anterior, and the dorsal appendages, two tubes which allow for gas exchange. These structures also conveniently provide markers for the polarity of the egg.

Oocyte determination and differentiation

The process of oocyte determination requires several steps, including the formation of the sixteen cell cyst with its appropriate lineage, the establishment of a polarized array of microtubules emanating from the oocyte and the localization of important RNAs and proteins via that array. The establishment of the sixteen-cell cyst is required for oocyte determination, as described above. As soon as the complete cyst forms, in region 2 of the germarium, it manifests signs of oocyte determination: the centrioles from the nurse cells move to the presumptive oocyte, a MTOC forms in that cell (Theurkauf et al., 1993), and the two cells with four ring canals enter meiosis (Carpenter, 1975). All sixteen cells go through an initial, long premeiotic S phase. The two cells with four ring canals begin to condense their chromosomes, producing synaptonemal complex (Carpenter, 1975). The two cells with three ring canals also produce synaptonemal complex that quickly dissipates. As the centrioles from the fifteen nurse cells move into one cell, the synaptonemal complex in the other cell breaks down. Only one of the cells with four ring canals maintains its state of chromosome condensation; that cell becomes the oocyte and becomes arrested in prophase of the first meiotic division.

egalitarian and *BicaudalD* in oocyte determination

This work is mainly concerned with two genes that affect the process of oocyte determination. In females mutant for *egalitarian* (*egl*) (Schüpbach and Wieschaus, 1991) or *BicaudalD* (*BicD*) (Mohler and Wieschaus, 1986; Suter et al., 1989; Wharton and Struhl, 1989) loss-of-function alleles, the cystoblast divisions occur normally, but the oocyte fails to develop; the cell that would normally become the oocyte instead forms an additional nurse cell. This phenotype will be referred to as the sixteen nurse cell phenotype. All sixteen nuclei in the mutant cluster become polyploid and the cyst develops to approximately stage 6 of oogenesis before degenerating. Reconstruction of serial electron microscopic sections of *egl* mutant germaria (Carpenter, 1994) has revealed that in *egl* mutants, one of the cells with four ring canals does move

to the posterior. Interestingly, this analysis also showed that in *egl* mutants, all sixteen cells enter meiosis, forming synaptonemal complex. The mutant germ cells then lose their synaptonemal complex and become polyploid; this behavior resembles that of the wild type cell that has four ring canals but does not become the oocyte. Unfortunately, the electron microscopic examination of *BicD* mutants has not been done. Examination of the ring canal structure of *BicD* null mutants has shown that the largest ring canal, that connecting the two cells with four ring canals, stays in the center of the cluster, indicating that neither of the cells with four ring canals moves to the posterior in this genotype (Ran et al., 1994).

BicD has been cloned (Suter et al., 1989; Wharton and Struhl, 1989) and its RNA localizes to a single cell in the cyst as early as stage one of oogenesis. The BicD transcript accumulates at the posterior of the oocyte from stage 2 to stage 7 and then shifts to form an anterior ring at stage 8 and 9. This distribution resembles the distribution of many RNAs, such as *gurken* and *oskar* (Ephrussi et al., 1991; Kim-Ha et al., 1991; Neuman-Silberberg and Schüpbach, 1993), that become enriched in the early oocyte, then form an anterior ring at later stages of oogenesis.

The BicD protein is similar to myosin tails and is predicted to form a coiled-coil structure (Suter et al., 1989; Wharton and Struhl, 1989). The similarity to myosin tails may only be an indication of the coiled-coil structure of BicD protein, rather than a functional homology. Indeed, the Paircoil program (Berger et al., 1995) predicts that approximately three quarters of the BicD protein forms blocks of coiled-coil. BicD protein is phosphorylated (Suter and Steward, 1991). Also, BicD protein accumulates in the oocyte very early in oogenesis, almost as soon as the sixteen-cell cluster forms. It localizes to the posterior cortex of the oocyte from stage 2 to stage 7. At stage 8, BicD protein accumulates in an anterior ring. Thus, BicD protein localization in the oocyte resembles the localization of many RNAs.

In *BicD* dominant mutants ($BicD^{D}$), the oocyte develops but the polarity of the embryo is altered by mislocalization of the *oskar* (*osk*) mRNA (Ephrussi et al., 1991; Mohler and Wieschaus, 1986). Females heterozygous for a *BicD* dominant allele ($BicD^{71.34}$ or $BicD^{IIIE}$) produce embryos that develop with reduced head structures due to a partial mislocalization of *oskar* mRNA to the anterior of the oocyte (Ephrussi et al., 1991). In the most extreme case double-abdomen (bicaudal or "two-tail") embryos develop, in which ectopic posterior structures replace anterior structures (Mohler and Wieschaus, 1986). Antibody staining reveals that BicD protein accumulates more strongly in the early oocyte in $BicD^{D}$ mutants than in wild type. Also, wild-type BicD protein is uniformly distributed in the early embryo, but in $BicD^{D}$ mutants, BicD protein accumulates at the anterior of the embryo (Wharton and Struhl, 1989). Despite this change in protein localization, the $BicD^{D}$ alleles behave as antimorphs; females carrying a dominant allele with extra copies of the wild-type gene ($BicD^{D}$/+/+) produce fewer bicaudal embryos than produced by females heterozygous for $BicD^{D}$(Mohler and Wieschaus, 1986).

Another gene, *stonewall* (*stwl*), also affects the process of oocyte determination, producing a sixteen nurse cell phenotype (Clark and McKearin, 1996). The sequence similarities, in a helix-turn-helix motif, between Stwl and known transcription factors indicate that Stwl may act by regulating the expression of genes required for oocyte determination. Stwl protein is localized to the nuclei of the oocyte and the nurse cells from stage 1 to stage 7, and BicD protein is expressed, but unlocalized, in *stwl*$^{-}$. Thus, *stwl* may regulate the transcription, not of BicD itself, but of another factor required for BicD localization.

Cell cycle control in oocyte determination

The nurse cell and the oocyte have very different cell cycles. The oocyte arrests in prophase of meiosis I; the nurse cells enter a cycle of endoreplication, becoming polyploid by continuing to replicate their DNA without dividing. Although the *egl* mutant defect has primarily been described as a defect in oocyte determination, the observation that all sixteen *egl*$^{-}$ germ cells transiently enter meiosis (Carpenter, 1994) shows that all sixteen cells express an *egl* mutant phenotype. Several mutations affect both the cystoblast divisions and the cell cycle of the cystoblast progeny. For example, *encore* mutant germ cells undergo an extra round of mitosis, forming a cyst with thirty-two cells. In these cysts, the oocyte develops a polyploid nucleus, like that of a nurse cell (Hawkins et al., 1996).

The phenotype of a hypomorphic allele of *cyclinE* provides another link between control of the cell cycle and oocyte determination. In wild type, CyclinE levels are high in the oocyte nucleus and oscillate in the nurse cell nuclei; in *cycE*^{01672} mutants, CyclinE levels are reduced and do not fluctuate in the nurse cells. In these mutants, one or two of the nurse cell nuclei do not become polyploid; instead they form additional oocyte nuclei (Lilly and Spradling, 1996). In contrast to the *egl* and *BicD* phenotypes, in which no oocyte forms, this hypomorphic allele of *cyclinE* causes additional cells to differentiate with some oocyte characteristics. Thus, one of the aspects of oocyte determination involves control of the cell cycle.

The meiotic phenotype of *egl* bears similarity to the phenotype of null mutations in the *germline development* mutation *gld-1* (Francis et al., 1995; Francis et al., 1995) of *C. elegans*. *gld-1* encodes a protein with similarities to known RNA-binding proteins (Jones and Schedl, 1995). Oocytes in *C. elegans* proliferate at the distal tip of the gonad, then enter meiosis and grow while arrested in the pachytene stage of meiosis I. In hermaphrodites mutant for some loss-of-function alleles of *gld-1*, the oocytes enter pachytene, but then instead of forming oocytes, they revert to the mitotic cell cycle. The initial proliferation of the gametes depends on the signal from the germline proliferation gene *glp-1*, but *gld-1* mutants divide even in the absence of the *glp-1* signal, forming what has been called ovarian tumors. Genetic analysis of double mutants with sex determination genes indicates that *gld-1*, unlike many *Drosophila* ovarian tumor mutants, acts

downstream of the sex determination pathway. Therefore, the *gld-1* phenotype resembles the *egl* phenotype in that the germ cells enter meiosis, but revert from the meiotic pachytene arrest to either mitosis in *gld-1*, or the endoreplication cell cycle in *egl*.

Spermatogenesis

The process of spermatogenesis in *Drosophila* also involves regulated divisions of germ line precursor cells and cooperation with somatic follicle cells in a process that has both significant similarities to and telling differences from oogenesis. For a review of spermatogenesis, see Fuller, 1993. The oogonial stem cells associate with *hh*-expressing follicle cells in the terminal filament; the spermatagonial stem cells associate with *hh*-expressing follicle cells in a structure called the hub. The divisions of the precursor cells in both oogenesis and spermatogenesis also are superficially similar. The primary spermatagonial cell goes through four rounds of division to form a cluster of sixteen cells that remain interconnected by ring canals. These ring canals contain filamentous actin and have other components in common with the ovarian ring canals. The spermatagonial divisions also involve the production of a fusome. *hu-li tai shao* (*hts*) mutations abolish the fusome (Lin et al., 1994); although the involvement of *hts* in male fertility has not been studied, at least one allele of *hts* is male sterile (H. Lin, personal communication), indicating that the fusome is important for spermatogenesis as well as oogenesis. Several other genes that control the cystoblast divisions in oogenesis, including *bag-of-marbles* (*bam*) and *benign gonial cell neoplasm* (*bgcn*) also affect the spermatagonial divisions.

One difference between spermatogenesis and oogenesis is in the behavior of the somatic follicle cells. A spermatogenic cyst associates with only two follicle cells which do not divide, unlike the oogenic cyst, which associates with many follicle cells that divide many times. The divisions of the oogenic stem cells are not coordinated with the divisions of the follicle cells (Margolis and Spradling, 1995), but the divisions of the germ line and soma are coordinated in spermatogenesis (Gönczy and DiNardo, 1996). The germ cells in spermatogenesis seem to repress the proliferation of the follicle cells, because in agametic testes, the cyst progenitor cells and the hub cells overproliferate (Gönczy and DiNardo, 1996).

Unlike *bam* and *bgcn*, however, *egl* and *BicD* mutations only affect female fertility. The *Drosophila* oocyte enters meiotic arrest and grows while its sister cells become polyploid nurse cells. In contrast, all sixteen primary spermatocytes enter meiosis, growing before prophase, in preparation for the meiotic divisions that will form sixty-four sperm. Thus, although many of the same players are involved in spermatogenesis and oogenesis, because all sixteen primary spermatids follow the same fate, there is no need for the establishment of asymmetry between the cells, and thus no need for the function of genes such as *egl* and *BicD*.

Microtubules and oocyte development

Microtubules are polar structures that serve as tracks for directional transport and are essential for the establishment and maintenance of polarity in many systems (reviewed in Drubin and Nelson, 1996). In *Drosophila*, the function of the microtubule cytoskeleton is crucial for oocyte determination as well as oocyte growth and polarity. The microtubule cytoskeleton undergoes complex rearrangements during oogenesis; these rearrangements correlate with its functions as defined by inhibitor studies. *egl* and *BicD* mutations affect the stability or establishment of the microtubule structure of the ovary.

Microtubule cytoskeleton

Microtubules have two characteristics that define the basis for their functions: dynamic instability and polarity. For a review of microtubule function, see Hyman and Karsenti, 1996. Microtubules are composed of α and β tubulin, which associate head-to-tail in a spiral to form a long, hollow tube. The γ tubulin protein, which forms part of a complex resembling a ring with a slight offset (Moritz et al., 1995; Zheng et al., 1995), nucleates microtubule formation by acting as a template for the assembly of α-β heterodimers. β tubulin associates with the γ tubulin template at the stable minus end of the microtubule, thus initiating the polarity of the microtubule. New heterodimers add on at the opposite, or plus, end; microtubules also tend to depolymerize from their plus ends. γ-tubulin is a component of the centrosome (centrioles and pericentriolar material), which acts to nucleate and organize spindle microtubules (Moritz et al., 1995; Zheng et al., 1995).

Unless stabilized by interaction with other proteins, microtubules tend to stochastically depolymerize with a high rate of turnover. Hydrolysis of GTP is required for the instability of the microtubule; this energy may be stored as mechanical strain on the lattice which is released when the microtubule depolymerizes (Drechsel and Kirschner, 1994). Although the basis for the change between the growth phase and the depolymerization phase is not known, the plus end of a growing microtubule may be protected by a cap of α-β heterodimers that have not hydrolyzed their bound GTP (Mitchison, 1993). Another theory for the stabilization of the growing end, based on cryo-electron microscopy of growing microtubules is that the end of a microtubule grows as a sheet, which then closes to form a tube (Chretien et al., 1995). The sheet is structurally more stable than the tube; when the microtubule closes completely, it becomes unstable and depolymerizes, releasing the stored energy. Because of their dynamic instability, the microtubules in a cell turn over frequently, catastrophically depolymerizing and then repolymerizing.

The polarity of microtubules not only reflects their structure and their stability, but is also used by microtubule motors to move cellular components (reviewed in Vallee and Sheetz, 1996). A specific microtubule motor, such as some types of kinesin, will move toward one end of the microtubule, in this case the plus end. Other motors, such as dynein or some members of the

kinesin family, move toward the minus end. By harnessing a load to a specific motor, a cell with a polarized cytoskeleton can direct the movement of cellular components. This mechanism of transport is important for chromosomal segregation, vesicle transport, secretion and endocytosis.

Microtubule dynamics in *Drosophila* oogenesis

In Drosophila oogenesis, the microtubule cytoskeleton is polarized along the oocyte nurse-cell axis (Figure 1-2); this polarity allows for the localization of specific RNAs and proteins to the oocyte. The dynamic instability of microtubules indicates that their stabilization might be important in the maintenance of this polarity, and thus in the formation of the oocyte. The function and distribution of microtubules in oogenesis have been examined by immunolocalization of microtubules, by depolymerization of microtubules, and by examination of the effects of different mutations on the microtubule organization of the ovary.

Theurkauf *et al.* (Theurkauf et al., 1992) examined the dynamics of the microtubule cytoskeleton during Drosophila oogenesis. They inferred the position of microtubule organizing centers (MTOCs) from the position of collections of microtubules. They also determined the position of the minus ends of microtubules in later oogenesis by treating briefly with colcemid to partially depolymerize the microtubules, then looking for short residual filaments marking the stable ends. After the divisions that establish the sixteen-cell cyst, a polarized array of microtubules forms from an MTOC in the presumptive oocyte at stage 1. This MTOC initially forms in the anterior of the oocyte, next to the ring canals, but moves to the posterior of the oocyte where it remains from stage 2 to stage 7. At stage 9, the location of the minus ends of the microtubules shifts to the anterior cortex of the oocyte, where the oocyte contacts the nurse cells.

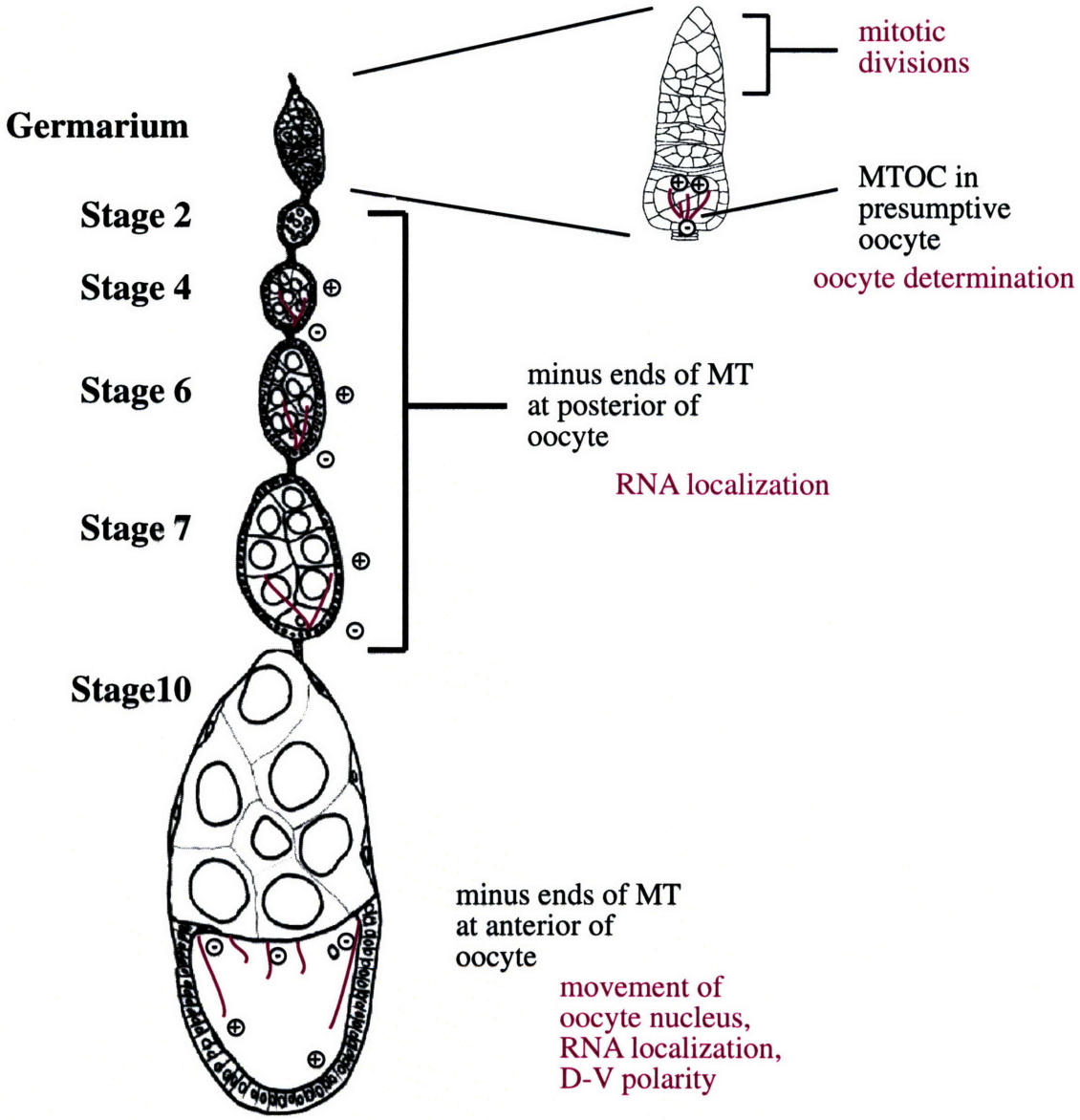
The localization of the microtubule motors kinesin and dynein during oogenesis partially supports this description of the polarity of the microtubule cytoskeleton during oogenesis. The plus-end directed motor kinesin fused to the reporter molecule β-galactosidase localizes transiently to the posterior of the oocyte at stages 8 and 9 (Clark et al., 1994). Antibodies to the minus-end directed motor cytoplasmic dynein show that it accumulates in the oocyte in region 2b of the germarium and localizes to the posterior of the oocyte by stage 3 (Li et al., 1994). Surprisingly, dynein localizes to the posterior of the oocyte at stage 9, when the plus-end directed motor kinesin also localizes to the posterior. This apparent contradiction shows the difficulty in inferring the orientation of microtubules from the accumulation of motors.

Figure 1-2 microtubule function in oogenesis

Microtubules are represented by magenta lines, with the locations of their plus and minus ends indicated. After the sixteen-cell cluster forms, a polarized array of microtubules forms from a microtubule organizing center (MTOC) in the oocyte. This MTOC remains at the posterior of the oocyte from stage 1 to stage 7 of oogenesis. At stage 8, the microtubule structure shifts so that microtubules are nucleated from the anterior cortex of the oocyte (Theurkauf et al., 1992).

Inhibitor studies (Koch and Spitzer, 1983; Theurkauf et al., 1993)Pokrywka and Stephenson, 1991 have shown that microtubules are required for several processes during oogenesis, described in magenta type. The microtubules of the spindle are required for the stem cell and cystoblast divisions. The polarized array of microtubules is required for oocyte determination, RNA localization, movement of the oocyte nucleus and dorsoventral polarity of the oocyte.

Figure 1-2 Microtubule function in oogenesis



Microtubule functions in oogenesis

Inhibitor studies have defined several functions for microtubules in *Drosophila* oogenesis, including oocyte determination, transport into the oocyte, movement of the oocyte nucleus and establishment of oocyte dorsoventral polarity (Koch and Spitzer, 1983; Theurkauf et al., 1993). Localization of kinesin and cytoplasmic dynein, which correlates with the pattern of localization of some RNAs in wild type and mutant ovaries, provides circumstantial evidence for a role in localization of RNAs in oogenesis. Unfortunately, little genetic evidence exists for the importance of microtubules in oogenesis, aside from the intriguing observation that some cytoplasmic dynein mutations cause the oocyte to form a sixteenth nurse cell, a phenotype similar to that of *egl* mutations (Li et al., 1994; McGrail and Hays, 1996).

The establishment of the MTOC in the developing oocyte is one of the key events of early oogenesis. Because centrosomes nucleate microtubules, the oocyte centrosome may initiate the formation of the MTOC. However, the nurse cells also contain centrosomes and the mechanism that prevents these from also nucleating microtubules is unknown. Theurkauf has proposed that the semiconservative replication of centrioles may provide a marker for the oocyte, analogous to the methylation of DNA (Theurkauf et al., 1992). Only the centriole bearing modifications made in the stem cell would be capable of forming the MTOC in the oocyte. Lin and Spradling have proposed that the fusome may also play a role in selectively activating the oocyte centrosome as it is required for oocyte formation and associates with the centrosomes at each cystoblast division (Lin and Spradling, 1995). The mechanism by which the MTOC forms specifically in the oocyte remains unknown.

BicD and *egl* mutations also affect the microtubule organization of the cyst and the localization of RNAs to the developing oocyte. One of the earliest signs of oocyte determination is the formation of a microtubule organizing center (MTOC) in the oocyte (Theurkauf et al., 1992). In *BicD* loss-of-function mutants, the MTOC never forms; in *egl* mutants the MTOC forms, but subsequently dissipates. Since BicD protein localizes to a single cell in one of these *BicD* alleles, BicD can localize without the formation of an MTOC. Localization of BicD to a single cell may thus initiate the asymmetry that leads to the formation of the MTOC in one cell, the future oocyte.

In *egl* mutants the MTOC forms and then immediately dissipates, suggesting that *BicD* and *egl* act sequentially and that *egl* may play a direct role in differentiation of the oocyte. Although this seems to indicate that *BicD* acts upstream of *egl*, the two BicD alleles used in this study were not null alleles. For example, $BicD^{r26}$, shows enhanced localization of BicD protein to the oocyte (Suter and Steward, 1991; Wharton and Struhl, 1989) Both alleles allow localization of *oskar* RNA to a single cell (Suter and Steward, 1991); null mutations *of BicD* abolish localization of

oskar RNA to the oocyte (Ran et al., 1994). Thus, the exact effect of *BicD* mutations on the formation of the MTOC and the order in which *BicD* and *egl* act remains unclear.

Establishment of oocyte polarity

Movement of the oocyte to the posterior of the cyst is required for oocyte polarity

Just as the cyst has polarity with the oocyte at the posterior, so the oocyte has polarity, with specialized regions of cytoplasm or special structures at the anterior, posterior and dorsal sides. These regions contain localized RNAs and proteins, such as *gurken* mRNA at the dorsal-anterior side, that contribute to oocyte polarity, and thus to embryo polarity. The anterior-posterior axis of the oocyte is marked by the localization of *bicoid* mRNA to the anterior (Berleth et al., 1988; St Johnston et al., 1989) and *oskar* mRNA to the posterior (Ephrussi et al., 1991; Kim-Ha et al., 1991).

Movement of the oocyte to the posterior of the cyst, which happens between regions 2 and 3 in the germarium, is required for proper polarity of the oocyte. Mutants in which the oocyte fails to move to the posterior also cause perturbations of embryonic polarity. For example, in *spindleC* (*spnC*) mutants, the oocyte remains centrally located and develops with a cluster of nurse cells at either end (González-Reyes and St Johnston, 1994). These oocytes localize *bicoid* mRNA to both ends of the oocyte and localize *oskar* mRNA to the center of the oocyte. Mutations in *dicephalic* (Frey et al., 1984), in *homeless*, a putative RNA-binding protein (Gillespie and Berg, 1995), and in *armadillo*, which is related to components of adherens junctions (Peifer et al., 1993), also affect the movement of the oocyte to the posterior. The phenotype resulting from failure of oocyte movement indicates that contact between the oocyte and the posterior follicle cells is required for the establishment of the oocyte posterior.

gurken signalling between the oocyte and the follicle cells establishes oocyte polarity

Specialized follicle cells communicate with the oocyte both in establishment of the posterior of the oocyte and later in oogenesis in the establishment of the dorsal region of the oocyte (Figure 1-3; reviewed in Ray and Schüpbach, 1996). These specialized follicle cells, polar cells, are at the anterior and posterior poles of each egg chamber. Both anterior and posterior polar cells will develop as anterior cells, but a signal from the oocyte induces the nearby polar cells to change to a posterior cell fate (González-Reyes et al., 1995; Roth, 1995). This signalling proceeds via *gurken* (*grk*), which encodes a protein similar to TGF-α and *torpedo* (*tor*), the Drosophila EGF receptor (Clifford and Schüpbach, 1992; Neuman-Silberberg and Schüpbach, 1993). Later in oogenesis, *grk* modulates a second set of signals between the oocyte and the follicle cells, this time in the establishment of the dorsal side of the oocyte. At stage 8 of oogenesis, the oocyte nucleus moves

from the posterior of the oocyte to the anterior. This process requires microtubule function; females treated with microtubule-depolymerizing drugs can produce eggs that have a loss of dorsal structures, similar to those produced by *grk* mutant females (Koch and Spitzer, 1983). *grk* RNA localizes proximal to the oocyte nucleus, where Grk protein signals to the overlying follicle cells to promote dorsal cell fate (Neuman-Silberberg and Schüpbach, 1993).

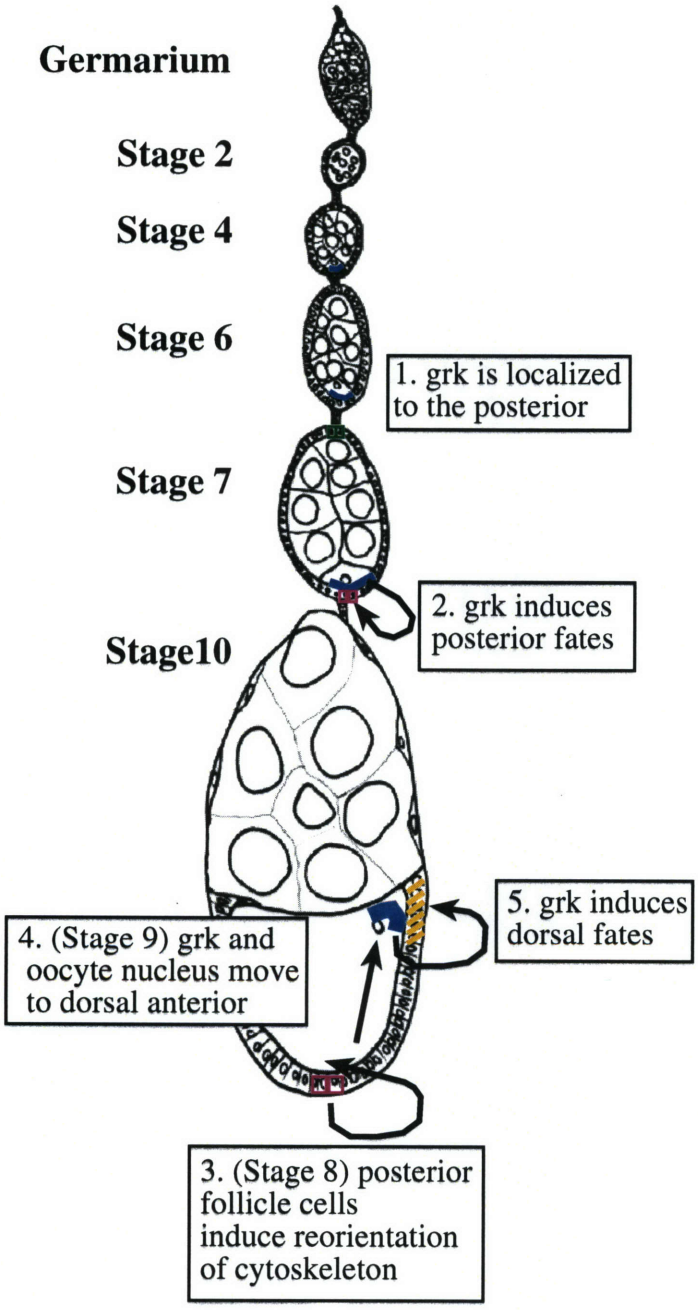
In the absence of *grk* function in the germ line, the follicle cells at the posterior express anterior markers such as *slow border cells*, migrate centripetally as if they were closing the anterior of the oocyte, and produce an ectopic micropyle at the posterior (González-Reyes et al., 1995). This change of follicle cell fate also causes a failure in oocyte polarity. Although the nature of the reciprocal signal from the germ cells to the oocyte is unknown, the activity of Protein Kinase A is required in the germ line to transduce this signal (Lane and Kalderon, 1994). In *grk* mutants, which fail to produce the signal from the oocyte, the absence of the follicle cell signal causes a failure in the reorganization of the microtubule cytoskeleton, such that the minus ends of the microtubules remain at the posterior instead of shifting to the anterior cortex (González-Reyes et al., 1995). This causes the oocyte nucleus to remain at the posterior instead of migrating to the dorsal anterior. RNAs that localize to the anterior or posterior poles also mislocalize. For example, *bicoid* mRNA, which usually localizes to the anterior of the oocyte, now localizes to the anterior and the posterior. *oskar* mRNA, which usually localizes to the posterior, now localizes to the center of the oocyte . Thus, the oocyte behaves as if it had two anterior poles, with the minus ends of microtubules at both ends.

The elaboration of pattern in the *Drosophila* oocyte proceeds in three steps: oocyte specification, anterior-posterior axis formation and dorso-ventral axis formation. These steps require the localization of specific RNAs and the function of the microtubule cytoskeleton. Genes required for processes common to all three steps may link these three sequential cellular asymmetries. In order to examine the molecular basis of oocyte determination, I have cloned the *egl* gene. In this thesis, I show that Egl protein and BicD protein colocalize during oogenesis. Initially both proteins accumulate in the future oocyte; they then concentrate at the posterior cortex of the oocyte before they transiently localize to the anterior margin of the oocyte. At each step the localization pattern resembles that of the presumed minus-ends of microtubules. Immunoprecipitation experiments demonstrate that Egl and BicD are part of a protein complex. Analysis of mutations in *egl* and *BicD* suggest that this complex is required for the specification of the oocyte and the establishment of oocyte polarity.

Figure 1-3 *grk* signalling in oogenesis

grk-mediated signalling causes polarization of the oocyte along both the anterior-posterior and dorso-ventral axes, at two different stages of oogenesis (González-Reyes et al., 1995). During early oogenesis, *grk* transcript (blue) is localized to the posterior cortex of the oocyte (1 Neuman-Silberberg and Schüpbach, 1993), where it signals to the posterior follicle cells, inducing them to develop as posterior cells (2, magenta) instead of anterior cells (green). During later stages of oogenesis, the posterior follicle cells signal to the oocyte, inducing a reorientation of the microtubule network (3) so that the minus ends of the microtubules are now at the anterior. The oocyte nucleus and *grk* RNA move to the dorsal anterior (4 Neuman-Silberberg and Schüpbach, 1993) where *grk* signals the overlying follicle cells to develop as dorsal cells (5, yellow).

Figure 1-3 *gurken* signalling in oogenesis



Specific Aims

The aims of this thesis are to describe my cloning and characterization of the gene *egalitarian*, including its requirement in oocyte determination, its localization during oogenesis, its interaction with *BicaudalD*, its dependence on the microtubule cytoskeleton, and its function in effecting the dorsoventral pattern of the oocyte. In Chapter 2, I describe my characterization of the *egl* phenotype and the requirement for *egl* function in the germ line to specify the oocyte. This chapter also describes my cloning of the *egl* locus. As part of the subsequent analysis, I describe the localization of the *egl* protein product within the developing oocyte. In Chapter 3, I describe the interaction between *egl* and *BicD*, including their colocalization, genetic interaction, and biochemical interaction. In Chapter 4, I examine the requirement for the function of the microtubule cytoskeleton in *egl* protein localization and the requirement for *egl* function in later oogenesis, specifically in localization of the *gurken* mRNA. Finally, in Chapter 5, I discuss the conclusions that can be drawn from this work, speculate about models for *egl* function and propose future experiments to elucidate the role of *egl* in oocyte determination.

CHAPTER 2:

Characterization of the *egalitarian* gene

Abstract

In this chapter, I describe the *egl* phenotype, show that *egl* function is required in the germ line cells of the ovary and explain the cloning of the *egl* locus. In *egl* mutant females, the oocyte does not form; instead, that cell develops as a nurse cell. Despite this malfunction in oocyte determination, the cluster of sixteen cells has formed normally, as indicated by the presence of the proper ring canal connections. Also, the follicle layer develops normally, until the mutant chambers degenerate. By transplanting germ cell precursors, I show that *egl* function is required in the germ line cells, not in the somatic follicle cells. Lastly, I describe the cloning of the *egl* locus and characterization of the Egl protein. The Egl protein is expressed in the germ line cells, as predicted by the transplantation experiments, and localizes to the developing oocyte in three stages.

Introduction

egl function is essential for formation of the oocyte; in *egl* mutants, the cell that would normally become the oocyte instead becomes a sixteenth nurse cell. Since microtubules and BicD protein also act in this process, *egl* might be acting in the same pathway as these proteins. Indeed, as the oocyte MTOC forms in *egl* mutants, but then immediately dissipates, *egl* may stabilize the polarized microtubule network that is essential for oocyte determination. Also, many RNAs fail to localize to a single cell in *egl* mutants; *egl* may localize these RNAs. Although consideration of these questions motivates much of the work presented in this thesis, this chapter only contains the initial characterization of the *egl* gene and the cloning of the *egl* locus, which provides reagents for further study of *egl* function in oocyte determination.

In the following analysis, I would like to answer these questions:

- What is the primary defect in *egl* mutants and where does the *egl* gene function? Do *egl* mutations affect the development of the follicle cells, which then must signal the developing oocyte, or do *egl* mutations directly affect the oocyte?
- What is the null phenotype of *egl*?
- What is the nature of the *egl* gene product? Does the sequence of the *egl* protein have any similarities to any previously identified proteins? Does it have any discernable structures or motifs?
- What is the distribution of the *egl* gene product? Is the mRNA or the protein localized within the cells of the ovary? Is its localization affected by *egl* mutations?

Results

egl mutations cause a failure of oocyte determination

Drosophila oocytes are produced in strings of maturing egg chambers, the ovarioles (reviewed in King, 1970; Spradling, 1993). At the anterior tip of each ovariole, in the germarium, stem cells divide to produce a cystoblast and a new stem cell. Each cystoblast undergoes four rounds of mitosis, thereby generating a 16 cell cyst. At each division, incomplete cytokinesis produces an actin-rimmed cytoplasmic bridge, the ring canal, which connects mother and daughter cell. Because all previously made ring canal connections segregate to one cell at each division, the two products of the initial cystoblast division have four ring canals; one of these two cells becomes the oocyte. At the posterior end of the germarial region, the complete sixteen cell cluster is surrounded by somatic follicle cells and the future oocyte moves to the posterior of the cluster. At this point the future oocyte and its sisters follow strikingly different fates: the oocyte arrests in prophase of the first meiotic division while the remaining fifteen cells continue to replicate their DNA and become polyploid nurse cells.

In *egl* mutants, cystoblast divisions proceed as in wild type. However, none of the cystoblast daughters develops as an oocyte (Figure 2-1) and all 16 cells become polyploid nurse cells (compare nuclei indicated by arrows in Figure 2-1 A and B). Despite the lack of oocyte determination, the initial formation of the cluster proceeds normally and one of the cells with four ring canals is usually located at the posterior (Carpenter, 1994). Further development of the sixteen cell cluster ceases at about stage 6 of oogenesis, no cell accumulates yolk, and eventually the cluster degenerates.

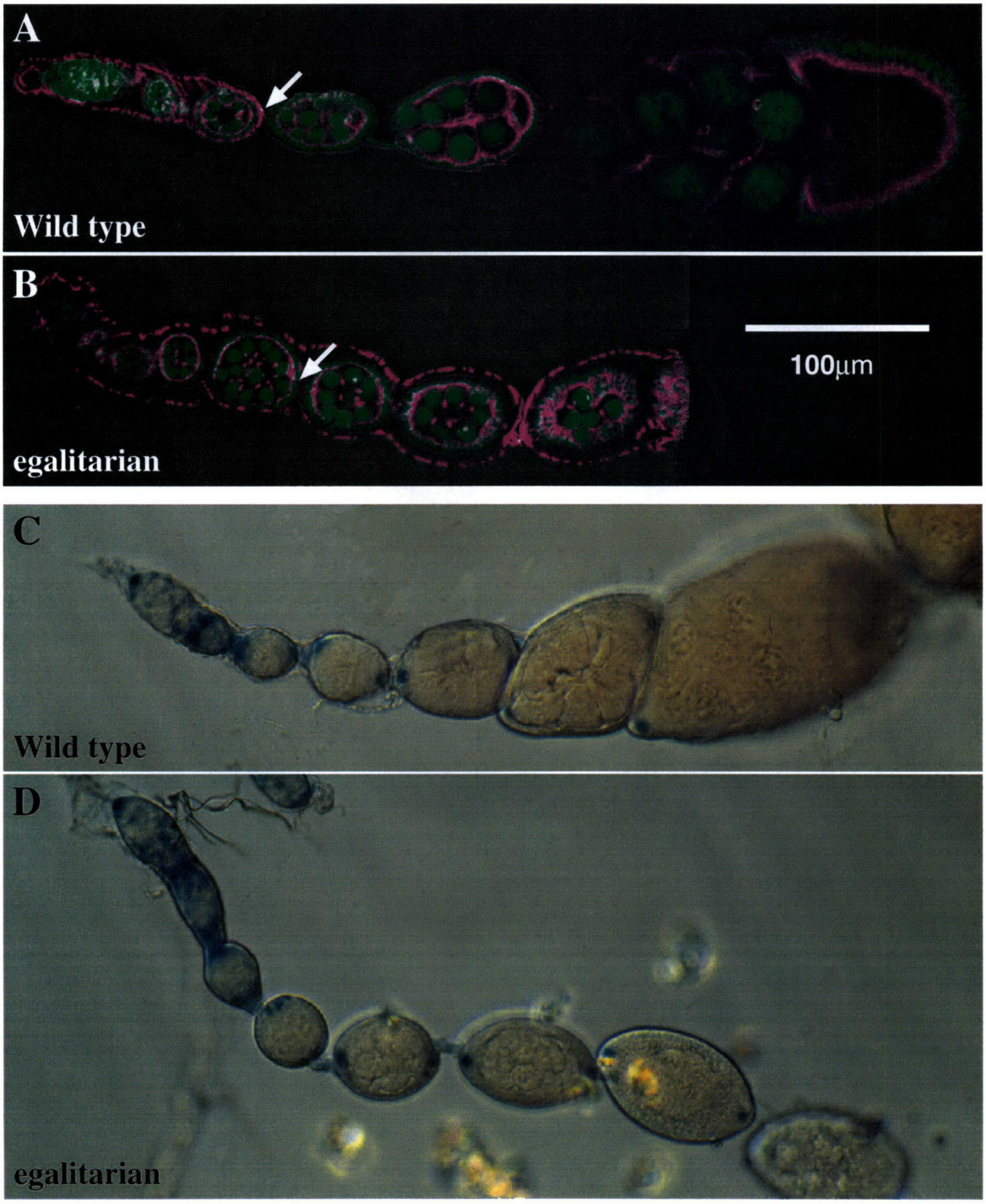
The malfunction of germ line development in *egl* mutants also affects the development of the somatic follicle cells (Figure 2-1 C and D). Although the follicle cells initially develop normally, they cease development at approximately stage 6. They show normal early expression of enhancer trap lines specific to certain follicle cell lineages, but do not migrate off of the nurse cells and do not form migratory border cells. It is likely that the follicle cell phenotype is a secondary consequence of the lack of the oocyte, as the oocyte determination phenotype is manifested much earlier.

Figure 2-1 *egl* mutations cause a failure of oocyte determination

(A and B) show ovarioles stained for DNA in green and filamentous actin in purple. Filamentous actin, stained by phalloidin, is found in the ring canals that connect the germ cells. These are most easily visible in the stage 10 egg chamber at the right side of the top panel. The nuclei indicated by arrows show the difference between wild type and *egl* mutants. (A) Wild-type oogenesis. This figure is a composite of three scans in different focal planes, to show the oocyte nucleus in each egg chamber. (Compare to Figure 1-1) In this ovariole, one cell at the posterior of the cluster follows a different developmental pathway from the others. That cell, the developing oocyte, arrests in meiosis I. Its nucleus stains much less intensely than the nuclei of its polyploid sister cells. (B) Oogenesis in an *egl* mutant. In this ovariole, all sixteen germ cells in each egg chamber form polyploid nuclei and oogenesis does not proceed past approximately stage 6. In the wild-type ovariole, stage 6 is represented by the egg chamber to the right of the arrow.

(C and D) show ovarioles stained for β-galactosidase activity, showing the activity of an enhancer trap line that is expressed in several subpopulations of follicle cells. This line stains cells at the anterior tip of the germarium, shown at the left side of each panel. The next two egg chambers to the right show staining in the stalk cells that connect the egg chambers. Later in oogenesis, this line stains the polar follicle cells at the anterior and posterior end of each egg chamber. At stage 10 (not shown) this line stains the border cells, the migratory follicle cells that move from the anterior of the egg chamber, between the follicle cells, to the anterior of the oocyte. In the *egl* mutant ovariole (D), the follicle cells develop normally, but do not form border cells that migrate. The egg chamber shown out of focus at the far right of panel (D) is degenerating; the *egl* mutant egg ovarioles develop no further.

Figure 2-1 *egalitarian* mutations cause the oocyte to form a sixteenth nurse cell



egl functions in the germ line cells of the ovary

The *Drosophila* ovary is composed of two tissues, the mesodermal follicle cells and the germ-line oocyte and nurse cells, that communicate during the development of the oocyte. Although *egl* mutations affect primarily the germ line, it is possible that *egl* could be involved in signalling to the developing oocyte from the follicle cells. Alternatively, *egl* could be required in the germ line cells for oocyte determination. In order to determine whether *egl* function is required in the germ cells or in the somatic follicle cells, or in both, I made chimeric flies in which the germ line cells were of a different genotype than the somatic cells, either wild-type germ cells in an egl^- soma, or egl^- germ cells in an egl^+ soma (Figure 2-2).

Making chimeras by transplantation of the germ line precursors, or pole cells, requires donor embryos that are marked so that the introduced germ cells can be identified. In designing this experiment, I hypothesized that *egl* function would be required in the germ line. Thus, the chimeras with wild-type germ cells were assayed by the phenotype of their progeny, but the chimeras with *egl* mutant germ cells were assayed histochemically. Making chimeras by pole cell transplantation also requires recipient embryos without a functional germ line in order to simplify the analysis and allow for the proliferation of the introduced germ cells. I used embryos mutant for ovo^D, a dominant female sterile mutation that causes extremely stunted ovaries, as recipients for *egl* mutant germ cells. Since *egl* mutant females also do not produce eggs, I used them as recipients for wild-type pole cells.

To produce chimeric females with wild-type germ cells in an *egl* mutant soma, I transplanted egl^+ pole cells marked genetically with the adult eye color and body color markers *scarlet* (*st*) and *ebony* (*e*) into embryos from the cross *cn bw* egl^{WU50}/*CyO* x *cn bw* egl^{RC12}/*CyO*. I identified the *egl* homozygous females by the eye markers *cinnabar* (*cn*) and *brown* (*bw*) and assayed the females for fertility after mating them to *st e* males. Two of fifty-five *egl* homozygous females produced progeny; all progeny were *st e*, indicating that they were derived from the introduced pole cells. After collecting the eggs from these females for several days, I dissected and stained their ovaries to show the eggs developing (Figure 2-2). This experiment shows that egl^+ germ cells can form eggs, even when surrounded by egl^- follicle cells. Thus, *egl* function is required in the germ line cells of the ovary, not in the somatic cells.

To produce chimeric females with *egl* mutant germ cells in a wild-type soma, I used recipient embryos from the cross +/+ x ovo^D/Y. Because ovo^D maps to the X chromosome, all the recipient females will be ovo^D. However, because the mother of the donor embryos must be heterozygous for *egl*, half of the time the introduced pole cells will

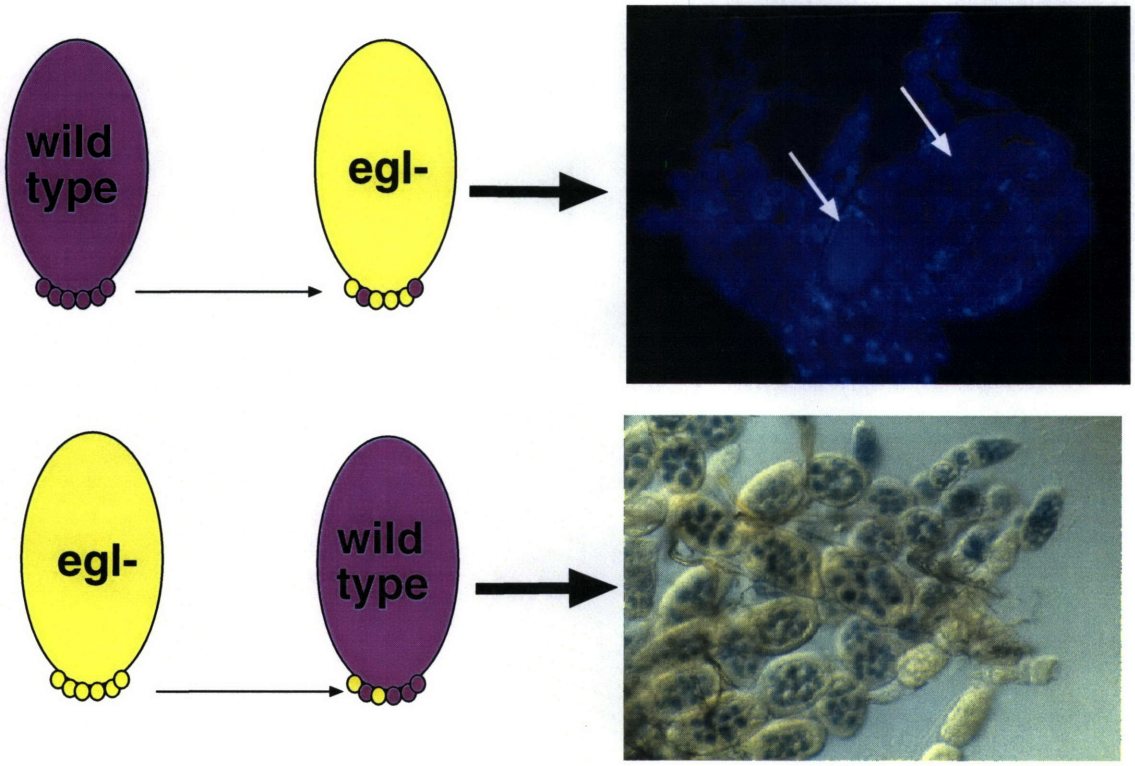
also be heterozygous for *egl*. I did not mix pole cells from different donors during this transplantation. The transplanted *egl*$^-$ pole cells were marked histologically with the nuclear lacZ germ cell enhancer trap line *BG07* (gift of Sarah Cramton and Frank Laski). So that only the germ cells homozygous mutant for *egl* would express the lacZ marker, I used donor embryos from the cross *BG07* egl^{WU50}*/CyO* females x egl^{WU50} /egl^{WU50} males. One of the eight resultant females was fertile; some of her progeny had curly wings, indicating that the introduced pole cells were *egl/CyO*. None of the remaining females was fertile; two of these had germ cells that stained for β-galactosidase, indicating that the introduced germ cells were homozygous for egl^{WU50}. None of the lacZ$^+$ germ cells formed an oocyte: all the cysts had the *egl* mutant phenotype. This experiment shows that *egl*$^-$ germ cells fail to form eggs, even when surrounded by *egl*$^+$ follicle cells, confirming the expectation that *egl* function is required in the germ line cells of the ovary, not in the somatic cells.

Figure 2-2 *egl* function is required in the germ line cells of the ovary

The upper panel represents the process of making chimeric flies by transplanting wild-type pole cells (purple) into an *egl* mutant recipient (yellow). The photograph shows a dissected chimeric ovary that has been stained for DNA with Hoechst 33258 to show the germ line nuclei. The endogenous *egl* mutant pole cells form mutant ovarioles; the introduced pole cells form wild-type ovarioles with developing eggs (arrows).

The lower panel represents the reciprocal transplantation, with wild type in purple and *egl* mutants in yellow. The *egl* mutant germ cells are marked with an enhancer trap that expresses β-galactosidase in the germ line nuclei. The photograph shows a dissected chimeric ovary that has been stained for β-galactosidase activity. The *egl* mutant germ cells do not form eggs and thus show the *egl* mutant phenotype.

Figure 2-2 egl function is required in the germ line cells of the ovary



Molecular isolation of the *egl* gene

I cloned the *egl* locus by standard *Drosophila* positional cloning methods, first mapping it to a cytological region by analysis of chromosomal deletions, then isolating that region as a series of overlapping cosmids. Next, I selected candidate transcripts from the *egl* region based on their germ line expression. One of the candidate transcripts was altered in an *egl* mutant allele. A transgene expressing that transcript complements the *egl* mutant phenotype, thereby proving that this transcript corresponds to the *egl* locus .

By cytology, *egl* maps to polytene bands 59F5-8 on the right arm of the second chromosome and is not complemented by several chromosomal deletions, including *Df(2R) HB132* and *Df(2R)* bw^{S46}. The breakpoints of two chromosomal deletions that complement *egl* mutations, *Df(2R)* bw^{DRj} and *Df(2R)* $or^{BR\text{-}11}$ (Reed, 1992), define the centromere-proximal and -distal limits of the *egl* region (Figure 2-3). Because these deletions complement *egl*, all sequences required for *egl* function must be in the region between the deletion breakpoints.

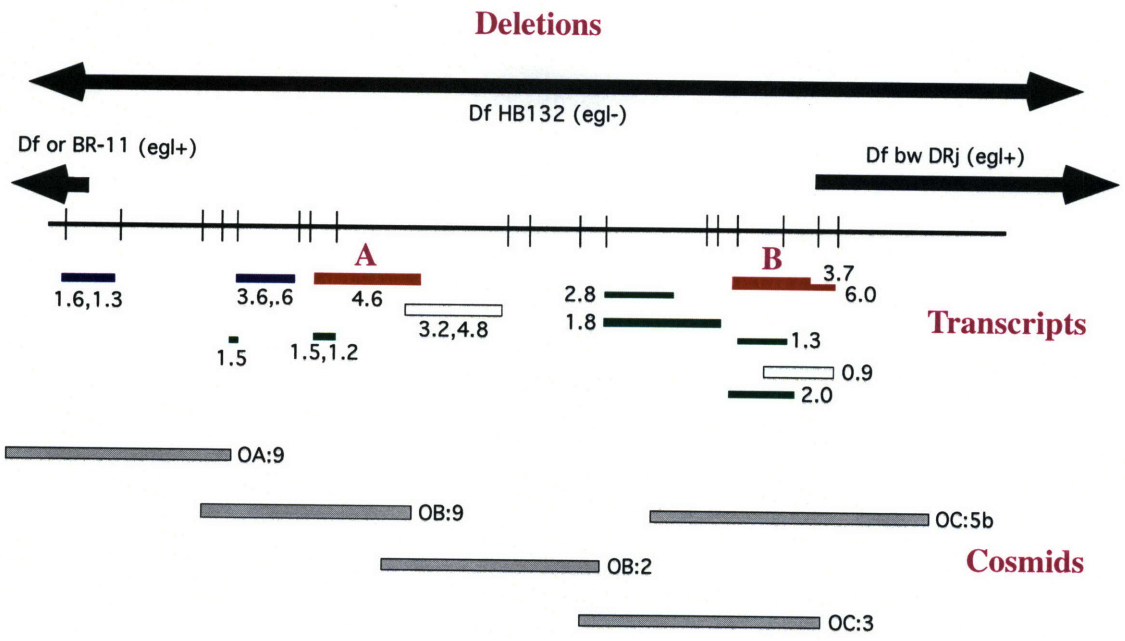
To provide entry into the region, I isolated a junction clone containing sequences from the *egl* region fused to sequences from the distal end of the or^{BR11} deletion. A probe from the distal end of or^{BR11} was provided by Linda Hall and used to screen an or^{BR11}/+ genomic library (Materials and Methods). Using the junction fragment to screen a wild-type library yielded clones from the *egl* region, which I differentiated from clones from the distal end of or^{BR11} by in situ hybridization to salivary gland polytene chromosomes.

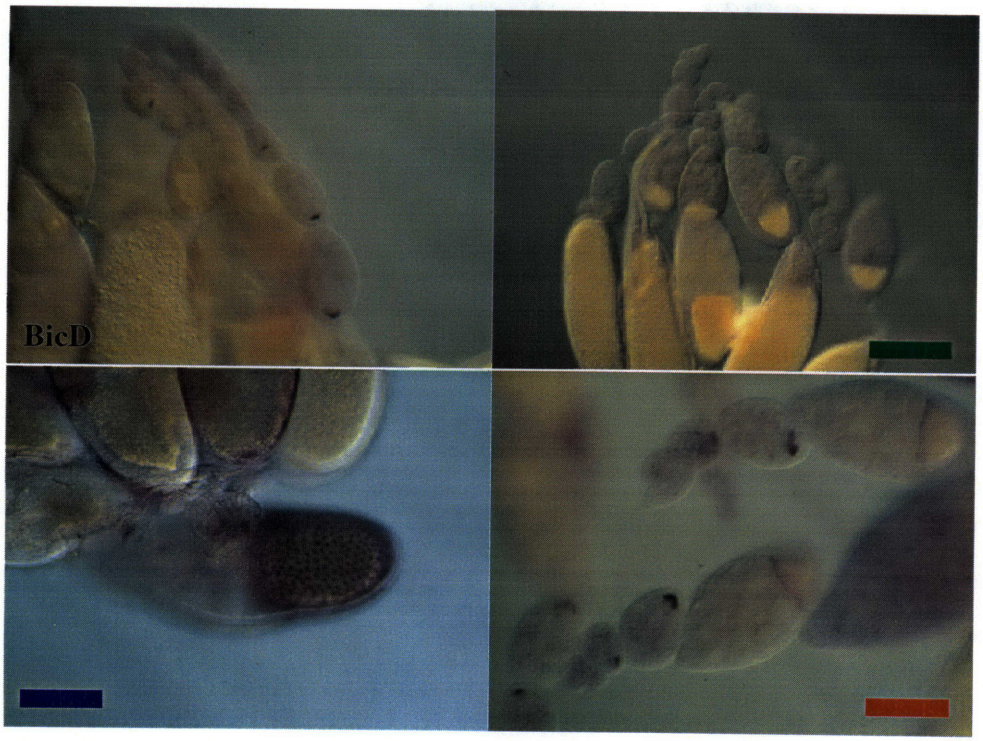
Figure 2-3 Transcripts in the *egl* region

The chromosomal deficiencies, or deletions, that define the *egl* region are shown as thick bars (designating the deleted region) at the top. Arrows indicate that the deleted region continues beyond the edge of the diagram. The region is oriented with the centromere to the right. The *egl* genomic region is represented as a thin bar; EcoRI sites are designated by vertical lines. The cosmids spanning the region are indicated by grey boxes.

The transcripts within this region are represented by colored boxes that span the extent of the genomic fragment used to detect them. The numbers refer to the size of the transcript on a Northern blot. The colors refer to the hybridization patterns indicated below; white indicates a fragment that was not analysed. Two different genomic fragments hybridize to transcripts that localize to the oocyte in a pattern similar to the pattern of *BicD* mRNA.

Figure 2-3 Transcripts in the *egl* region





I isolated the rest of the region, using the end of one cosmid to isolate another, to produce a series of overlapping cosmids (Tamkun et al., 1992). To determine when the walk had crossed the breakpoint of the bw^{DRj} deletion, and thus contained the complete *egl* region, I hybridized each cosmid to polytene chromosomes from bw^{DRj}/+ larvae. Hybridizing each cosmid to quantitative DNA blots prepared from wild-type and deletion-heterozygous DNA showed the exact location of the deletion breakpoint. The *egl* region spans 80 kb region between the deletion breakpoints of *Df(2R)* bw^{DRj} and *Df(2R)* or^{BR-11}.

To identify candidate transcripts within this 80 kb region, I made three suppositions about the nature of the *egl* transcript. First, *egl* mutants affect the ovary, so this transcript should be expressed in the ovary. Second, *egl* function is required in the germ line cells, so this transcript should be expressed in the nurse cells and the oocyte. Third, *egl* has the same phenotype as *BicD*, so this transcript might be localized to the developing oocyte in the same pattern as the *BicD* transcript. Northern blot analysis with genomic fragments across the region identified sixteen transcripts that were expressed in both ovaries and 0-2 hour embryos. No transcript was specific to the ovary. Ovary in situ hybridization with the same genomic fragments showed three patterns of expression. The transcripts are represented in Figure 2-3 and are color coded to represent their patterns of expression.

Two genomic fragments, designated 'A' and 'B', hybridize to RNA strictly localized to the developing oocyte in a pattern very similar to that of *BicD* mRNA (red in Figure 2-3). I isolated cDNAs corresponding to the 'A' and 'B' transcripts from a cDNA library from 4-8 hr embryos (Brown and Kafatos, 1988). The 'A' fragment hybridizes to a 4.6 kb transcript and the 'B' fragment hybridizes to 3.7 kb and 5.9 kb transcripts. Although no transcript could be formally eliminated by the analysis of its expression pattern, 'A' and 'B' became the leading candidates to be the *egl* transcript.

To differentiate between the two candidates, I examined the 'A' and 'B' transcripts in *egl* mutant alleles. Northern blot analysis showed that the 'A' transcript showed a slight, but reproducible, decrease in mobility in egl^{PB23}, indicating an increase in the size of this transcript associated with a mutation in *egl* (Figure 2-4). The 'B' transcripts were unaltered in all alleles of *egl* (data not shown).

The next sections discuss the proof that the 'A' transcript is the *egl* transcript. I have sequenced a partial cDNA that hybridizes to the 'B' transcript. Computer database searches with this sequence reveal that the 'B' transcript corresponds to the *angel* gene (Kurzik-Dumke and Zengerle, 1996).

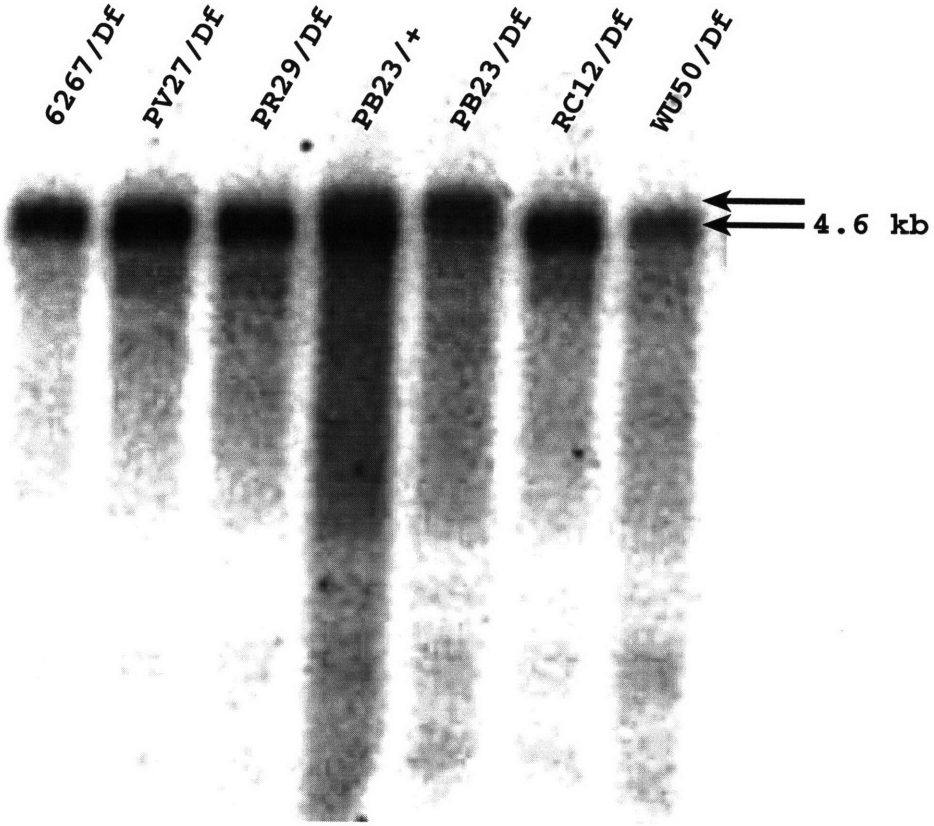
Figure 2-4 *egl* mutations perturb the *egl* RNA and protein

(A) Northern blot analysis of *egl* mutant alleles. The wild-type *egl* RNA migrates at approximately 4.6 kb. In egl^{PB23}, the *egl* RNA increases in size, as indicated by the upper arrow. Sequence analysis, shown in Figure 2-5 and the Appendix, shows that the egl^{PB23} mutation causes the 201 nucleotide Intron #3 to fail to excise.

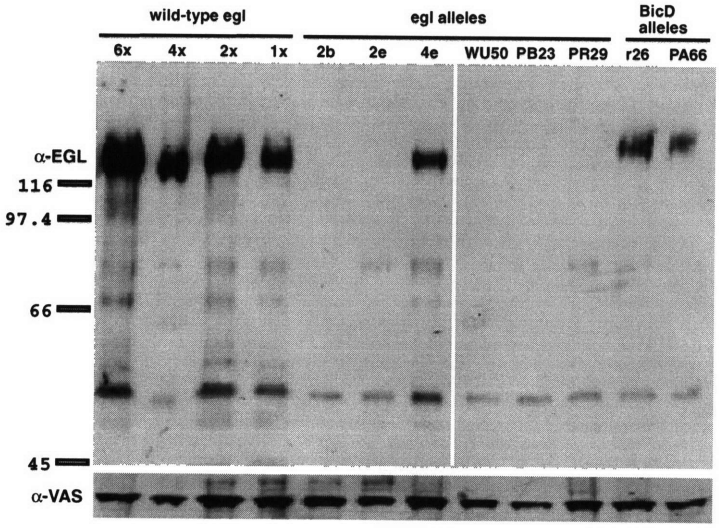
(B) Western blot analysis of *egl* mutant alleles. The Egl protein migrates at approximately 125 kD; the positions of the Bio-Rad high molecular weight size markers are shown at left. The intensity of the Egl signal increased with increasing *egl* gene dosage, provided by the egl^{+} transgenes used to complement the *egl* mutants. The "6x" lane contains ovarian extract from flies homozygous for two different egl^{+} transgenes (CA 11C and CA 8B); the number six derives from the two endogenous copies plus the four transgene copies. Similarly, the "4x" lane contains extract from females homozygous for one egl^{+} transgene, the "2x" lane is from wild-type flies, and the "1x" lane is from females heterozygous for a deletion that removes the *egl* locus, *Df(2R)* bw^{S46}. All of the *egl* mutant extracts are from females heterozygous for the specified mutation and *Df(2R)* bw^{S46}. The following *egl* alleles were also tested and show no detectable Egl protein: RC12, 1286 and 1287. The *BicD* mutant extracts are from females heterozygous for the specified mutation and *Df(2L) TW119*. For comparison, the blot shown in the top panel was stripped and reprobed with anti-Vasa serum, showing that all lanes contain ovarian protein and allowing comparison of loading levels in the lanes.

Figure 2-4 *egl* mutations perturb *egl* RNA and Egl protein

A. egl Northern



B. Egl Western



Complementation of *egl* mutations

To prove that the 'A' transcript corresponds to the *egl* locus, I used cloned DNA to complement the *egl* mutants. In my initial characterization of the *egl* region, I had made P-element transformants with five cosmids from across the *egl* region (Figure 2-3). None of these transgenic lines complemented the *egl* mutants. Later PCR analysis showed that the OB:9 cosmid contains the full 'A' transcript, to within 300 bases of the beginning of the cDNA. This cosmid may lack essential regulatory sequences, or it may have undergone a rearrangement on transformation. To circumvent these possibilities, I made a minigene construct, fusing the 'A' cDNA to exogenous promoters specific for early oogenesis.

Two criteria were essential in the design of the minigene constructs. First, the promoters must produce expression in the germ line during early oogenesis. I used the *ovarian tumor* (*otu*) promoter, which expresses very early in oogenesis, as *otu* mediates germ line sex determination (Mulligan et al., 1988). I also used the 'germ' promoter, which contains the *Sgs3* (*Salivary gland secretion* protein) promoter fused to the *hsp26* nurse cell enhancer, and had been shown to mediate lacZ expression in stage 6 and older egg chambers (Serano et al., 1994). Although stage 6 seems to be too late for *egl* expression, this promoter was reputed to express much earlier (R. Cohen, personal communication).

The second criterion in design of the minigene constructs is that the cDNA must contain sequences sufficient for activity, including a complete open reading frame (ORF) and adequate 5' and 3' untranslated regions (UTRs). The longest 'A' cDNA, A9, matches the length of the 'A' transcript seen by Northern blot analysis; the cDNA is 4,555 bases and the transcript is approximately 4.6 kb. Also, sequence analysis, discussed below, showed that the A9 cDNA encodes a complete ORF with stop codons in all three reading frames in the 5' UTR and a polyadenylation sequence and polyA tail in the 3' UTR (see Appendix). Thus, the *otu* and germ promoters should provide early expression and the A9 cDNA should provide gene function.

I introduced the constructs containing the A9 cDNA downstream of the *otu* or germ promoters into flies by P-element mediated transformation (Spradling, 1986) and introduced the transgenes into an *egl* mutant background by genetic crosses. Several lines of each construct were tested for complementation of egl^{WU50} and egl^{PB23}. I characterized a number of transgenic lines which show varying degrees of complementation (below), presumably due to their insertion site. Most lines fully complement the *egl* mutant phenotype such that *egl* homozygous flies carrying one copy of the transgene are fertile. This proves that the 'A' cDNA encodes *egl* gene function.

Numbers of *egl*$^+$ transgenic lines giving varying degrees of complementation in an *egl*$^-$ background:

promoter	many eggs	few eggs	no eggs
otu (CA)	16	6	3
germ (ga)	5	6	6

The germ constructs do not complement as well as the *otu* constructs; expression of the germ promoter may indeed be too late to provide adequate *egl* function. For future analyses, strong complementing lines CA 11C and CA 8B were used. Females homozygous for CA11C and CA8B (6x *egl*$^+$) have no obvious oogenesis phenotype. In Chapter 4, the weakly complementing lines CA 3B and CA 13F were used.

Sequence of the Egl protein

The immediate goal of cloning the *egl* locus was to determine the nature of the *egl* gene product. To that end, I sequenced the A9 cDNA, which was shown by transformation to encode complete *egl* function, using the sonication sequence method (described in detail in Bankier et al., 1987). Briefly, I excised the cDNA insert and ligated it in a dilute reaction to circularize it. I then sonicated the insert into random fragments of at least 200 bp, which were cloned into Bluescript KS(-) and sequenced using primers from the vector. Assembly of the random segments into a contiguous sequence provided the complete cDNA sequence. Thus, the cDNA was mechanically broken into fragments that were sequenced and conceptually reassembled. The complete sequence of the *egl* cDNA designated A9 is shown in the Appendix.

To predict the sequence of the Egl protein, I looked for an open reading frame within the cDNA sequence. The largest open reading frame contains 11 possible translational initiation sites in the first 800 nucleotides; the first four possible initiation sites, within 45 nucleotides of each other, are indicated in bold in the Appendix. The consensus for *Drosophila* translational initiation is C/A A A A/C AUG(Cavener, 1987);. The first four AUG's are preceded by: AGCA, GTCC, CGAG and CAAC, in that order. Because only the fourth AUG has a 4 out of 4 match to the consensus, as well as having the A codon at the -3 position that is found in 82% of Drosophila initiation sequences (Cavener, 1987), I selected that AUG as the probable initiation site. Conceptual translation from the selected start codon predicts an 874 amino acid, 98kD protein (Figure 2-5). The predicted

772 base 5' UTR contains stop codons in all three reading frames, indicating that the beginning of the ORF is contained within the sequenced cDNA.

Comparison of the predicted Egl protein to database sequences show that Egl has significant similarity to the predicted gene product C10G6.1 from *Caenorhabditis elegans* (Wilson et al., 1994). The similarity lies in three blocks of approximately 65 amino acids each, with 46% identity. The third block, from amino acids 599-739, also shows similarity to expressed sequence tags (EST's) from *Arabidopsis thaliana* (58% identity) and to *ribonucleaseD* (Zhang and Deutcher, 1988) from *Haemophilus influenzae* (38% identity) (Figure 2-6). The significance of these similarities is unclear.

Other computer analyses predict possible aspects of Egl protein structure. The Paircoil program (Berger et al., 1995) predicts a 67% probability that residues 744-775 (bold in Figure 2-5) form a coiled-coil structure. The predicted Egl protein also contains multiple possible phosphorylation, glycosylation and myrystylation sites. Interestingly, BicD protein has been shown to be phosphorylated and is predicted to form coiled-coil (Suter et al., 1989; Suter and Steward, 1991; Wharton and Struhl, 1989). A detailed analysis of Egl protein structure and function will be required to determine whether the observed similarities to other proteins or putative modification sites have functional significance.

egl mutations cause alterations in the Egl sequence

In order to correlate protein sequence with *egl* function, I analyzed four strong *egl* mutant alleles by sequencing PCR product from genomic DNA. The amino acid alterations in these alleles are shown in Figure 2-5; the coding alterations are shown in the Appendix. Three mutations predict protein truncations. egl^{PB23}, whose alteration in transcript size initially identified the *egl* transcript, has a splice donor site mutation. Sequencing of RT-PCR product from egl^{PB23} mRNA confirms that the third intron is not excised; the 200 base increase in size accounts for the aberrant mobility of the *egl* transcript in egl^{PB23} mutants. Translation of this message should produce 126 amino acids of Egl, followed by 22 intron-encoded amino acids before reaching a stop codon in the intron. In egl^{WU50} the splice acceptor site of the fourth intron is mutated, causing a frameshift after residue 777. This allele also has an amino acid substitution at residue 399. egl^{PR29} has a stop codon after amino acid 279.

In contrast to these three protein truncation mutations, egl^{4e} has a single amino acid substitution, a cysteine to tyrosine change at amino acid 24. Interestingly, the C-Y change in egl^{4e} is in the first block of homology found in *egl* and the *C. elegans* C10G6.1. This result lends strong support to the significance of the sequence homology observed between the *Drosophila* and *C. elegans* proteins.

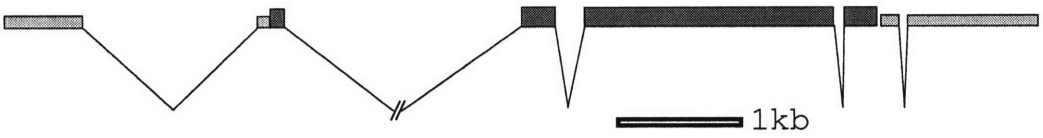
Figure 2-5 Egl protein sequence

(A) *egl* genomic organization. The *egl* coding sequences are represented by dark grey bars and the untranslated regions are represented by light grey bars. The introns are represented by thin lines, and are to scale, except for intron #2, which is approximately 6 kb.

(B) Egl protein sequence. The putative coiled-coil is represented in bold type. The lesions in the *egl* alleles are indicated by a triangle above the line, with a filled triangle representing stop codons or single amino acid changes and an open triangle representing splice site mutations.

Figure 2-5 Egl protein sequence

A. *egl* genomic organization



B. Egl protein sequence

```
                                ▼
MTLLFFLERL LDKGEPRTVH DLSCQFGNKE FTKEMRQIAG GSQSGLKKFL 50
                                Y (4e)
AQYPAIFLVD GDYVQVNAYQ HHNADDGGCG GKRDYIQEAK DYFKNKMLQY 100

                                ▽
GAAAEVPVRS LLGHRSQASP QVRHISGQHI KEFTDFLMKH TDTFKVTDDY 150
                                (PB23)
VMLVGCENMT DLPARDRLHL PQSNIDTRGT QQMLDFFAQC IEVKGPLLVD 200

QLFHLLTTNF PQDQWLRMFK TPGDLSSFLK LFADCFHIQA NLVTLLQKPK 250

                                ▼
LSDTHIQQAQ AQTREQFNAL NNNNSASIRK QEPTPGGGGV GGVSSVQQRL 300
                                STOP (PR29)
QSPALRTNGH TNNNNGSNGS NNNNNNNNSI ACPNFKLNAP VSNVMGGQSQ 350

                                                ▼
GFGQPKSEPS SGFDSYVPMS ELKLENLCEN NYPSANTCYG PINNSSQQAQ 400
                                                T (WU50)
QVQTQQQQQP QHATQNPAEQ RLNSVNQTLK QRINTLVIRT LAENLEKDKQ 450

SLANQQGGPI SPHASPVHSI ANSSSNQNAG SAANNANSNS NANPNNANHS 500

PSHSYFVGDT WKIKVLQNTT VIANVKQSVF VTDIILKYAA KNESIVVSLD 550

CEGINLGLKG EITLIEIGTT RGEAFLFDVQ SCPAMVTDGG LKTVLEHDQV 600

IKVIHDCRND AANLYLQFGI LLRNVFDTQA AHAILQYQES GKQVYKAKYI 650

SLNSLCEQYN APCNPIKDQL KQIYRRDQKF WAKRPLTREM MLYAAGDVLV 700

LIHDQLFGNL ARQIKPENRA LFSELCTEQI LMQIKPNEVK IRKKQRKVST 750

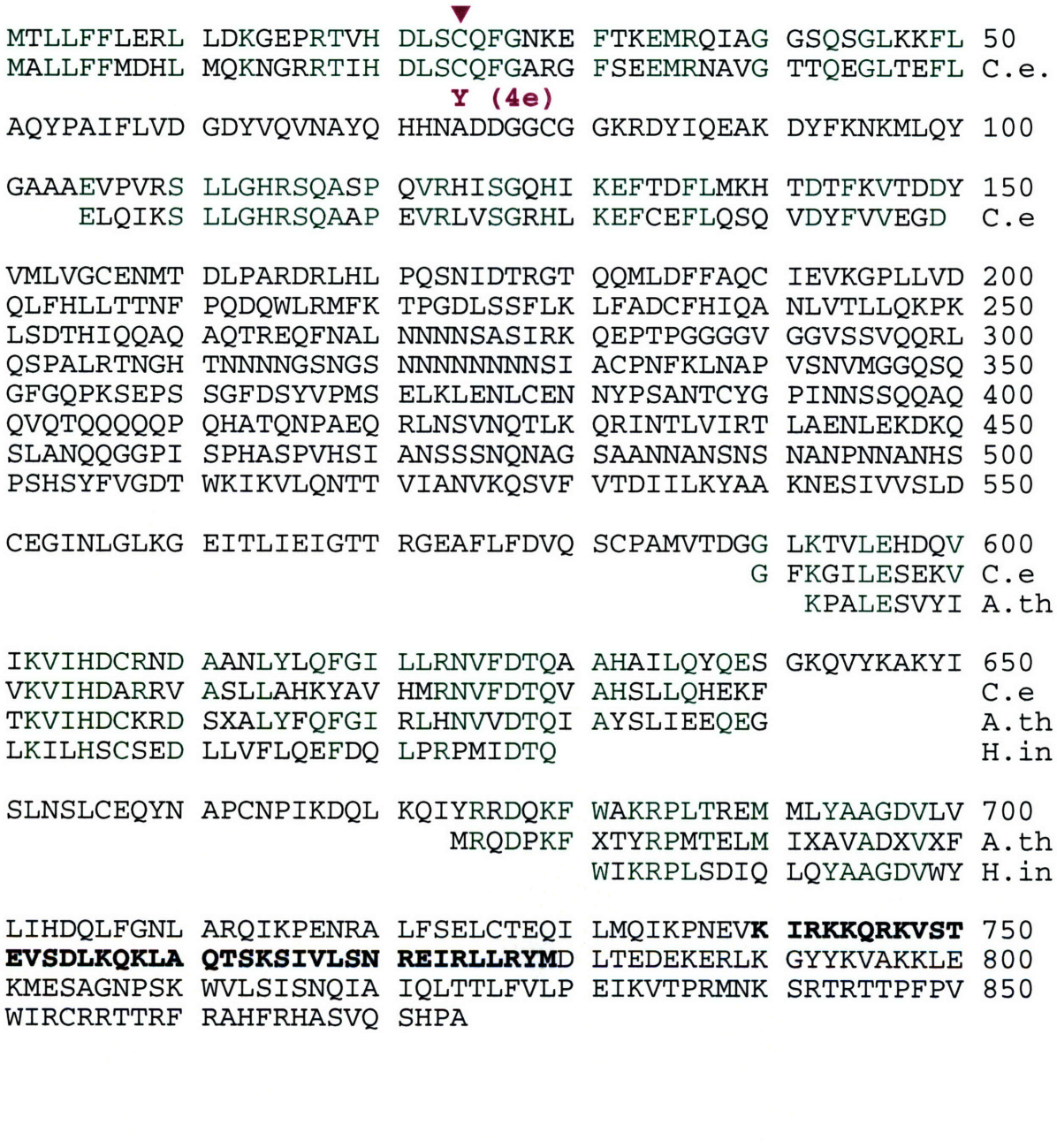
                                ▽
EVSDLKQKLA QTSKSIVLSN REIRLLRYMD LTEDEKERLK GYYKVAKKLE 800
                                (WU50)
KMESAGNPSK WVLSISNQIA IQLTTLFVLP EIKVTPRMNK SRTRTTPFPV 850

WIRCRRTTRF RAHFRHASVQ SHPA
```

Figure 2-6 Egl protein shows similarities to proteins from other species

The Egl protein sequence is shown in black or green type, in lines that are numbered at the end. The aligned sequences are shown in green where they are identical to Egl and in black where they differ. The lines ending in 'C.e' contain sequence from predicted gene product C10G6.1 from *Caenorhabditis elegans* (Wilson et al., 1994). The similarity lies in three blocks of approximately 65 amino acids each, with 46% identity. The lines ending in 'A.th' contain sequence from an expressed sequence tag (EST) from *Arabidopsis thaliana* (58% identity). The lines ending in 'H.in' contain sequences from *ribonucleaseD* (Zhang and Deutcher, 1988) from *Haemophilus influenzae* (38% identity). As in Figure 2-5, the putative coiled-coil is shown in bold type. The position of the lesion in egl^{4e} is shown in magenta.

Figure 2-6 Egl protein shows similarities to proteins from other species



KEY **C.e** **C. elegans**
A.th **A. thaliana**
H.in **H. influenzae ribonuclease D**

possible coiled-coil: in bold

Immunoblot analysis of Egl protein

To analyze the function of the Egl protein further, I generated antibodies against the N-terminal half of the Egl protein (Experimental Procedures). The Egl antiserum detects a single band of 125 kDa in ovary extracts. Specificity of this antiserum for Egl is shown by two tests (Figure 2-4). First, the band detected on Western blots increases in intensity with increasing *egl* gene copy number. Second, Egl protein is not detected in extracts from several *egl* mutants. The lack of protein in these mutants cannot be explained by the failure of *egl* mutants to develop an oocyte, because Egl protein is detected in one *egl* mutant and two *Bic-D* alleles (Figure 2-4), all of which have a strong sixteen nurse cell phenotype.

Sequence analysis predicts that egl^{WU50} and egl^{PR29} mutants produce truncated proteins which should be detected at a smaller size than the wild-type protein on a protein blot. However, although the Egl antigen used for immunization included the protein regions theoretically present in the mutants, no Egl positive protein bands were detected in these alleles, nor was Egl protein detected by whole mount immunofluorescence in these mutants (Figures 2-4 and 2-7). In egl^{PB23}, longer exposures of Western blots show a small amount of protein of wild-type size; the mutant intron in this allele may occasionally be spliced correctly. The following *egl* alleles were tested and show no detectable Egl protein: 2b, 2e, WU50, PR29 (Figure 2-4) and RC12, 1286 and 1287 (not shown). It seems likely that the protein truncations lead to protein instability. In this case, the phenotype of these alleles should represent the *egl* null phenotype. In contrast to these three protein truncation mutations, egl^{4e} has a single amino acid substitution, a cysteine to tyrosine change at amino acid 24. Immunoblotting with anti-Egl serum detects Egl protein in ovary extracts from egl^{4e} mutants (Figure 2-4).

***egl* mRNA localizes to the developing oocyte**

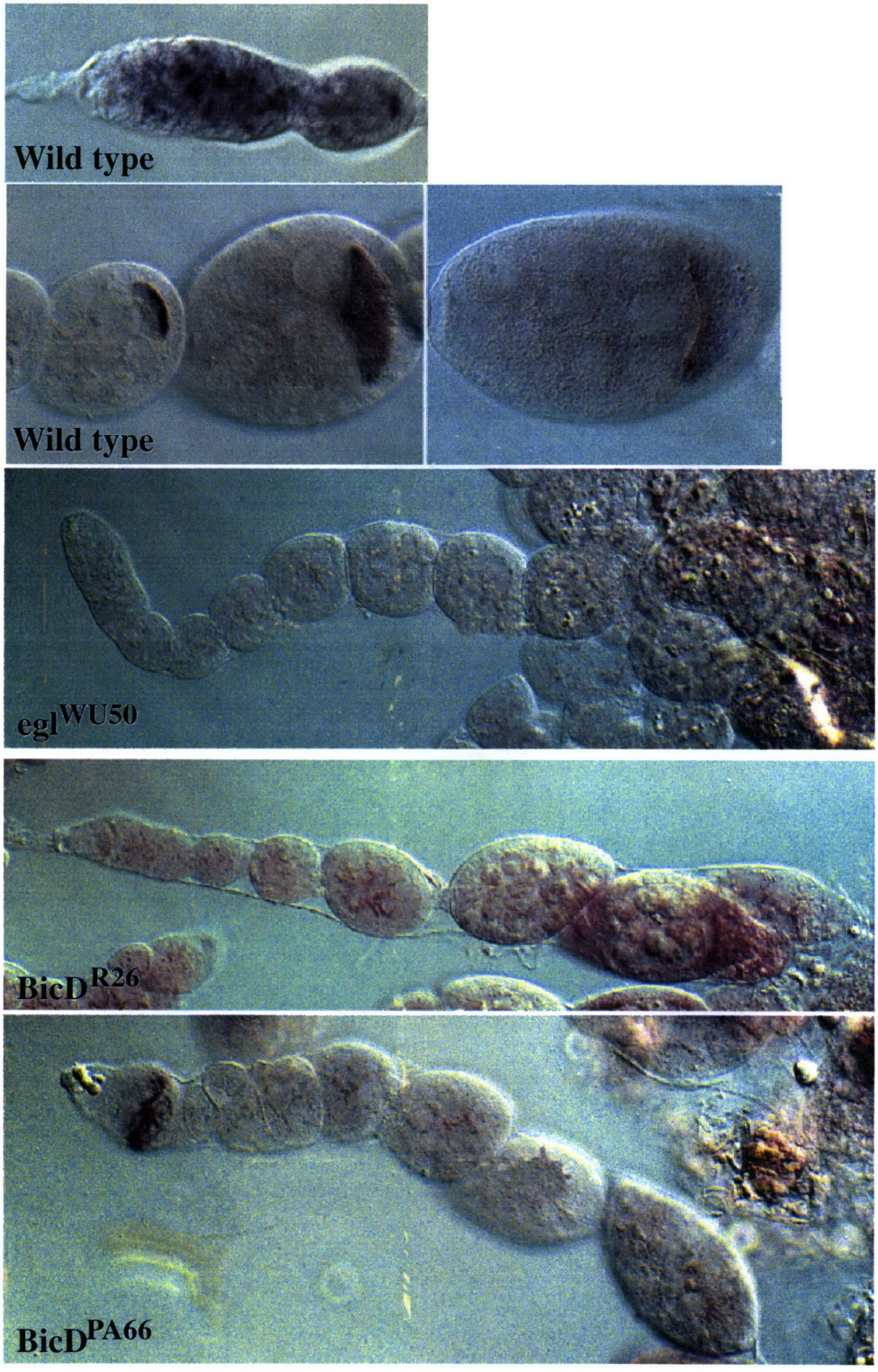
Whole mount in situ hybridization shows that *egl* mRNA localizes to a single cell of the 16-cell cyst in the germarium (Figure 2-7). Until stage 7 of oogenesis, *egl* RNA is enriched in the posterior cortex of the oocyte. As the oocyte starts to accumulate yolk, *egl* transcript accumulates in an anterior ring. This distribution is similar to that of other RNAs such as *K10*, *BicD* and *oskar* (*osk*) (Ephrussi et al., 1991; Kim-Ha et al., 1991; Serano and Cohen, 1995; Suter et al., 1989; Wharton and Struhl, 1989) that accumulate early in the oocyte and somewhat later form an anterior ring. However, unlike *osk*, *egl* RNA does not localize to the posterior pole at stage 9 of oogenesis; *egl* RNA remains evenly distributed throughout the oocyte after stage 10, where the transcript persists into early embryogenesis.

Figure 2-7 *egl* mRNA localization

Wild type, upper panel: The *egl* mRNA is enriched in a single cell in the germarium in region 2. This sample was hybridized by the method of Suter and Steward, 1991; the other samples in this figure were hybridized by the method of Ephrussi et al., 1991. Wild type, lower panel: From left to right, *egl* mRNA localizes to the posterior cortex in a stage 3 egg chamber. At stages 8 and 9, *egl* mRNA localizes to the margin of the anterior cortex.

Mutant genotypes: $egl^{WU50}/Df(2R)\ bw^{S46}$ or $BicD^{R26}/Df(2L)\ TW\ 119$ or $BicD^{PA66}/Df(2L)\ TW\ 119$. No localization of *egl* RNA is detected. Only in $BicD^{PA66}$ can RNA expression be seen in the germarium, where it seems to be expressed in a flattened cyst at the end of region 2.

Figure 2-7 *egl* mRNA localization



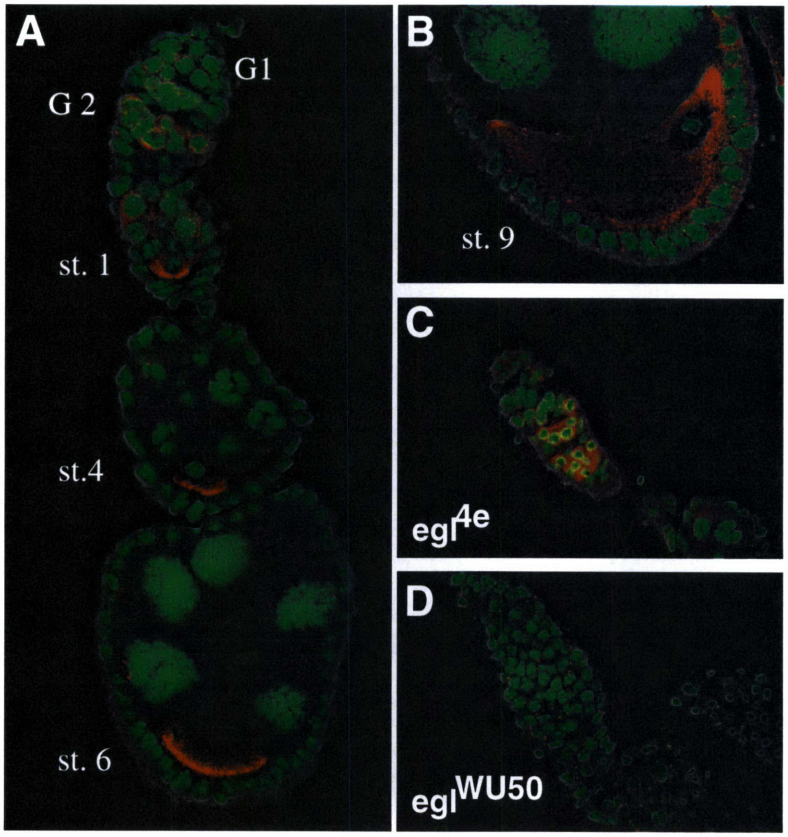
Egl protein localizes to the developing oocyte in three stages

In the germarium, Egl protein is expressed throughout the 16 cell cyst, becoming enriched in one or two cells in region 2. By stage 1 of oogenesis Egl protein is concentrated in a single cell, the future oocyte. From stage 2 to stage 7, Egl protein is enriched at the posterior cortex of the oocyte, wherever the oocyte abuts the follicle cells. At stage 8 Egl protein shifts to the anterior cortex in what appears to be a ring along the margin of the oocyte where the oocyte, nurse cells and follicle cells meet (Figure 2-8). By stage 10, Egl protein is no longer detectable in the ovary. This may be due to technical difficulties in getting antibodies to penetrate the developing egg shells. Immunoblot analysis shows Egl protein in the oocyte through early embryogenesis. In *egl* mutants that have no Egl protein detectable by Western blotting, no Egl protein is detectable by in situ immunofluorescence. In egl^{4e} mutants, Egl protein is detectable, but not localized to a single cell. Instead, the protein is distributed uniformly throughout the sixteen cells. Egl localization in wild type and *egl* mutants is shown in Figure 2-8.

Figure 2-8 Egl protein localization in wild type and in *egl* mutants

Egl protein is shown in red; DNA is shown in green. (A) In germarial region 2, a complete cyst can be seen spanning the width of the germarium. At stage 1, Egl protein can be detected in all the cells of the cyst, but is enriched at the posterior of the oocyte. At stage 4 and stage 6, Egl is localized at the posterior cortex of the oocyte. (B) At stage 9, Egl protein shifts in distribution so that it is localized to the anterior margin of the oocyte and is enriched at the dorsal anterior side, near the oocyte nucleus. (C) In egl^{4e}, Egl protein is present, but does not localize to a single cell. (D) In egl^{WU50}, no Egl protein is detectable.

Figure 2-8 Egl protein localizes to the oocyte in three stages



Discussion

egl mutant ovaries show several defects very early in oogenesis. Most significantly, the oocyte fails to develop and instead forms a sixteenth nurse cell. Others have shown that all sixteen cells transiently enter meiosis (Carpenter, 1994) and that the oocyte MTOC is not maintained in *egl* mutants (Theurkauf et al., 1993). This analysis shows that the *egl* mutant alleles used in these two studies contain do not produce detectable amounts of Egl protein; thus, the conclusions of these studies probably apply to the *egl* null phenotype.

The *egl* gene product shows no informative similarities to any previously isolated proteins, although it does show similarity to predicted gene products from across three kingdoms. Doubtless any investigator working on those gene products will look to biochemical studies of Egl for information. Other analyses of the Egl sequence provide more tantalizing, but equally uninformative, results. For example, a short segment of the Egl protein is predicted to have a 67% probability of forming a coiled-coil. This probability is in the low range for known coiled-coils, but higher than other proteins from the non-coiled-coil database (Berger et al., 1995). Because the probability is marginal and the putative coiled-coil is short, only a biochemical study of Egl structure will determine whether this region forms a coiled-coil. However, it is interesting to note that the coiled-coil motif is implicated in protein-protein interactions and BicD protein shows a very high probability of forming a coiled-coil. If these two proteins interact directly in oocyte determination, this interaction may be mediated through a coiled-coil interaction.

One clue as to how *egl* functions comes from looking at where *egl* acts. By making chimeric flies, I have shown that *egl* function is required in the germ line cells of the ovary, but is dispensable in the somatic follicle cells. Consistent with this finding, and with its role in oocyte determination, Egl protein localizes to the developing oocyte in three stages. In the germarium, Egl protein is present in all sixteen germ cells, but becomes concentrated in a single cell. During early stages of oogenesis after the cyst has budded off from the germarium, Egl localizes to the posterior cortex of the oocyte. Three-dimensional analysis of Egl distribution shows that it localizes, not to a spot at what might be the posterior pole of the oocyte, but to one entire face of the oocyte, wherever the oocyte contacts the follicle cells. At later stages of oogenesis, as the oocyte starts to fill with yolk, the distribution of Egl protein shifts so that Egl is now present in an anterior ring and is enriched around the oocyte nucleus.

The pattern of Egl distribution is intriguing for three reasons. First, it is very similar to the distribution of BicD protein. The interaction between these two proteins is explored in Chapter 3. Second, the distribution of Egl protein follows the observed pattern of the location of the minus ends of microtubules: first at the posterior of the oocyte, then at the anterior cortex. The connection between Egl localization and microtubule function is explored in Chapter 4. Since both Egl and microtubules are required for oocyte determination, they may be acting in the same pathway. Microtubules are also required for movement of the oocyte nucleus to the dorsal anterior side of the oocyte and the elaboration of dorso-ventral polarity of the oocyte (Koch and Spitzer, 1983). The third intriguing aspect of Egl localization is that it is localized at later stages of oogenesis, suggesting that *egl* may act in microtubule-dependent processes both early and later in oogenesis. This possibility is examined in Chapter 4.

Materials and Methods

Fly Stocks

egl^{WU50}, *egl*^{RC12}, *egl*^{PB23}, *egl*^{PR29}, *egl*^{PV27}, and *BicD*^{PA66} are described in Schüpbach and Wieschaus, 1991 ; the *egl* alleles are referred to in Lindsley and Zimm, 1992 as *egl*^{1}, *egl*^{2}, *egl*^{3}, *egl*^{4}, and *egl*^{5}, in that order. *egl*^{2b}, *egl*^{2e}, *egl*^{3e} and *egl*^{4e} were isolated by Anne Ephrussi and Francisco Pelegri (Pelegri, 1994) as suppressors of *BicD*$^{71.34}$. *egl*^{1287} and *egl*^{6267} were isolated by the late Laura Kalfayan. *BicD*^{r26} was isolated by Mohler and Wieschaus, 1986. All other strains are as described in Lindsley and Zimm, 1992.

Staining ovaries for DNA and actin

Ovaries were dissected in cold PBS (130mM NaCl, 7mM Na_2HPO_4, 3mM NaH_2PO_4, pH 7.2), fixed for 10' in 8% EM grade formaldehyde (Ted Pella) and extracted for 1-2 hours in 1% Triton X100 in PBS. The ovaries were labelled with BODIPY 581/591 phalloidin (Molecular Probes) as described for embryos in Wieschaus and Nüsslein-Volhard, 1986. Briefly, the ovaries were washed twice in PBS for 10 minutes each. The methanol was evaporated from a 10µl aliquot of phalloidin stock solution for 5 minutes in a Speedvac, then the phalloidin was resuspended in 200µl PBS. The ovaries were incubated with this solution for 20 minutes, washed with PBS for 10 minutes, then incubated in 1:5000 OliGreen (Molecular Probes), 5µg/ml RNAseA and 0.1% Triton X100 in PBS for 30'. After being rinsed twice in PBS to remove the detergent, the ovaries were mounted in 90% glycerol, 1X PBS, 1mg/ml phenylenediamine.

Staining ovaries for β-galactosidase

Ovaries were dissected in cold PBS and fixed for 4 minutes in 0.5% gluteraldehyde in PBS, then rinsed in PBS. The ovaries were stained in 1 ml 7.2mM Na_2HPO_4, 2.8mM NaH_2PO_4, 150mM NaCl, 1mM $MgCl_2$, 3.05mM $K_3Fe(CN)_6$, 3.05mM $K_4Fe(CN)_6$ plus 15µl 10% X-Gal in DMF or in DMSO. The ovaries were stained overnight at room temperature, rinsed in PBS and mounted in 50% glycerol, 1XPBS.

Genomic DNA preparation

Genomic DNA was isolated from 50-200 flies by a protocol developed by Doug Barker (Barker et al., 1992). The flies were homogenized in 5 ml 0.35M sucrose, 0.1M EDTA and 50mM Tris pH 8 and the nuclei were spun down and resuspended in 3 ml 0.1M NaCl, 20mM Tris pH 8 and 10mM EDTA. The nuclei were treated with 10μg/ml RNAseA for 15 minutes at room temperature, then proteinase K was added to 100μg/ml and SDS to 0.5%. This mixture was incubated at 65° for 1 hour, phenol extracted and ethanol precipitated. The precipitated DNA was collected with an inoculating loop into cold 70% ethanol, pelleted and resuspended at 42° overnight in 1μl TE per fly. For higher quality DNA, the samples were phenol extracted and precipitated again.

Genomic phage library construction

To isolate a junction fragment containing sequences from both ends of *Df(2R) or^{BR-11}*, a genomic library was made from DNA from *Df(2R) or^{BR-11}/+* flies, prepared as above. Fifteen micrograms of the genomic DNA was digested with either 0.15U or 0.30U Sau3a for one hour at 37°. The fragments were separated over a 5 ml 5%-30% sucrose gradient and fractions were collected. Fractions containing DNA fragments of approximately 15-20 kb were selected and ethanol precipitated. These fragments were ligated to prepared BamH1-digested lambda DASH2 vector arms (Stratagene). This library was packaged and plated using standard techniques (Sambrook et al., 1989). The junction clones were selected by hybridization to a 4.1 kb EcoR1 fragment from cosmid SD-1, provided by Linda Hall.

Polytene chromosome in situs

Salivary glands were dissected from wandering third instar larvae. Glands were dissected in 45% acetic acid and fixed in fresh 1:2:3 lactic acid:water:acetic acid until the tissue began to disintegrate. The tissue was then covered with a siliconized coverslip and squashed by tapping the coverslip, then wrapping the slide and banging the bottom with a flat pen. The sample was then compressed by pressing the bottom with the pad of the thumb and each slide was frozen in liquid nitrogen. The coverslip was removed quickly with a razor blade, the slide was dehydrated in 95%, then 100% ethanol and then stored dry at 4°. The slides were hybridized as described (Rubin lab manual II, 1990) using nick-translated biotin-labelled DNA.

Ovary RNA preparation and Northern analysis

RNAs were extracted by the method of Barker *et al.* (Barker et al., 1992) and analysed by Northern analysis as in Gavis and Lehmann (Gavis and Lehmann, 1992). Each lane contains 10μg of poly(A) plus mRNA selected from total fly RNA using the Oligotex mRNA kit (Quiagen).

P element constructs

To express the 'A' cDNA under the control of the *otu* promoter, the 'A' cDNA was ligated into the pCOG construct (kind gift of D. Robinson and L. Cooley). This construct has the *otu* promoter upstream of a polylinker and the K10 3' UTR, all in the transformation vector pCaSpeR2 (D. Robinson and L. Cooley, personal communication). A fragment of the 'A' cDNA from the EcoRV site 70 nucleotides upstream of the putative start of translation to the NotI site downstream of the cDNA in the NB40 vector was ligated into the HpaI and NotI sites of pCOG. The 'A' cDNA in the pCOG vector forms the 'CA' construct.

To express the A cDNA under the control of the germ promoter, the A cDNA was ligated into the germ10 construct (Serano et al., 1994; kind gift of R. Cohen). First, the germ10 construct was modified to remove the hsp26 3'UTR by cutting the vector with PstI and BglII, filling the ends and recircularizing the vector. Then, a fragment of the A cDNA, from the NheI site in the 5' UTR to the NotI site in the NB40 vector, was ligated into the NheI and NotI sites of the modified vector, germ10a. The A cDNA in the germ10a vector forms the 'gA' construct.

PCR sequencing of alleles

To sequence the *egl* alleles, PCR products spanning the coding sequence were generated, purified with Centricon 100 columns and sequenced by Liuda Ziaugrua at the Whitehead Sequencing Facility.

Computer methods for database, motif searching

To compare the *egl* sequence to previously identified sequences, BLAST (Atschul et al., 1990) algorithms were used through the Baylor College of Medicine Search Launcher (http://dot.imgen.bcm.tmc.edu:9331/seq-search/protein-search.html).

Generation of anti-Egl antisera

To generate antibodies, four different protein expression constructs were made. The first step in the construction of these constructs was to generate a BamHI site in frame just after the ATG at the start of translation by PCR with the primer ANB (5' CCC GGG CCA TGG CAAC ATG GGA TCC ACG TTG CTC TTC TTC CTG 3') and the internal primer AAN (Appendix). The PCR product was cloned blunt into BluescriptSK and sequenced, then cut with NcoI and BstXI. This fragment was cloned into the NcoI and BstXI sites of the A9 cDNA to form the construct A9B.

For the next step, a BamHI linker (New England Biolabs #1021, pCGGATCCG) was inserted to the MscI site at amino acid 415, or into the NruI site downstream of the coding sequence. Since a second NruI site exists in the coding sequence, this was done by partial digestion. The fragment from the artificial BamHI site to the MscI site is referred to as the 'BM' fragment; the fragment from the artificial BamHI site to the NruI site, containing the full Egl coding region, is referred to as the BN fragment.

To make protein expression constructs, the BM or BN fragment was fused in frame to PET3a (Rosenberg et al., 1987) at the BamHI site, forming constructs PBM and PBN. The BM and BN fragments were also cloned in frame into the BamHI site of pGEX2t (Pharmacia), forming constructs XBM and XBN. PBM was expressed in bacterial strain BL21(DE3) PlysS after induction with 0.5mM IPTG. The antigen was purified as inclusion bodies, gel-purified and sent as a gel slice to HRP, Inc. (Denver, PA) for production of rabbit sera as per their standard protocol. Serum from the third bleed was used for all analyses.

Western blotting

For Western blotting, all gels were 8% acrylamide run on the Hoefer Tall Mighty Small apparatus, blotted semi-dry onto PVDF membrane (Millipore) using the Hoefer Semi-Phor semi-dry transfer apparatus. Anti-Egl serum was used at 1:5000, anti-Vas at 1:50,000 and anti-BicD at 1:2000 in Superblock blocking agent (Pierce) with 0.05% Tween-20, as described in the manufacturer's instructions. HRP-conjugated secondary (anti-rabbit from Amersham or anti-mouse from Jackson labs) was used at 1:5000 and the blot was developed with chemiluminescent detection reagents (Pierce) as described in the manufacturer's instructions.

Antibody staining

Ovaries were dissected in cold PBS, fixed for 10' in 8% EM grade formaldehyde (Ted Pella) and extracted for 1-2 hours in 1% Triton X100 in PBS. The ovaries were blocked in 1X PBS, 1% BSA, 0.1% Triton X100 and 2% normal donkey serum for one hour. The ovaries were then incubated overnight at 4^{o} in the same solution containing primary antibody, anti-Egl at 1:1000, anti-BicD monoclonal 1B11 (kind gift from Beat Suter) at 1:500 or FITC anti-alpha tubulin from Terry Orr-Weaver at 1:250. The ovaries were washed four times, fifteen minutes each in PBT (PBS containing 0.1% v/v TritonX100), blocked again and then incubated in secondary antibody for 2 hours at room temperature. Cy3 donkey anti-rabbit or FITC donkey anti-mouse IgG secondary antibodies (Jackson Labs) were used at 1:500. The ovaries were then washed as above and incubated in 1:5000 OliGreen (Molecular Probes), 5µg/ml RNAseA and 0.1% Triton X100 in PBS for 30'. After washing for 15 minutes in PBT, then rinsing three times in PBS, the ovaries were mounted in 90% glycerol, 1X PBS, 1mg/ml phenylenediamine. All fluorescence images were made with the BioRad MRC 600 confocal with a krypton/argon laser, mounted on a Zeiss Axioskop microscope. Images were processed with the Adobe Photoshop program. All composites were made with the Aldus Freehand program.

Ovary in situ hybridization

All RNA in situs on ovaries and embryos were performed as described by Ephrussi et al., 1991, except where noted. DNA probes were labelled using the Genius DIG DNA labelling and detection kit from Boehringer Mannheim.

Chapter 3:

Interaction between *egalitarian* and *BicaudalD*

Abstract

In this chapter, I show that *egl* and *BicD* interact in oocyte determination. BicD and Egl proteins localize to the same regions of the oocyte during oogenesis and each protein requires the function of both *BicD* and *egl* genes for its localization. *egl* also shows a genetic interaction with dominant alleles of *BicD*. Decreasing the amount of *egl* suppresses the $BicD^{D}$ phenotype; increasing the amount of *egl* enhances the $BicD^{D}$ phenotype, both in penetrance and severity. Coimmunoprecipitation experiments show that Egl and BicD proteins form part of a complex. The formation of this complex, the localization of this complex, and the function of this complex in oocyte determination are separately affected by mutations in *egl* and *BicD*. Thus, as suggested by their colocalization and genetic interaction, BicD and Egl form part of a protein complex that is necessary for oocyte determination.

Introduction

Several results indicate a connection between *egl* and *BicD*, two genes essential for oocyte determination. Analysis of the BicD protein showed that it localizes to the developing oocyte in a pattern very similar to the pattern of Egl protein localization (Suter et al., 1989; Wharton and Struhl, 1989). Moreover, *egl* mutations have been shown to suppress *BicD* dominant alleles (Mohler and Wieschaus, 1986).

The *BicD* loss-of-function mutant phenotype is almost identical to the *egl* mutant phenotype. However, several studies have revealed intriguing differences in the position of the cell that would normally become the oocyte and in the formation of the oocyte microtubule organizing center (MTOC). As in *egl* mutants, *BicD* mutants cause a failure of oocyte determination such that the cell that would normally become the oocyte instead becomes a sixteenth nurse cell (Mohler and Wieschaus, 1986; Ran et al., 1994). Reconstruction of serial electron microscope sections showed that in *egl* mutants, one of the two cells with four ring canals moves to the posterior of the cluster (Carpenter, 1994). Analysis of the pattern of ring canals showed that in *BicD* null mutants, the two cells with four ring canals remain in the center of the cluster (Ran et al., 1994).

Examination of the microtubule structure of the early cyst also revealed a difference between *egl* and *BicD* (Theurkauf et al., 1993). This analysis showed that in two loss-of-function *BicD* alleles, $BicD^{PA66}$ and $BicD^{R26}$, the oocyte MTOC never forms. In *egl* mutant ovaries, the MTOC forms, but subsequently dissipates. I have shown that the two *egl* alleles used in this study, egl^{WU50} and egl^{RC12}, do not produce detectable amounts of Egl protein; although the exact mutation egl^{RC12} is unknown, it seems likely that these alleles show the null phenotype. In contrast, the two *BicD* alleles used are not null mutations. In $BicD^{R26}$, which is a revertant of the dominant allele $BicD^{71.34}$, BicD protein localizes to the oocyte more strongly than in wild type (Suter and Steward, 1991; Wharton and Struhl, 1989). In $BicD^{PA66}$, BicD protein is unlocalized. However, this allele is sterile in trans to the dominant allele $BicD^{71.34}$; the *BicD* deletion *Df(2L)TW119* is fertile in trans to $BicD^{71.34}$ (Ran et al., 1994). True *BicD* null alleles were only recently isolated and are semi-lethal (Ran et al., 1994). Results using the non-null alleles do indicate that *BicD* may be acting upstream of *egl* in oocyte determination. For example, in $BicD^{R26}$, BicD protein localizes to a single cell in the absence of the polarized microtubule array; BicD may be involved in initiating the formation of that array. However, *egl* is required for the localization of BicD protein (Suter and Steward, 1991), indicating that any hierarchy must be tempered by a feedback mechanism.

The *BicD* gene was originally described as a dominant, gain-of-function mutation that caused a maternal-effect perturbation of embryonic polarity (Mohler and Wieschaus, 1986). Females heterozygous for either $BicD^{71.34}$ or $BicD^{IIIE}$ produce embryos in which head structures are replaced by ectopic posterior structures, thus producing the bicaudal, or "two tail" phenotype. These $BicD^{D}$ alleles behave as antimorphic mutations, in that the frequency of bicaudal embryos decreases with increasing wild-type *BicD* gene dosage. Genetic studies showed that the $BicD^{D}$ phenotype can be suppressed by mutations in *tudor*, *vasa*, or *egl* (Mohler and Wieschaus, 1986; Pelegri and Lehmann, 1994). Indeed, egl^{2b}, egl^{2e}, and egl^{4e} were isolated by F. Pelegri and A. Ephrussi as suppressors of $BicD^{71.34}$ (Pelegri, 1994).

Molecular analysis of the $BicD^{D}$ mutations showed that BicD protein accumulation in the oocyte is more pronounced than in wild type; also, in the embryo, the protein accumulates at the anterior, instead of being uniformly distributed as in wild type (Wharton and Struhl, 1989). *osk* RNA is also mislocalized in the embryos produced by $BicD^{D}$ females. In these embryos, *osk* RNA is localized to the posterior, as in wild type, but shows an additional abnormal accumulation at the anterior (Ephrussi et al., 1991). This mislocalization serves to nucleate the localization of the determinant *nanos*, which then causes the pattern duplications (Wang and Lehmann, 1991).

In this chapter, I explore the connection between *egl* and *BicD* with the aim of answering the following questions:

- Is the localization of BicD and Egl proteins identical?
- How do *BicD* mutations affect Egl protein localization?
- Can adding additional copies of egl^{+} enhance the $BicD^{D}$ phenotype?
- How does changing the copy number of *egl* affect the mislocalization of *osk* RNA in $BicD^{D}$ mutations?
- Do BicD and Egl proteins form part of a protein complex? If so, is formation of this complex affected by *egl* or *BicD* mutations?

Results

Egl and BicD localization requires the function of both genes

egl and *BicD* have very similar loss of function phenotypes: both genes are essential for oocyte determination. This suggests that the two genes act in the same pathway, but precludes the ordering of these two genes with respect to each other. Experiments in which the same egg chamber is stained for both Egl and BicD proteins reveal that Egl and BicD have an identical distribution during all stages of oogenesis (Figure 3-1 A). Analysis of Egl and BicD distribution in *egl* and *BicD* mutant ovaries showed that there is no hierarchical relationship in the localization of these two proteins; instead, localization of Egl depends on BicD and vice versa (Figure 3-1 B and C). For example, BicD does not accumulate in a single cell either in the absence of Egl protein (egl^{WU50}) or in the presence of Egl mutant protein which does not localize (egl^{4e}) (Figure 3-1 B). Furthermore, in a *BicD* mutant in which BicD protein is not localized ($BicD^{PA66}$), Egl does not accumulate in a single cell (Figure 3-1 C). In $BicD^{r26}$, mutant BicD accumulates in a single cell in the cyst, as does Egl (Figure 3-1 C). These data suggest that localization of these two proteins is interdependent; whenever one fails to localize, the other also fails to localize. Moreover, in $BicD^{r26}$ mutant oocytes both mutant BicD protein and the wild-type Egl protein localize very strongly to one cell (Figure 3-1 C; Suter and Steward, 1991; Wharton and Struhl, 1989). Thus, the ability of Egl and BicD to localize to the oocyte can be mutated separately from the ability to determine the oocyte. Also, localization of wild-type Egl protein to a single cell in the cyst is not sufficient for oocyte determination.

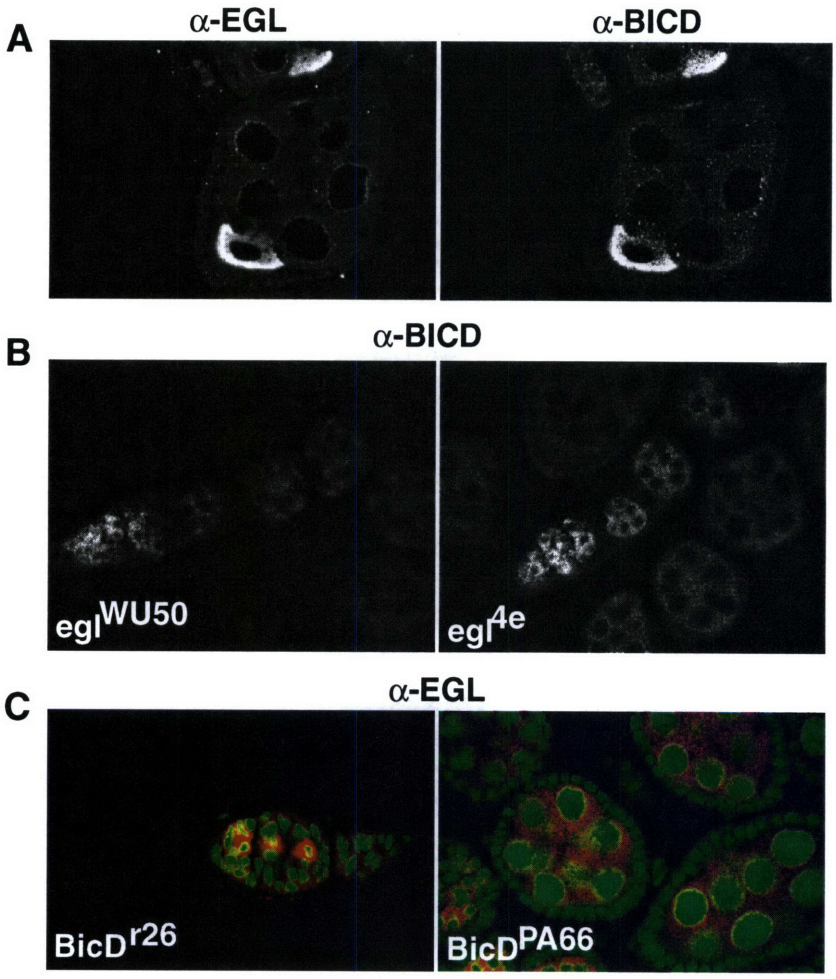
Figure 3-1 Colocalization of Egl and BicD proteins requires the function of both genes

(A) Double staining for Egl and BicD. This sample was stained with both anti-Egl and anti-BicD. Anti-Egl was detected with Cy3 donkey anti-rabbit and BicD was detected with FITC donkey anti-mouse. To collect the image, the fluorescent tags were excited individually; no bleedthrough between channels was observed.

(B) BicD localization in *egl* mutants. BicD protein does not localize to a single cell in *egl* mutants. Each *egl* allele was used heterozygous to $Df(2R)bw^{S46}$.

(C) Egl localization in *BicD* mutants. Egl protein is shown in red and DNA is shown in green. Egl protein does not localize to a single cell in $BicD^{PA66}$. Egl protein does localize to a single cell in $BicD^{r26}$. *BicD* alleles were heterozygous to *Df(2L) TW119*.

Figure 3-1 Colocalization of Egl and BicD requires the function of both proteins



Genetic interaction between *egl* and dominant alleles of *BicD*

Genetic interactions indicate further functional relatedness between the two genes. Females carrying a *BicD* dominant mutation (*Bic-D*^{D}) produce eggs, in contrast to the loss-of-function phenotype. However, the eggs have a defect in oocyte polarity, causing the embryos to develop with reduced head structures, often including ectopic posterior structures replacing the anterior structures, forming bicaudal ("two-tail") embryos. The embryonic phenotypes range in severity, including symmetric bicaudal embryos, which have a complete duplicated abdomen at the anterior, and asymmetric bicaudal embryos, which have a lesser degree of duplication. The least severely affected embryos have no duplications of posterior structures, but have a reduction of head structures. These defects in embryonic polarity can be attributed to a partial mislocalization of *oskar* mRNA to the anterior of the oocyte (Ephrussi et al., 1991). To examine the genetic interaction between *egl* and *BicD*^{D}, I examined the effects of increasing and decreasing the *egl* gene dosage in a *BicD*^{D} mutant background, both on the polarity of the embryos produced, and on the localization of *osk* RNA in those embryos.

The amount of *egl* product in the ovary dramatically affects the severity of the dominant *BicD*^{D} phenotype (Mohler and Wieschaus, 1986). About 33% of embryos from *Bic-D*^{D}/+ females show some loss of head structures; in contrast, only 1% of embryos from females that in addition are heterozygous mutant for *egl* (*BicD*^{D} +/+ *egl*$^{-}$) develops abnormally (Table 3-1). Increasing *egl*$^{+}$ copy number in a *BicD*^{D} background dramatically enhanced the penetrance and severity of the *BicD*^{D} phenotype such that two-thirds of the embryos show anterior defects, with the frequency of the most severe bicaudal phenotype increasing from 11% to 43% (Table 1).

To determine if *egl* directly affects the extent to which *oskar* RNA mislocalizes in embryos from *BicD*^{D} females, I examined the distribution of *oskar* RNA by in situ hybridization on embryos from different genetic backgrounds. I found that in *BicD*^{D} lowering the amount of *egl* wild-type product decreased ectopic localization of *osk* to the anterior and increasing the amount of *egl* wild-type product increased the mislocalization of *osk* to the anterior (Table 3-1). Thus, changing the amount of *egl* in the ovary changes the penetrance and severity of the *BicD*^{D} phenotype. Reducing *egl* suppresses the phenotype; increasing *egl* enhances the phenotype.

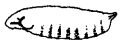
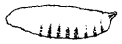
Thus, not only do *egl* and *BicD* share a loss of function phenotype, colocalize and show interdependence in their localization but in addition *BicD*^{D} requires *egl* activity for mislocalization of *osk* RNA. Taken together these results strongly suggest that the two proteins act within a protein complex.

Table 3-1: Genetic interaction between *egl* and *BicD* dominant alleles

The BicD dominant alleles $BicD^{71.34}$ (top) or $BicD^{IIIE}$ (bottom) were used for $BicD^{D}$; similar results are seen for both alleles. egl^{WU50} was used for egl^{-}. '4x egl^{+}' flies contain the two endogenous copies of the *egl* gene and two additional copies of an egl^{+} transgene. For cuticle phenotype, all embryos that hatched were assigned to the 'normal' class and at least 150 eggs were examined. At least 25 cuticles were examined for each class, with more being examined for those classes that produced very few hatching embryos. The unfertilized, or undeveloped class was not included in this table. For *osk* in situs, at least 35 embryos were counted. The class with no *osk* signal was not included in this table.

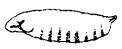
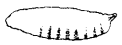
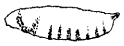
Table 3-1 Genetic interaction between egl and BicD

A. BicD 71.34

Cuticle phenotype		wild type	*BicD^{D} /egl^{-}*	*BicD^{D} /+*	*BicD^{D}*; 4x *egl^{+}*
normal		97%	94%	64%	20%
headless		1%	1%	17%	20%
asymmetric		0%	0%	5%	11%
symmetric bicaudal		1%	0%	11%	43%

***osk* RNA localization**		wild type	*BicD^{D} /egl^{-}*	*BicD^{D} /+*	*BicD^{D}*; 4x *egl^{+}*
normal		100%	86%	51%	43%
weak anterior		0%	14%	29%	37%
strong anterior		0%	0%	12%	15%

B. BicD IIIE

Cuticle phenotype		wild type	*BicD^{D} /egl^{-}*	*BicD^{D} /+*	*BicD^{D}*; 4x *egl^{+}*
normal		97%	95%	45%	9%
headless		1%	2%	19%	22%
asymmetric		0%	0%	14%	18%
symmetric bicaudal		1%	0%	18%	31%

***osk* RNA localization**		wild type	*BicD^{D} /egl^{-}*	*BicD^{D} /+*	*BicD^{D}*; 4x *egl^{+}*
normal		100%	83%	57%	35%
weak anterior		0%	14%	37%	38%
strong anterior		0%	3%	0%	25%

Egl and BicD proteins form part of a protein complex

In order to test whether Egl and BicD form part of a protein complex, I used antisera to immunoprecipitate Egl or BicD from ovary extracts and looked for coimmunoprecipitation of Egl and BicD proteins from wild-type and mutant ovary extracts. For each immunoprecipitation, homogenized ovaries were spun to produce two fractions, an insoluble pellet, which was discarded, and the supernatant, which was used for the immunoprecipitation reaction. Antiserum specific to either Egl or BicD protein was added to bind one of the two proteins, then the antibody was precipitated, along with any bound or associated proteins, by the addition of Protein A sepharose. Anti-Egl antiserum immunoprecipitates both Egl and BicD proteins (Figure 3-2 A) and anti-BicD antiserum (kind gift of Robin Wharton) also immunoprecipitates both proteins (Figure 3-2 B). Less than 50% of Egl protein is immunoprecipitated by anti-Egl antibody (A), while the anti-BicD antibody is very efficient (90%) in immunoprecipitation of BicD protein (B), and in coimmunoprecipitation of Egl protein. Coimmunoprecipitation is specific and is not observed in extracts from ovaries that lack Egl protein (from egl^{WU50} mutants, Figure 3-2 C) nor when nonspecific antibodies such as anti-gamma tubulin (Figure 3-2 A and B; Zheng et al., 1995), anti-Vasa or Egl preimmune serum are used (data not shown).

To determine whether mutations in either protein affect this interaction, I also examined the coimmunoprecipitation of Egl and BicD from extracts of mutant ovaries that contain both BicD and Egl proteins. In egl^{4e} mutants neither BicD nor Egl proteins localize to the oocyte (Figures 2-8 C and 3-1 B). In these mutants, BicD and Egl proteins fail to coimmunoprecipitate (Figure 3-2 C), suggesting that the egl^{4e} mutation interferes with association of the two proteins. In $BicD^{PA66}$ mutants, as in egl^{4e}, BicD and Egl proteins do not localize to a single cell (Figure 3-1C, Suter and Steward, 1991; Wharton and Struhl, 1989). In $BicD^{PA66}$ extracts, BicD and Egl proteins coimmunoprecipitate (Figure 3-2 D), indicating that this mutation does not affect complex formation but rather a common function of the two proteins that is required for localization and function of the protein complex. In $BicD^{r26}$ mutants, BicD protein localizes more strongly to one cell of the sixteen cell cyst than in wild type (Suter and Steward, 1991; Wharton and Struhl, 1989). In this mutant, Egl protein colocalizes with the mutant BicD protein (Figure 3-1 C) and Egl and BicD are coimmunoprecipitated from $BicD^{r26}$ ovary extracts (Figure 3-2 D). This result suggests that $BicD^{r26}$ mutant protein can form a complex with Egl; this complex localizes to one cell in the 16 cell cyst, but is unable to promote oocyte differentiation despite having wild-type Egl protein localized to a single cell.

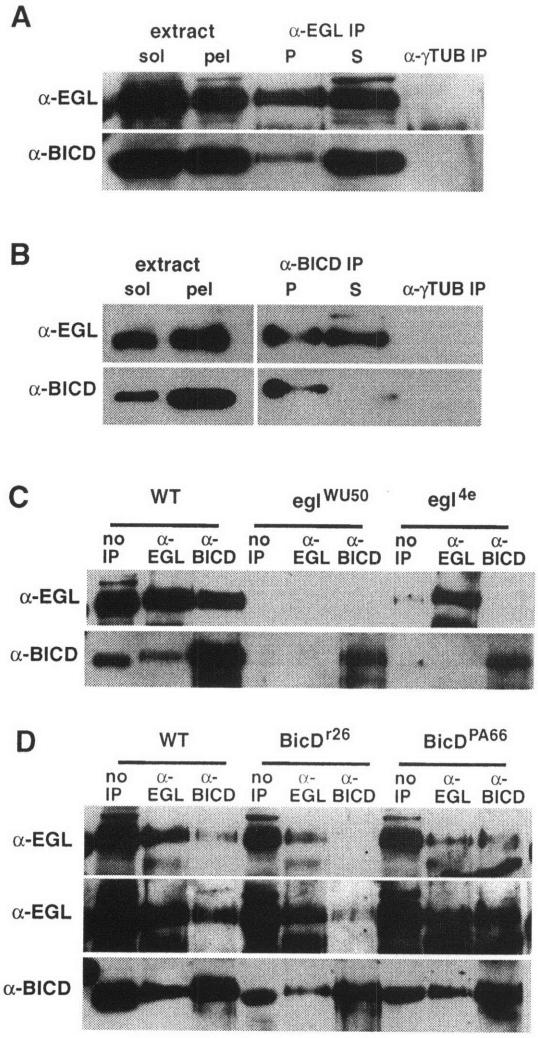
Figure 3-2 Egl and BicD proteins coimmunoprecipitate

In each panel, the antisera used for immunoprecipitation are indicated above the lanes and the antibodies used for Western blotting are indicated to the left. For each immunoprecipitation, homogenized ovaries were spun to produce two fractions, the insoluble 'pel' fraction, which was discarded, and the supernatant, labelled 'sol' or 'no IP', which was used for the immunoprecipitation reaction. For A and B, the proteins immunoprecipitated by the reaction are in the lanes labelled 'P' and the proteins remaining in the supernatant are in the lanes labelled 'S'. For C and D, the supernatants were discarded and only the precipitates were analysed.

(A) Anti-Egl immunoprecipitates both Egl and BicD proteins and (B) Anti-BicD immunoprecipitates both Egl and BicD proteins. For both of these experiments, the 'extract' lanes entitled 'sol' and 'pel' represent the equivalent of 7 µl of ovary extract. The immunoprecipitation lanes each contain the precipitate from 35 µl of the soluble fraction of the ovary extract. The 'S' lane contains an equivalent amount of the supernatant from the anti-Egl (A) or anti-BicD (B) immunoprecipitation. Note that less than 50% of Egl protein is immunoprecipitated by anti-Egl antibody (A), while the anti-BicD antibody is very efficient (90%) in immunoprecipitation of BicD protein (B). I find that anti-BicD antibody is consistently more effective in coimmunoprecipitation of Egl protein (B) than anti-Egl antibody is in coimmunoprecipitation of BicD protein (A).

(C) Immunoprecipitations from *egl* mutants and (D) Immunoprecipitations from *BicD* mutants. For each *egl* allele, females were heterozygous for the allele and chromosomal deletion *Df(2R) bw*^{S46}; *BicD* alleles were heterozygous to *Df(2L) TW119*. In (C) the 'no IP' lane contains the equivalent of 2 µl of ovary extract and the other lanes contain the immunoprecipitate from 35 µl of extract. In extract from *egl*^{WU50} ovaries, BicD protein is visible in a longer exposure in the 'no IP' lane and is clearly present and visible when immunoprecipitated with anti-BicD antiserum. However, no Egl protein is detectable and no BicD protein immunoprecipitates with anti-Egl antiserum. In (D) the middle panel is a longer exposure of the top anti-Egl Western, to show that Egl does coimmunoprecipitate with anti-BicD from *BicD*^{r26}. Also, the 'no IP' lane contains the equivalent of 10 µl of ovary extract and the other lanes contain the immunoprecipitate from 35 µl of extract.

Figure 3-2 Egl and BicD proteins coimmunoprecipitate



Discussion

egl and *BicD* are both required for oocyte determination; experiments in this chapter show that they act together in this process, as part of a protein complex. The interaction between Egl and BicD is suggested by their colocalization, which requires the function of both *egl* and *BicD* genes. Coimmunoprecipitation experiments show that BicD and Egl are part of a protein complex, but do not show whether Egl and BicD proteins interact directly. Both proteins are predicted to form coiled-coil structures, which can mediate protein-protein interactions. However, only biochemical experiments can determine whether Egl and BicD proteins interact directly and, if so, determine which domains are required for this interaction.

The analysis of *egl* and *BicD* mutant proteins reveals that complex formation, complex localization to the oocyte and complex function in oocyte determination are mutationally separable functions of the two proteins:

genotype	interaction	localization	oocyte determination
wild type	yes	yes	yes
egl^{4e}	no	no	no
$BicD^{PA66}$	yes	no	no
$BicD^{r26}$	yes	yes	no

These three mutations all produce approximately the same amounts of Egl and BicD proteins, so comparison of their effects may illuminate the functions of Egl and BicD and the nature of the complex. Unfortunately, immunoprecipitation experiments cannot be used to examine a quantitative effect on complex formation, but can reveal a large change in the amount of complex formed.

egl^{4e} has a cysteine to tyrosine change in a block of amino acids conserved between *Drosophila* and *C. elegans*, in the N terminus of the Egl protein (Figures 2-5 and 2-6). This mutant protein can perform none of the identified functions. That cysteine may be required for Egl folding, or it may be required specifically for complex formation, which may then be essential for localization and oocyte determination.

Two mutant forms of BicD still form some complex with Egl, but show very different effects on the functions of that complex. Examination of $BicD^{PA66}$ shows that maintenance of the interaction of Egl and BicD is not sufficient for localization. $BicD^{PA66}$ encodes an alanine to valine mutation that shows reduced phosphorylation (Suter and Steward, 1991); thus, phosphorylation may be involved in the control of BicD and Egl localization. $BicD^{r26}$ was generated as a reversion

of *BicD*$^{71.34}$ and therefore contains two lesions, the *BicD*$^{71.34}$ lesion, a phenylalanine to isoleucine change at amino acid 684, and the reversion, a deletion of four amino acids (VDAL) from codons 376-379 (Suter and Steward, 1991; Wharton and Struhl, 1989). Although the authors of these studies assign this region to a heptad repeat, and claim it may be involved in coiled-coil formation, analysis of BicD with the Paircoil program (Berger et al., 1995) assigns that region to a short gap between predicted coiled-coils and shows no difference in the predicted probability of forming coiled-coil between the wild-type and R26 BicD proteins.

Although the structural implications of the *BicD*^{r26} mutation will only become clear through further studies, its lesions have identified a region of BicD that is required not for interaction with Egl, nor for localization to the oocyte, but for determination of the oocyte. Because Egl protein localizes to a single cell in *BicD*^{r26}, concentration of Egl protein in a single cell is not sufficient for oocyte determination, making it unlikely that *egl* acts as the oocyte determinant. Theurkauf *et al.*(Theurkauf et al., 1993) have reported that *BicD*^{r26} mutants do not form the oocyte MTOC; this may be the oocyte determination function that *BicD*^{r26} lacks. If BicD protein can localize to a single cell in the absence of a polarized microtubule network, this suggests that BicD may be setting the location of the MTOC and specifying the oocyte. However, the minus end directed microtubule motor dynein transiently localizes to a single cell in *BicD*^{r26} mutants (Li et al., 1994), indicating that this mutant retains some microtubule structure.

My studies showing that BicD and Egl act together in oocyte determination are consistent with a model in which BicD localization specifies MTOC formation, which then leads to Egl localization. After Egl localization, the formation of a complex containing Egl and BicD stabilizes the MTOC and promotes oocyte determination. Another possible model is that Egl and BicD form a complex and localize together; although this model is consistent with the observations that their localization is interdependent and that the proteins form part of a complex, it fails to explain the different effects of *egl* and *BicD* mutations on the oocyte MTOC. *egl* mutants transiently form an MTOC in a single cell; *BicD*^{r26} and *BicD*^{PA66} fail to form the oocyte MTOC (Theurkauf et al., 1993). If the failure to form a visible oocyte MTOC is a gain-of-function aspect of the two *BicD* alleles used in this study, then *BicD* null alleles (Ran et al., 1994) may transiently form the MTOC, similar to *egl* mutants. Moreover, if *egl* function is required by these alleles, then *egl-BicD*^{r26} or *egl-BicD*^{PA66} double mutants may also transiently form the oocyte MTOC. Thus, resolution of these models awaits clarification of the microtubule structure in these genotypes.

The analysis of the dominant alleles of *BicD* shows two interesting features of *egl* and *BicD* function. First, the enhancement and suppression of *BicD*^{D} by differing gene dosages of *egl* supports the observation that Egl and BicD proteins form part of a complex. Second, the effect of *egl*, *BicD* and *BicD*^{D} mutations on *osk* RNA localization suggests that Egl and BicD may have a

role in RNA localization and that they may have a role in axis determination later in oogenesis. These topics are discussed in Chapter 4, which contains observations on the role of *egl* in RNA localization in dorsoventral axis formation. Also, Chapter 5 contains speculation about the role of *egl* and *BicD* in microtubule polarity and RNA localization as a link connecting oocyte determination, anterior-posterior axis specification, and dorsoventral axis formation.

Materials and Methods

Fly Stocks

BicD^{PA66} is described in Schüpbach and Wieschaus, 1991. *BicD*^{r26}, *BicD*$^{71.34}$, and *BicD*^{IIIE} are described in Mohler and Wieschaus, 1986. All other strains are as described in Lindsley and Zimm, 1992.

Cuticle preparations

Cuticles were prepared by dechorionating in 50% bleach, fixing in 1:4 glycerol:acetic acid and embedding in Hoyer's medium (Wieschaus and Nüsslein-Volhard, 1986).

Embryo in situs

Embryos were hybridized as in Gavis and Lehmann, 1992.

Immunoprecipitations

To prepare ovary extract, ovaries were dissected in cold 1X PBS. For each 50 µl ovaries, 180 µl buffer without detergent, but with protease inhibitors (final with detergent: 50 mM Tris pH 8.0; 150 mM NaCl; 10 µg/ml each leupeptin, Pefabloc and pepstatin from Boehringer Mannheim; 1% aprotinin and 0.1 mM PMSF from Sigma) were added. The extract was homogenized and spun at maximum speed in a microfuge at 4^{o} for 5'. The resulting pellet was discarded as the insoluble fraction. 10% NP-40 was added to the supernatant to a final concentration of 1%.

For each immunoprecipitation reaction, 75 µl of the soluble fraction of the extract was combined with 2 µl antibody (rabbit anti-Egl or rat anti-BicD, a kind gift from Robin Wharton) and incubated on ice for 1 hour. 30 µl of a 1:1 suspension of Protein A Sepharose (Pharmacia) was added and incubated on ice for 1 hour with occasional mixing. The beads were rinsed once with cold NP-40 buffer with protease inhibitors, then washed 3 times for 5' each. The beads were resuspended in 50 µl sample buffer (125 mM Tris pH 6.8, 4% SDS, 20% glycerol, 5M urea, 0.01% bromphenol blue) plus 10 µl 1M DTT and boiled for 5' before being loaded on a gel.

Chapter 4:

egalitarian, microtubules and oocyte polarity

Abstract

Cytological studies (Theurkauf et al., 1992) have shown that the microtubule cytoskeleton undergoes complex rearrangements during oogenesis; during these rearrangements, Egl protein localizes to the position of the minus ends of microtubules. In ovaries mutant for genes that affect the polarity of the oocyte, Egl localization changes to reflect the polarity of the mutant ovary. Moreover, examination of Egl localization in ovaries treated with microtubule depolymerizing drugs showed that Egl localization requires microtubule function. Inhibitor treatments (Koch and Spitzer, 1983; Theurkauf et al., 1993) have shown that microtubule function is important for several stages of oogenesis in *Drosophila*, including oocyte determination, transport into the oocyte, migration of the oocyte nucleus and formation of the dorsoventral axis of the oocyte. Both *egl* and microtubules are required for oocyte determination; to determine whether *egl* functions in later steps of oogenesis that also require microtubules, I examined the phenotype of eggs produced by females with reduced amounts of *egl*. These eggs show a perturbation of dorso-ventral polarity, indicating that *egl* is required for later oocyte polarity.

Introduction

The specification of pattern in the *Drosophila* oocyte proceeds sequentially (reviewed in Ray and Schüpbach, 1996). First, a single cell is specified as the oocyte and this cell moves to the posterior of the cluster of sixteen interconnected sibling germ cells. Second, RNAs, including *gurken* (*grk*) mRNA, are localized into the developing oocyte; Grk protein at the posterior of the oocyte signals to the follicle cells, delineating the anterior-posterior polarity of the oocyte. Third, a reciprocal signal from the follicle cells causes a shift in microtubule polarity, which results in movement of the oocyte nucleus to the anterior of the oocyte. *grk* RNA relocalizes in association with the oocyte nucleus and again signals oocyte polarity to the follicle cells, delineating the dorso-ventral polarity of the oocyte.

The oocyte must move to the posterior of the cyst for proper oocyte polarity, because only a subset of follicle cells is competent to respond to the *grk* signal. For example, in *SpindleC* mutants, the oocyte fails to move to the posterior of the cyst and an oocyte develops with a cluster of nurse cells at both the anterior and the posterior (González-Reyes and St Johnston, 1994). In the absence of contact with posterior follicle cells, the oocyte develops as if it had two anterior poles; *bicoid* RNA localizes to the anterior and posterior, at both nurse cell-oocyte boundaries, and *osk* RNA localizes to the center of the oocyte.

Microtubule function is essential for oocyte determination, transport into the oocyte, movement of the oocyte nucleus, and RNA localization (Koch and Spitzer, 1983; Pokrywka and Stephenson, 1991; Theurkauf et al., 1993). Also, the microtubule cytoskeleton reorients during development in a pattern correlated with its functions (Theurkauf et al., 1992). Initially, a polarized array of microtubules forms from an MTOC in the oocyte; during early oogenesis, the microtubules are oriented such that their minus ends are at the posterior of the oocyte. Later, the microtubules reorient in response to a signal from the posterior follicle cells; at stage 9, the minus ends of the microtubules are at the anterior cortex of the oocyte.

One interesting mutation that affects the microtubule organization of the ovary is the *morula* (*mr*) mutation (Reed, 1992). In *mr* mutants, oogenesis proceeds normally, but instead of accumulating yolk in the oocyte, the cluster breaks down and the nurse cells begin to condense their chromosomes and assemble spindles. Thus, these cells revert from the endoreplication cycle, causing perturbations of both the cell cycle and the microtubule structure of the cyst. Instead of the cyst having microtubules organized for transport and localization of oocyte components, the microtubules form inappropriate spindles in the nurse cells.

The *gurken* gene is essential for formation of both the anterior-posterior axis and the dorso-ventral axis (González-Reyes et al., 1995). Grk protein is a TGFα-like molecule secreted from the

oocyte to be received in the follicle cells by the Torpedo protein, the *Drosophila* EGF receptor (Clifford and Schüpbach, 1994; Neuman-Silberberg and Schüpbach, 1993). Grk signalling induces the polar follicle cells at the posterior to differentiate as posterior cells; these cells in turn signal back to the oocyte to induce the reorientation of the oocyte microtubule cytoskeleton. Later in oogenesis, the *grk-top* signalling pathway again polarizes the oocyte, by signalling to the dorsal follicle cells. In the absence of *grk* or *top*, the minus ends of the microtubules remain at the posterior, as well as forming at the anterior, *bicoid* mRNA localizes to both ends of the oocyte, and the oocyte develops with two anterior poles.

As described in Chapter 2, *egl* function is essential for the first step in oocyte polarity, the specification of the oocyte from its sister cells, the nurse cells. Microtubule function is also essential for oocyte determination, as well as being required for oocyte dorso-ventral axis formation. Moreover, the pattern of Egl protein localization shows a striking similarity to the distribution of the minus ends of microtubules during oogenesis. In this chapter, I examine the connection between Egl localization and microtubule function, with the aim of answering the following questions:

- Does Egl protein localization require microtubule function?
- Do mutations that affect the microtubule polarity of the oocyte affect Egl protein localization?
- Is *egl* function required after oocyte determination, in later oogenesis?

Results

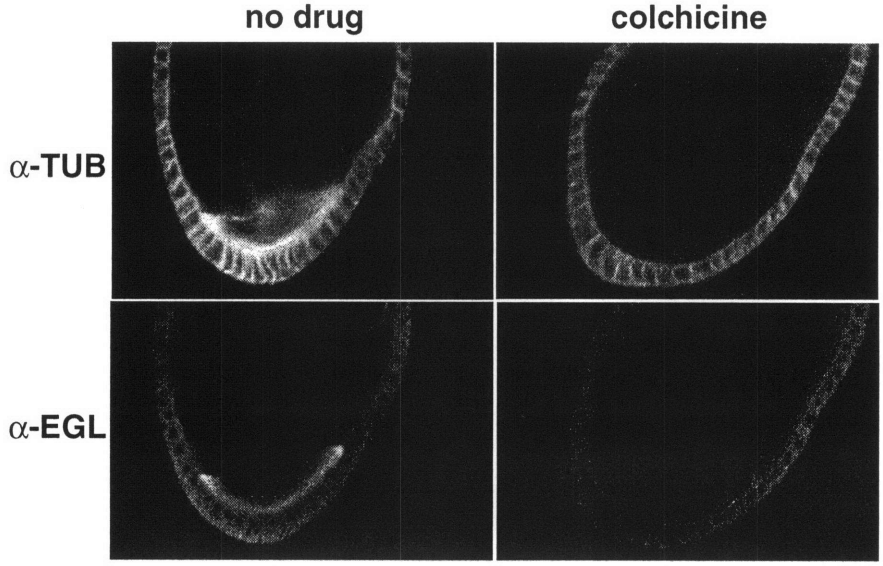
Egl localization requires microtubules

The distribution of Egl protein closely parallels the predicted orientation of the minus ends of the microtubules during oogenesis. Furthermore, treating wild-type flies with microtubule inhibitors causes a failure of oocyte determination, producing ovaries in which the oocyte has shifted to the nurse cell fate (Koch and Spitzer, 1983; Theurkauf et al., 1993). Thus, both microtubule structure and *egl* function are essential for oocyte determination. If the role of microtubules in oocyte determination is to transport or localize Egl, then the localization of Egl should be dependent on microtubules and should be disrupted when microtubules are absent. Alternatively, if *egl* is required for orientation of the microtubule network in the cyst, then Egl localization may be independent of microtubule integrity. To distinguish between these two models, I treated flies with colchicine to disrupt the microtubules and observed the distribution of Egl protein. I used a short colchicine treatment so that the ovaries would contain some already formed oocytes, allowing examination of all stages of Egl protein localization. This treatment disrupts microtubule organization in the oocyte and nurse cells and abolishes Egl localization in the oocyte at all stages of oogenesis (Figure 4-1). Thus, Egl localization requires an intact microtubule cytoskeleton.

Figure 4-1 Microtubules are required for Egl localization

These ovaries were double-stained with anti-tubulin, top, and anti-Egl, below. Flies were either fed plain yeast paste or yeast paste containing 50 µg/ml colchicine for 18 hours before being dissected and stained. This treatment disrupts the germ line microtubules without affecting the follicle cell microtubules (Theurkauf et al., 1993).

Figure 4-1 Microtubules are required for Egl localization



Egl localization in mutants that perturb oocyte polarity

In order to determine whether Egl can localize to the minus ends of other microtubules, I examined the distribution of Egl in *morula* (*mr*) mutants that form inappropriate spindles in the nurse cells. If Egl can localize to the minus ends of any microtubules, then it should accumulate at the spindle poles in these mutant ovaries, unless these inappropriate spindles also have an unusual microtubule organization. By staining mr^2 ovaries with anti-Egl and anti-tubulin, I found that Egl was uniformly distributed, not concentrated at the minus ends of the microtubules at the spindle poles (Figure 4-2 E and F). Thus, Egl does not associate with the minus ends of microtubules in the spindles of mr^2 mutant ovaries. Further study of the inappropriate spindles in *mr* mutants will be required to determine the significance of this result.

In *spindleC* (*spnC*) mutants, the oocyte is often positioned incorrectly, resulting in changes in its microtubule polarity, presumably due to its failure to communicate with the polar follicle cells (González-Reyes and St Johnston, 1994). In *spnC* ovaries, Egl protein accumulates in the oocyte but does not associate with the posterior cortex. Instead, it concentrates near the oocyte nucleus, often on the side away from the follicle cells (Figure 4-2 C). At later stages, it fills the oocyte but does not show a subcellular localization within the oocyte (Figure 4-2 C).

As the oocyte expands in *spnC* mutants, it often pushes apart the nurse cells, separating them into anterior and posterior clusters. The follicle cells migrate over the centrally located oocyte and the border cells migrate from both ends. At this stage, *bicoid* mRNA localizes to both ends of the oocyte and *osk* RNA localizes to the center (González-Reyes and St Johnston, 1994). Egl protein localizes to the entire oocyte cortex, where it is adjacent to nurse cells and to follicle cells (Figure 4-2 D).

In *gurken* (*grk*) mutants, the oocyte fails to signal the polar cells to form posterior polar cells. These cells develop as anterior polar cells and fail to signal the oocyte to form a posterior pole (González-Reyes et al., 1995). This causes the *grk* mutant oocyte to develop an ectopic anterior pole at the posterior. The oocyte nucleus fails to shift to the dorsal anterior side at stage 9, indicating that the rearrangement of the cytoskeleton at this stage fails to occur. Egl protein normally localizes to the anterior cortex at this stage. Egl localizes normally until stage 9, when it should shift to the anterior (Figure 4-2 A and B). In *grk* mutant ovaries, some Egl localizes to the anterior, but most of the Egl remains at the posterior pole. Thus, the failure of microtubule reorganization in *grk* mutants causes Egl localization to remain in a configuration typical of earlier stages of oogenesis, but inappropriate for a later stage of oogenesis.

Figure 4-2 Egl localization in *gurken*, *spindleC*, and *morula* mutants

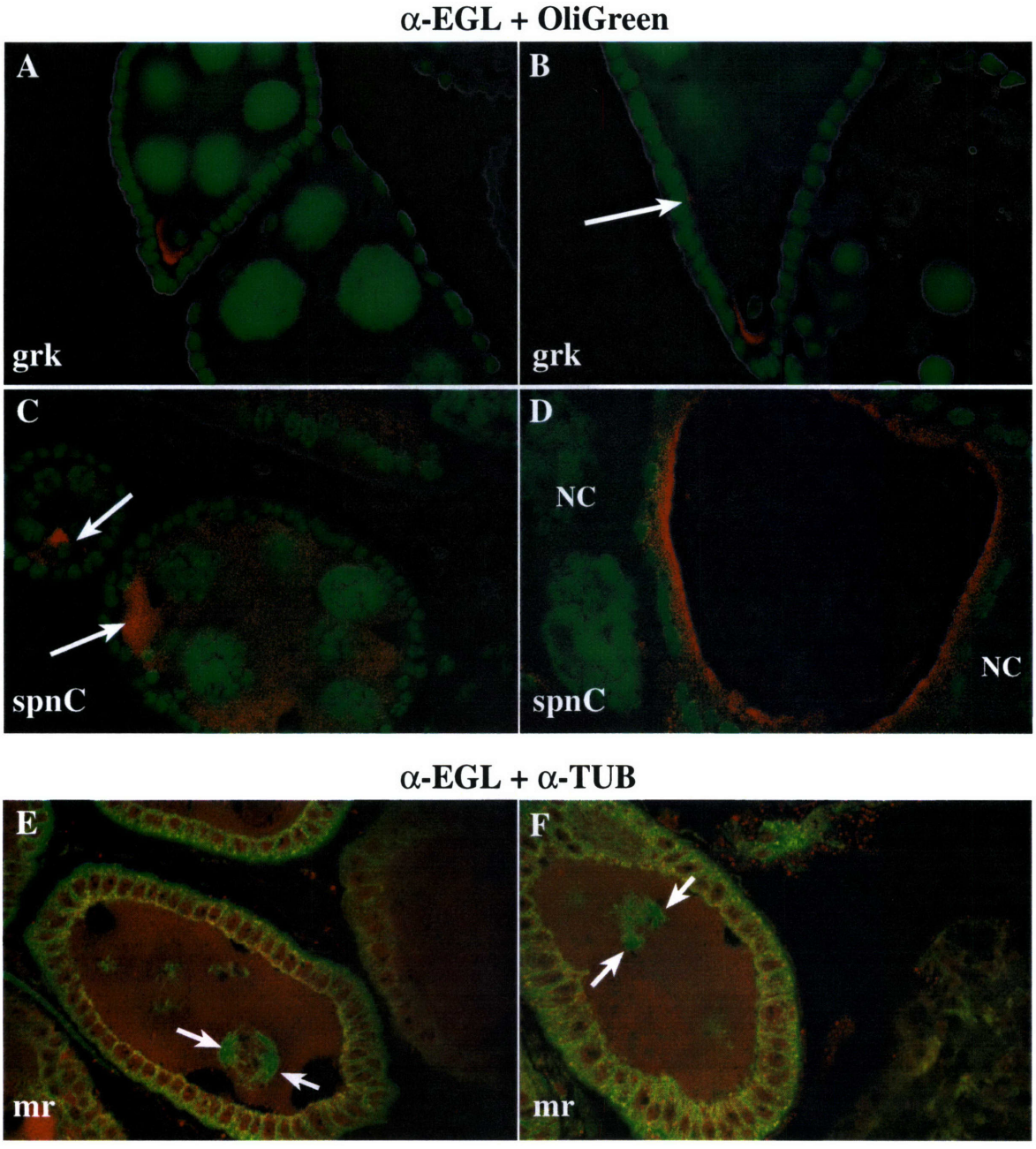
(A-D) DNA is shown in green; (E and F) tubulin is shown in green. Egl protein is shown in red.

(A) In *gurken* (*grk*) mutants, the absence of a signal from the oocyte causes the posterior follicle cells to develop as anterior follicle cells. These cells then fail to produce the reciprocal signal that induces the reorientation of the microtubule cytoskeleton of the oocyte. The early stages of Egl localization are unperturbed in *grk* mutants. Egl protein is visible at the posterior in this stage 6 egg chamber.
(B) At stage 9 in wild-type ovaries, the oocyte nucleus is at the dorsal anterior side of the oocyte and Egl protein localizes to the anterior. In this stage 9 *grk* mutant egg chamber, Egl protein and the oocyte nucleus both remain at the posterior. A small amount of Egl protein, visible as a faint patch of red in the upper left corner of the oocyte (arrow), localizes to the anterior at this stage.

(C) In *spindleC* (*spnC*) mutants, the oocyte is correctly specified, but fails to migrate to the posterior of the cluster. In the stage 4 egg chamber at left, the oocyte is in a lateral position (arrow). Egl localizes to the oocyte, but fails to associate with the posterior cortex. In the stage 6 egg chamber at the center of this panel, the oocyte is located anteriorly (arrow); Egl accumulates in the oocyte, but fails to form a posterior crescent on the oocyte cortex.
(D) In this stage 10 *spnC* egg chamber, the oocyte is located between two clusters of nurse cells (NC). Egl protein localizes to the entire margin of the oocyte.

(E and F) In *morula* mutants, the endoreplication cycle of the nurse cells fails and the chromosomes condense onto spindles. The spindles are indicated by pairs of arrows pointing at the spindle poles. Egl protein is uniformly distributed in these egg chambers and shows no concentration at the minus ends of the spindle pole microtubules.

Figure 4-2 Egl localization in gurken, spindleC, and morula mutants



gurken localization and oocyte dorso-ventral polarity require *egl*

Egl is enriched at the anterior pole of the oocyte in close proximity to the oocyte nucleus during later stages of oogenesis, suggesting that it may also play a role in later oocyte polarity. I could not address this question with the existing *egl* alleles, because strong *egl* mutants form no oocyte. Fortunately, several egl^{+} transgenes provide only partial complementation of the *egl* phenotype (Figure 4-3 A), possibly due to the site of transgene insertion. Seventy-seven percent of eggs produced from egl^{-}; $P[egl^{+}]^{wk}$ females display various degrees of egg shell ventralization, such as fusion of the two dorsal appendages and complete loss of dorsal appendages (Figure 4-3 B). To test whether this effect on egg shell morphology was caused by improper localization of *gurken* RNA I probed egl^{-}; $P[egl^{+}]^{wk}$ ovaries for *gurken* RNA distribution. *gurken* RNA localization is reduced or absent in these ovaries but the oocyte nucleus is usually correctly located at the dorsal anterior side of the oocyte (Figure 4-3 B). The correct location of the oocyte nucleus indicates that the microtubule structure of these ovaries is normal. Staining with anti-tubulin confirmed this indication and showed that the microtubule distribution in $P[egl^{+}]^{wk}$ ovaries is indistinguishable from wild type. The distribution of *oskar* RNA was unaffected in these oocytes (data not shown). These results suggest that *egl* is not involved in stabilizing microtubules during oogenesis but may instead be acting to reinforce the established cell polarity by localizing molecules such as *grk* mRNA.

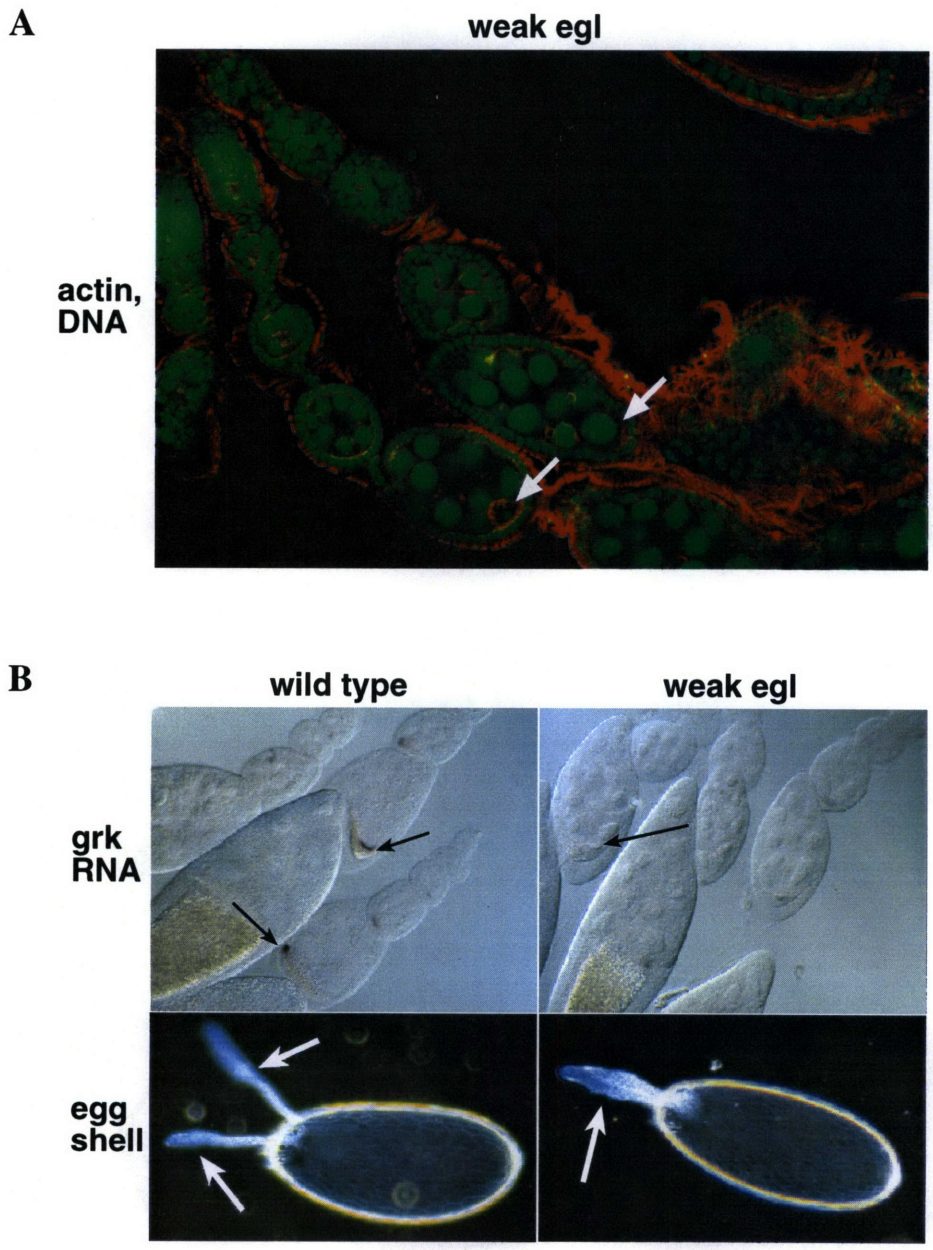
Figure 4-3 *egl* is required for oocyte dorsoventral polarity

(A) Some insertions of an egl^+ transgene provide variable complementation of the *egl* mutant phenotype. DNA is shown in green, filamentous actin is shown in red. This ovary, from a *yw*; egl^{WU50}; P[egl^+]/+ female, shows some egg chambers that form sixteen nurse cells and some egg chambers that form fifteen nurse cells and an oocyte. One of each kind of egg chamber is indicated by arrows.

(B) Reduced *egl* activity reveals a role of *egl* in dorsal-ventral polarity of the egg. In wild-type ovaries, *grk* RNA initially accumulates in the oocyte and by stage 9 of oogenesis *grk* RNA localizes to the anterior margin of the oocyte in close association with the oocyte nucleus (upper left panel, arrows). In ovaries that have reduced *egl* function, *grk* RNA is either only weakly localized or unlocalized (upper right panel). However, the oocyte nucleus does move to the dorsal anterior side of the oocyte in this genotype.

Presumably as a consequence of partial loss of *grk* function, the egg shells of eggs produced by females that have reduced *egl* function show a loss of dorsal fates resulting in fused dorsal appendages. The wild-type egg shell in the lower left panel has two distinct dorsal appendages (arrows), but the weak *egl* egg shell in the right panel has a single appendage (arrow). Females mutant for *egl* but carrying one of two different weak insertions of an egl^+ transgene produce eggs in the following distribution: 18% wild type, 77% fused dorsal appendages, 5% no dorsal appendages. The weak *egl* genotype used is *yw*; egl^{WU50} with one copy of an egl^+ transgene (CA3B or CA13F) that only partially complements the *egl* phenotype. *grk* in situs were hybridized as in Ephrussi et al., 1991.

Figure 4-3 *egl* function is required for dorsoventral polarity of the oocyte



Discussion

Egl protein localization depends on microtubule function. This result suggests that Egl does not establish the orientation of microtubules, but may reinforce or maintain the microtubule network once an initial asymmetry has been established. This result is consistent with the finding that in *egl* mutants the initial formation of the microtubule network is not affected but that the microtubule network disintegrates. Indeed, in *grk* and *spnC* mutants, Egl localization follows the location of the predicted minus ends of the microtubules.

In contrast, in *morula* mutants, which form microtubule spindles in the germ cells, Egl does not localize to the spindle poles, which are likely to be the location of the minus ends of the microtubules. The Egl localization process may be disrupted by the drastic rearrangements in *mr* mutants, or may be specific to the normal oocyte microtubule structures. It would be interesting to determine whether the spindle poles in *mr* mutants accumulate other minus-end directed oocyte components, such as *bicoid* RNA, or whether the oocyte microtubule transport system fails to function in these disrupted ovaries.

In *grk* mutants, the oocyte develops as if it had two anterior poles. However, the pattern of Egl localization indicates that the oocyte has retained an earlier pattern of microtubule organization that is inappropriate for later stages of oogenesis. However, the RNA sorting that happens at stage 9 of oogenesis happens normally, but the sorted RNAs are directed to the wrong cytoplasmic locations because of the misorientation of the microtubule cytoskeleton.

One interesting aspect of Egl localization in *grk* mutant ovaries is that most Egl remains at the posterior, rather than being segregated to both ends of the oocyte. Perhaps Egl must be released from the posterior before it can accumulate at the anterior. Indeed, Egl protein spreads more along the cortex of the oocyte at early stages of oogenesis than does cytoplasmic dynein. Egl may be anchored to the cortex, rather than localized to the MTOC. However, this hypothesis seems to be inconsistent with the loss of Egl localization in flies treated with microtubule inhibitors; if Egl protein is anchored to the cortex, then its localization should not be perturbed by microtubule inhibitors.

The activity of Protein Kinase A is required in the germ line to transduce the signal from the soma back to the germ line (Lane and Kalderon, 1994). Intriguingly, Egl contains five possible sites for phosphorylation by this cAMP-dependent kinase. Phosphorylation by Protein Kinase C has been shown to regulate the associtation of the MARCKS (myristoylated, alanine-rich C kinase substrate) protein with the membrane (Thelen et al., 1991). Phosphorylated MARCKS shifts to the cytosolic fraction; dephosphorylated MARCKS reassociates with the membrane. If the possible myristylation sites in Egl are used, then this modification, along with nearby basic amino

acids (Hancock et al., 1990; reviewed in Resh, 1994), may mediate association of Egl with the oocyte cortex. From this circumstantial evidence, one speculative hypothesis for Egl function later in oogenesis is that PKA phosphorylation causes the dissociation of Egl from the posterior cortex. Dephosphorylation of Egl at the anterior may cause its reassociation with the cortex. This hypothesis is consistent with the observation that in *grk* mutants, which fail to produce this signal, very little Egl protein shifts to the anterior cortex. If Egl were merely localizing to the minus ends of microtubules, it should be evenly distributed between the anterior and the posterior in this mutant.

egl is required, not only for oocyte determination, but also for oocyte dorsoventral polarity. Thus, egl may act with microtubules at several stages of oogenesis. Its possible roles in linking RNA localization and microtubule polarity are discussed in Chapter 5.

Materials and Methods

Fly stocks

morula mr^2 flies were provided by Bruce Reed (Reed, 1992). $SpnC^{442}$ and $SpnC^{660}$ are described in González-Reyes and St Johnston, 1994. grk^{HP48} and grk^{PC29} are described in Schüpbach, 1987.

Inhibitor treatments

For the inhibitor studies, flies were fed 50μM colchicine in yeast paste for 16 hours as described in Theurkauf et al., 1993.

Eggshell preparation

Whole eggs were mounted in 1:1 Hoyers:lactic acid and cleared overnight at 65°, as described in Brand and Perrimon, 1994.

Chapter 5:

Conclusions and future directions

Conclusions

I have identified a protein complex which contains Egl and BicD proteins and which is essential for the designation of one of sixteen sister cells as the oocyte: in the absence of either *egl* or *BicD* function, no oocyte develops and all sixteen cells become polyploid nurse cells. The existence of a complex containing these proteins is strongly suggested by the genetic interaction between the loci and by the colocalization of the protein products. Concomitant with the first morphological signs of oocyte differentiation, the formation of synaptonemal complex in the pro-oocyte (Carpenter, 1975), both Egl and BicD become enriched in a single cell. Mutations in either *egl* or *BicD* affect the oocyte localization pattern of the other, suggesting that complex formation precedes or coincides with localization of the proteins to a single cell and subsequent determination of the oocyte. Complex formation, complex localization and complex function can be affected separately by different *egl* or *BicD* mutations providing evidence for the existence of distinct functional domains in the two proteins.

A role for egl *and* BicD *in oocyte determination*

Egl is localized to a single cell and is required for the development of that cell as the oocyte; however, three observations indicate that *egl* is not the oocyte determinant. First, increasing the copy number of egl^{+} to increase the levels of Egl protein within the germarium does not cause multiple cells within the cluster to form oocytes. Second, in *egl* null mutations all sixteen cells initially follow the pro-oocyte fate and form synaptonemal complex before reverting to the nurse cell fate (Carpenter, 1994). Third, in $BicD^{r26}$ mutants, Egl protein is highly enriched in a single cell but this cell does not follow the oocyte fate. Thus, Egl protein alone is not sufficient to control oocyte fate. I therefore favor the idea that the Egl /BicD protein complex promotes oocyte fate by controlling the distribution of molecules that regulate oocyte and nurse cell differentiation.

The germ cells adjacent to the oocyte exhibit one sign of oocyte determination, formation of synaptonemal complex; this may reflect the distribution of an oocyte determinant. An early sign of oocyte determination is the formation of the synaptonemal complex, however cells with three or four ring canals transiently form synaptonemal complexes but eventually follow the nurse cell fate (Carpenter, 1975). At this stage of oogenesis, in germarial region 2A and 2B, Egl and BicD proteins become enriched in the future oocyte. If Egl and BicD are involved in localizing oocyte determining factors, lower amounts of these factors in cells adjacent to the oocyte may account for this transient entry into meiosis. Consistent with this hypothesis, in *egl* mutants all sixteen cells transiently form synaptonemal complexes (Carpenter, 1994). According to this model, *egl* may be required not for the activation or synthesis of oocyte determining factors, but rather for producing a critical concentration of these factors in the future oocyte.

Differentiation of the nurse cell-oocyte cluster requires the establishment of two cell fates: the premeiotic oocyte and polyploid nurse cells. In *egl* and *BicD* mutant ovaries the cell that would normally become an oocyte develops as a sixteenth nurse cell; thus, the same factors that promote oocyte determination may also repress nurse cell fate. Common to both oocyte and nurse cell fate decisions is a change from normal cell cycle regulation: the oocyte arrests in meiotic prophase and the nurse cells become polyploid by DNA replication without mitosis. A *cyclinE* mutation perturbs this decision, causing one of the cells that would normally become a nurse cell to develop as a second oocyte (Lilly and Spradling, 1996); thus, determination of oocyte and nurse cell fate may be linked to the differential distribution or activation of cell cycle regulators. It is therefore intriguing to speculate that the Egl/BicD complex is involved in the distribution of these regulators.

egl *and* BicD *in axis determination*

Specification of the oocyte as different from its fifteen sister cells and positioning of the oocyte posterior to the nurse cells are essential for the subsequent polarization of the oocyte. Oocyte determination requires *egl* and *BicD*; oocyte positioning requires the function of a number of genes such as *dicephalic*, *homeless*, and *spindleC* (Frey et al., 1984; Gillespie and Berg, 1995; González-Reyes and St Johnston, 1994). Oocyte determination leads to microtubule-mediated transport of RNA from the nurse cells into the future oocyte (Theurkauf et al., 1993). Among these RNAs are *gurken*, *oskar* and *bicoid* which encode essential regulators of the two embryonic axes (Pokrywka and Stephenson, 1991; Pokrywka and Stephenson, 1994). The anterior-posterior axis is thought to be set when Gurken protein, a TGFα-like molecule synthesized from *grk* RNA in the oocyte and presumably secreted from the oocyte, signals to the underlying follicle cells to set posterior follicle cell fate (González-Reyes et al., 1995; Roth and Schüpbach, 1994; reviewed in Lehmann, 1995; Ray and Schüpbach, 1996). A yet unknown signal returned from the follicle cells leads to the repolarization of the microtubule network in the oocyte such that the MTOC at the

posterior cortex is lost and microtubules now extend with the minus end from the anterior toward the posterior. This repolarization has two consequences; first microtubule polarity leads to the sorting of RNA molecules along the anterior-posterior axis, i.e. *bicoid* RNA becomes localized to the anterior pole and *osk* RNA becomes localized to the posterior pole of the oocyte. Second, *grk* RNA moves in close association with the oocyte nucleus to the dorsal anterior margin of the oocyte. In another intercellular signaling step Gurken protein secreted from the oocyte now promotes dorsal follicle cell fate.

Each sequential specification, first of the oocyte, then of the anterior-posterior axis, and finally of the dorso-ventral axis, requires both RNA transport along a polarized microtubule network and the function of the Egl/BicD complex. The distribution of Egl and BicD proteins resembles the localization of both the minus ends of microtubules and many of the RNAs involved in these specifications. Also, many RNAs that are transported into the oocyte during early oogenesis do not accumulate in a single cell in *egl* and *BicD* mutants. Mutations in either *egl* or *BicD* disrupt microtubule stability or the initiation of the microtubule organizing center in the oocyte.

The role of the Egl/BicD complex in anterior-posterior axis formation is suggested by its effect on localization of *osk* RNA. *egl* and *BicD* mutants abolish transport of *osk* into the oocyte (Ephrussi et al., 1991). In $BicD^{D}$ mutants, the BicD/Egl complex directs ectopic localization of *osk* RNA to the anterior of the oocyte (Ephrussi et al., 1991). The *osk* localization signal in the *osk* 3'UTR contains separable regions for oocyte and posterior localization of *osk* (Kim-Ha et al., 1993). The Egl/BicD complex affects the initial localization of *osk* to the oocyte; in $BicD^{D}$ mutants, the complex may mediate ectopic localization via the oocyte localization signal. This hypothesis would explain why in these mutants *osk* is localized to the anterior pole independent of the posterior localization machinery (Ephrussi et al., 1991; Lehmann and Nüsslein-Volhard, 1991; Manseau and Schüpbach, 1989).

The Egl/BicD complex is also involved in establishment of the dorso-ventral axis. I show that *egl* affects egg shell morphology and that this phenotype can be attributed to a defect in *grk* RNA localization. Similarly, Mohler and Wieschaus (Mohler and Wieschaus, 1986) observed that 90% of eggshells produced by $BicD^{D}$ mutant females have fused dorsal appendages, an indication of ventralization due to reduced function of the *grk* pathway. Thus the Egl/BicD complex may not only affect initial transport of *grk* RNA into the oocyte where Gurken sets the anterior-posterior axis but may also affect *grk* RNA localization to the anterior during mid-oogenesis when Gurken sets the dorso-ventral axis.

Although it is possible that the Egl/BicD complex affects RNA localization solely by stabilizing microtubule structure, I favor the hypothesis that association of the Egl/BicD complex with microtubules stabilizes microtubules and that the complex also acts as a link between

microtubules and the RNA localization machinery. If *egl* and *BicD* act directly to localize RNAs, these proteins may either bind RNA or associate with an RNA-binding protein, such as Orb, which has a distribution strikingly similar to that of Egl and BicD proteins and a localization that depends on Egl and BicD function (Lantz et al., 1994).

Future directions

In writing this thesis, I have had the chance to consider both the experiments that didn't work for me and the experiments that I wish I had done. This section contains a miscellaneous collection of the experiments I think should be done, in the hope that someone will try at least one of my favorites.

Loose ends

localization of *egl* RNA

Many RNAs localize with a pattern similar to the *egl* mRNA, first in the oocyte, then at the posterior of the oocyte, then in an anterior ring at stages 8-9. However, at stage 9 different RNAs localize with different patterns, each reflecting their later role in oocyte polarity. Although some progress has been made in determining what domains of a particular RNA are required for what phase of localization (Kim-Ha et al., 1993), the mechanisms by which RNAs that share an early localization pattern become sorted to different patterns at later stages is unclear. By determining what domains of the *egl* mRNA are important for localization and by comparison of these domains, both in primary and in secondary structure, to those of other localized mRNAs, one may gain a hint at whether the mechanisms of RNA localization are independent or shared.

What is the function of the *egl* worm homolog?

One way to address the significance of the similarity between *egl* and C10G6.1 from *Caenorhabditis elegans* is to determine biochemically whether the conserved residues have any functional importance; another way is to examine the function of C10G6.1 in *C. elegans*. Many unassigned mutations with various phenotypes, including sterile mutations, map to the region of C10G6.1. Assigning this predicted gene product to a mutant phenotype could provide some information about its function. If this mutation does not map to a complementation group, then its mutant phenotype could be determined by the injection of antisense constructs to reduce gene function.

egl and the cell cycle

As described above, results with both *egl* mutants and with a hypomorphic allele of *cyclinE* indicate that the control of the cell cycle is integral to the process of oocyte determination. Unfortunately, the meiotic phenotype, in which all sixteen cells form synaptonemal complex, has only been described for *egl*; knowing whether other genes that share the sixteen nurse cell phenotype also share the meiotic phenotype would help in our understanding of oocyte determination. If the *egl* mutants enter meiosis because of an unlocalized oocyte determining factor, then other genes may share this phenotype. Only in mutants in the oocyte determinant

itself, or genes downstream of its function will all sixteen germ cells enter the endoreplication cycle directly, rather than transiently entering meiosis.

Many experiments can be visualized to explore the relationship between *egl* and *cycE* and to test the hypothesis that *egl* localizes a factor that stabilizes CyclinE in the oocyte. First, the experiments described above, to find molecules that associate with Egl, may identify a factor that regulates *cycE* in the germ line. Second, *cyclinE* and *egl* mutants may show a genetic interaction; specifically, $cycE^{01672}$ may suppress *egl* mutants, or *egl* may be epistatic. Also, overexpression of either CyclinE or Egl may affect the phenotype of mutants in the other gene. Third, *egl* mutants may affect the expression of *cyclinE* or vice versa. Lastly, if regulation of CyclinE levels requires protein instability, then deletion of the cyclinE PEST sequence may cause a gain-of-function phenotype.

BicD

Null phenotype

Much of the characterization of *BicD* has used two alleles, PA66 and R26, which are not nulls. To determine the exact requirement for *BicD* in oocyte determination, many of these characterizations will have to be redone using the *BicD* null alleles isolated by Ran et al., 1994. These alleles were describes as producing a semi-lethal phenotype; in my hands, they were completely lethal. To make an analysis of these alleles possible, they should be examined as germ line clones, produced by the FRT-mediated recombination (Harrison and Perrimon, 1993).

Three analyses of these null alleles will add to our understanding of oocyte determination. First, if these alleles do not form a microtubule organizing center in the oocyte, then *BicD* is essential for that process and *egl* acts in a subsequent step, the maintenance of that MTOC. Second, if all sixteen *BicD* null germ cells form synaptonemal complex, then this phenotype may be a consequence of an unlocalized oocyte determinant. If none of the cells form synaptonemal complex, then *BicD* may be the oocyte determinant, or this phenotype may be specific to *egl*. Third, if Egl protein is unlocalized in these *BicD* null ovaries, then BicD function is truly required for Egl localization. Because the two mutant forms of BicD examined in this study still form a complex with Egl protein, they may change its localization. Thus, only examination of the null alleles of *BicD* will reveal its exact realtionship with *egl*.

BicD dominant alleles

The phenotype of the dominant alleles of *BicD* can be explained by the mislocalization of *oskar* RNA to the anterior of the oocyte. Several questions about this process remain unanswered. First, BicD protein is also mislocalized in these alleles, where it is concentrated at the anterior

instead of being uniformly distributed as in wild type. If Egl and BicD always colocalize, then it would be expected that Egl protein distribution changes in the embryos from *BicD*^{D} females. Second, *egl* mutations suppress the *BicD*^{D} phenotype; they may suppress by reducing the amount of mutant BicD protein that can accumulate at the anterior. Third, *BicD*^{D} causes the relocalization of *osk* RNA. The localization signal in the 3' UTR of the *osk* RNA has been divided into domains, each required for a different phase of *osk* localization (Kim-Ha et al., 1993). If *BicD* acts only on early *osk* localization to the oocyte, then the dominant phenotype may be due to a failure to release the early localization signal of *osk*. This can be tested by examining the effect of *BicD*^{D} on the localization of chimeric mRNAs carrying only one of the *osk* localization signals linked to a marker sequence. Although no commonalities have been found between the *osk* localization signals and the 3' UTR sequences of other RNAs, *BicD*^{D} may also cause the mislocalization of other RNAs, if its affect on *osk* localization is not specific.

Role of *egl* in oocyte polarity

egl and microtubules

The oocyte MTOC forms in *egl* mutants, but is not maintained. The resultant failure of oocyte determination can be phenocopied by treatment of wild type flies with microtubule depolymerizing drugs such as colchicine. It would be interesting to determine whether the failure of oocyte determination in *egl* mutants can be suppressed by microtubule-stabilizing drugs such as taxol.

The link connecting *egl*, *BicD* and microtubules will have to be explored biochemically, as discussed below. However, examining the effect of inhibitor treatments on the formation of the complex containing Egl and BicD proteins may provide some information about Egl and BicD localization and interaction. Egl and BicD coimmunoprecipitate from *BicD*^{PA66} and from *BicD*^{R26} mutant extracts; also, the oocyte MTOC does not form in either of these mutants. To be consistent with this result, the coimmunoprecipitation of Egl and BicD should be not be affected by treatment with microtubule inhibitors. However, Egl localization requires microtubules; if BicD sets the location for the MTOC, which then causes the localization of Egl, then the coimmunoprecipitation of Egl and BicD should be abolished by treatment with microtubule inhibitors.

Mislocalization of Egl

The clearest way to determine whether *egl* sets the location of the minus ends of microtubules, or whether *egl* sets the location of *gurken* RNA, is to change the subcellular localization of Egl. Since Egl localizes to the anterior of the cyst at stage 9, it should be relocalized to the posterior of the cyst. Two protein fusions can localize β-galactosidase to the posterior at this stage, fusions with kinesin (Clark et al., 1994) or with the first 400 amino acids of the *fat facets*

protein (Fischer-Vize et al., 1992). Constructs expressing the *egl* coding sequence fused to either of these localized molecules could be introduced into flies by P-element mediated transformation. The mislocalization of *egl* may cause mislocalization of *gurken* RNA, possibly resulting in an alteration of embryonic polarity. If the mislocalization of *egl* causes a phenotype, it will be interesting to determine whether that phenotype requires the function of other genes such as *orb* or *BicD*. If *BicD* acts upstream of *egl*, then the *egl* mislocalization phenotype will be independent of *BicD*, but if *egl* and *BicD* act together, then this phenotype will require *BicD* function. Although examining the requirement of *BicD* in *egl* mislocalization will require early expression of *BicD* to allow oocyte determination, this experiment could show whether *egl* and *BicD* act together, at least in late oogenesis.

Attempting to mislocalize *egl* has at least three potential pitfalls. First, the mislocalization of *egl* may have no phenotype. Second, localization of these fusion partners depends on the microtubule cytoskeleton; if egl relocalization changes the polarity of the cytoskeleton, then its mislocalization may be perturbed. Third, a fusion with the *egl* coding sequence may inactivate the Egl protein. For example, I constructed an *egl* fusion to the Green Fluoresecent Protein; for unknown reasons, this transgene neither glowed nor complemented *egl* mutants. Since the domain conserved between *egl* and C10G6.1 from *Caenorhabditis elegans* extends to the N terminus of Egl, this region may be important for *egl* function. The mislocalization of Egl experiment carries a significant risk of failure, but a high reward for success; it is my hope that someone will try it.

Biochemistry of Egl

The answers to the questions raised by this study and the tests of the hypotheses outlined above will only come through a complete characterization of the biochemistry of the *egl* protein. Since the sequence of the Egl protein provides few clues as to possible *egl* function, only educated guesses verified by biochemical studies can hope to explain the role of *egl* in oocyte determination. The preliminary biochemical experiments in this study have shown that Egl and BicD proteins form part of a complex. The logical next step from this study is to further characterize this interaction by determining whether Egl and BicD interact directly and what other molecules associate with them.

To understand the interaction between Egl and BicD, one must know whether the proteins interact directly or through intermediate proteins. If Egl and BicD interact directly, then determination of the domains that are required for this interaction will explain the significance of two observations. First, the egl^{4e} mutation, a Cys to Tyr change at amino acid 23, causes Egl to no longer coimmunoprecipitate with BicD. This region at the N terminus of Egl is also conserved in a predicted gene product in *C. elegans*, another indication that this region is important for Egl function. Second, the BicD protein contains long stretches of predicted coiled-coil sequence. Egl

contains a short region that may form a coiled coil. Either the putative coiled-coil or the N-terminal conserved domain that is mutated in egl^{4e} is a reasonable candidate for a region that interacts directly with the BicD protein.

Analysis of the *egl* sequence indicates that the protein may be post-translationally modified. The *egl* protein migrates substantially slower than other proteins of similar size on an acrylamide gel and contains several possible phosphorylation, glycosylation and myristylation sites. To determine the significance of these sequences, one must determine whether the Egl protein is modified and whether this modification has any significance. For example, one could assay for modification by determining whether treatment with phosphatases or endoglycosidases alters the mobility of Egl in a Western blot assay. In this manner, BicD has been shown to be phosphorylated (Suter and Steward, 1991). To determine whether any modifications have functional significance, one could assess the state of modification in mutants that affect oocyte determination, or determine whether the modifications are required for coimmunoprecipitation of Egl and BicD, or determine whether *egl* mutants that cannot be modified can provide *egl* function. The action of Egl and BicD may be controlled by modulation of phosphoryation. Interestingly, Protein Kinase A is required in the germ line to transduce the signal from the posterior follicle cells that induces the reorientation of the cytoskeleton (Lane and Kalderon, 1994).

Another way to determine the function of *egl* in oogenesis is to determine what molecules it associates with during oogenesis. Simply conceived, this means that if Egl is acting to stabilize the MTOC, then it should associate with microtubules, perhaps even with γ-tubulin. If Egl acts to localize RNAs, then it may bind directly to RNA, or to a known RNA-binding protein. There are two ways to approach the investigation of the past associations of Egl in oogenesis. First, one can attempt to use their association with Egl to purify and identify unknown components of the Egl-BicD complex. Second, one can make educated guesses about proteins and RNAs likely to be present in the complex and look for those. Although the first method successfully identified an interaction between Egl and BicD, the lack of bias in the second method should eventually yield more information.

In this study, I have laid the groundwork for an extensive biochemical characterization of Egl. Simply looking at what other proteins coimmunoprecipitate with Egl and BicD, by Westerns with antibodies to known proteins such as Orb, or by RT-PCR to look for associated RNAs, or by characterization and identification of Egl-specific bands on a silver-stained gel, should provide information about other members of the complex. In addition, other analyses that do not depend on immunoprecipitation are possible, using bacterially expressed Egl protein or examination of the Egl-BicD complex on a sizing column, or the yeast two-hybrid system.

Appendix

egalitarian cDNA sequence

This appendix provides a guide to the sequence of the *egl* cDNA A9, the exact position of the introns, the exact alterations in the mutant alleles, and the sequence and position of the oligos.

Key

Introns are designated by the two flanking exon nucleotides, in bold. Where mutant alleles alter intron sequence, the intron sequence is designated in small type.

Oligonucleotide primers are designated by two- or three- letter codes beginning with the letter "A", as in "A9 cDNA", as well as an arrowhead denoting their direction. In the sequence, all primers are underlined; reverse primers are also in italics. All primers, except AAI2, are 18 nucletides long. This should clarify any ambiguities in overlapping sequences.

Mutations are indicated beneath the affected sequences.

Translational start sites are indicated in bold.

```
GGTTCGTTGGTGTTTTTCGCTGCGCTGGTGTTCGTGTGTTCGCGTTGGCGCTGTCATCAT    60

CATATCGTATCGCATCGCTCCGCTCCGAAAACCGTGGCAATTGTCTGTTTTTTTACCTGC   120

CAGCGAGTGCGTTTCAGTTGCCCGCCAACCGGCAGGAATCGCAAATTCACGTGCGCCACT   180
 < AW

CTCCAATTGGACGAGTGCCTTTAAAACAGTGCAACAAGTGGTTTTTACAATTCTTACCAG   240
                                                        AE >

CATTCAGTTCAAAGTGCCTAACAAACACACGCACACACATACGTAACCTACCTGACCAGT   300
         < AX

GCGCATATGTGTGTGCATGAGTGTGAGTGATACAACAACAATACAATAGCAGCAACACAG   360

CTACGAAAATAACTAACTGCTTTCGTCGCAGTTTTTCTGCAAGTGTATGGGTTAATTGCG   420

CCGAAAAATGTTAATTAATTGAAGCCCAATTGGAAATCGCCAGTGGCAAGAAAAGCTGAA   480

ACTAAGATCTGAACGCAGATCTGGTGAGAAATCCCAAACGCGAGGATCTGTATCGCGTGT   540
                                          < AB
```

```
GTGTCAATCGGTTTAAATATAGATCAGATCACAAGTGCACTTAAACAAACAGAAGGGAAA  600

GAAACAAACAAGTGATTAACCGACAGATAAGCAGCCAACGTGACGTACTCGGGCGGATTA  660
              (Intron #1 GA)  AY >

ATAATTGAGCAGATAGCGGATAGCAGAGAGCAGCTAGCAGATATCAGATAGCGAATAGCG  720

GACACAGCAGGCCCAGCAATGGAGTCCATGGAGTACGAGATGGCACGCACGCAACATGAC  780
                          AAK >                              M  T    2

GTTGCTCTTCTTCCTGGAGCGACTGCTGGACAAGGGCGAGCCCCGCACCGTCCACGACCT  840
  L  L  F  F  L  E  R  L  L  D  K  G  E  P  R  T  V  H  D  L   22
                                                 AA >

GTCCTGCCAATTCGGCAACAAGGAGTTCACCAAGGAGATGCGCCAAATCGCCGGTGGCAG  900
  S  C  Q  F  G  N  K  E  F  T  K  E  M  R  Q  I  A  G  G  S   42
                                  < AAM

TCAGTCGGGTCTGAAGAAGTTCCTCGCCCAGTACCCGGCCATATTTCTGGTTGACGGCGA  960
  Q  S  G  L  K  K  F  L  A  Q  Y  P  A  I  F  L  V  D  G  D   62
      (GG Intron #2; 6kb)

CTACGTCCAGGTGAACGCCTATCAGCACCACAACGCGGACGATGGCGGCTGCGGGGGCAA 1020
  Y  V  Q  V  N  A  Y  Q  H  H  N  A  D  D  G  G  C  G  G  K   82
      < AAN                        AJ >

GCGGGACTACATTCAAGAGGCTAAAGACTACTTCAAGAACAAGATGCTGCAGTACGGAGC 1080
  R  D  Y  I  Q  E  A  K  D  Y  F  K  N  K  M  L  Q  Y  G  A  102

GGCTGCCGAGGTGCCCGTCCGCAGTCTCCTGGGCCACCGGTCGCAGGCGTCGCCCCAAGT 1140
  A  A  E  V  P  V  R  S  L  L  G  H  R  S  Q  A  S  P  Q  V  122
                       < AAZ

GCGTCACATATCGGGCCAGCACATCAAGGAGTTCACCGACTTCCTGATGAAGCACACGGA 1200
  R  H  I  S  G  Q  H  I  K  E  F  T  D  F  L  M  K  H  T  D  142
 (Intron #3  GG)      < AZ                              AAA >
 (201 nt; PB23 ...ATCGGgt -> ATCGGga  translation of intron:
  R  H  I  S  DEFETKTDGGVHNHGCHSKLMT.)

CACCTTCAAGGTGACGGACGACTATGTGATGCTGGTGGGCTGCGAGAACATGACGGATCT 1260
  T  F  K  V  T  D  D  Y  V  M  L  V  G  C  E  N  M  T  D  L  162

GCCGGCGCGGGATCGCCTCCACCTGCCGCAGTCGAACATCGATACTCGAGGCACGCAGCA 1320
  P  A  R  D  R  L  H  L  P  Q  S  N  I  D  T  R  G  T  Q  Q  182

GATGCTGGACTTCTTTGCGCAGTGCATCGAGGTCAAGGGACCGTTGCTGGTGGACCAGTT 1380
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```
  M  L  D  F  F  A  Q  C  I  E  V  K  G  P  L  L  V  D  Q  L  202

GTTCCACCTGCTGACCACCAATTTCCCGCAGGACCAGTGGCTGCGCATGTTCAAGACGCC 1440
  F  H  L  L  T  T  N  F  P  Q  D  Q  W  L  R  M  F  K  T  P  222
   < AAS

GGGCGACCTGAGCTCATTCCTCAAGCTCTTCGCCGACTGCTTCCACATACAGGCCAATCT 1500
  G  D  L  S  S  F  L  K  L  F  A  D  C  F  H  I  Q  A  N  L  242

AGTCACCCTGCTGCAGAAGCCCAAGCTCAGCGACACGCACATTCAGCAGGCACAGGCTCA 1560
  V  T  L  L  Q  K  P  K  L  S  D  T  H  I  Q  Q  A  Q  A  Q  262

AACCCGGGAGCAATTTAATGCGCTGAACAATAACAACAGTGCCAGCATTCGAAAGCAGGA 1620
  T  R  E  Q  F  N  A  L  N  N  N  N  S  A  S  I  R  K  Q  E  282
            < AAB                                    TGA in PR29
                                                     STOP

GCCAACACCTGGAGGTGGAGGCGTTGGAGGTGTCAGCTCGGTGCAGCAGAGACTGCAGTC 1680
  P  T  P  G  G  G  G  V  G  G  V  S  S  V  Q  Q  R  L  Q  S  302

GCCGGCACTGCGGACTAATGGTCACACGAACAACAACAATGGGAGCAACGGCAGCAACAA 1740
  P  A  L  R  T  N  G  H  T  N  N  N  N  G  S  N  G  S  N  N  322
            < AI

TAACAACAACAACAACAACAGCATTGCCTGCCCGAACTTCAAGCTAAATGCGCCCGTTTC 1800
  N  N  N  N  N  N  S  I  A  C  P  N  F  K  L  N  A  P  V  S  342
(one N deleted in 4e, 2b, 3e)   AAC >

GAATGTGATGGGTGGACAGAGCCAAGGGTTCGGGCAGCCCAAGTCGGAGCCCAGCTCTGG 1860
  N  V  M  G  G  Q  S  Q  G  F  G  Q  P  K  S  E  P  S  S  G  362

CTTTGACAGCTACGTGCCCATGTCGGAGCTGAAGCTGGAGAATCTGTGCGAGAACAACTA 1920
  F  D  S  Y  V  P  M  S  E  L  K  L  E  N  L  C  E  N  N  Y  382
                       AAD >              < AAE

TCCCAGTGCGAACACCTGCTACGGGCCCATTAACAACTCCAGCCAGCAGGCGCAGCAAGT 1980
  P  S  A  N  T  C  Y  G  P  I  N  N  S  S  Q  Q  A  Q  Q  V  402
                                                (ACG in WU50)
                                                   T

GCAAACGCAGCAGCAGCAGCAGCCGCAGCATGCGACACAGAATCCGGCGGAGCAGCGCTT 2040
  Q  T  Q  Q  Q  Q  Q  P  Q  H  A  T  Q  N  P  A  E  Q  R  L  422

GAATAGTGTTAACCAAACGCTGAAGCAACGCATCAACACCCTGGTTATACGCACTCTGGC 2100
  N  S  V  N  Q  T  L  K  Q  R  I  N  T  L  V  I  R  T  L  A  442
```

*CGAGAACCTGGAGAAG*GACAAGCAATCGCTGGCCAACCAGCAAGGCGGGCCCATTTCCCC 2160
E N L E K D K Q S L A N Q Q G G P I S P 462
< AAF

GCACGCCAGTCCCGTGCATTCCATAGCCAATTCGAGTTCGAACCAAAATGCCGGTAGTGC 2220
H A S P V H S I A N S S S N Q N A G S A 482

TGCAAATAACGCCAATAGCAACTCGAATGCGAATCCGAACAATGCCAACCA*CTCACCTAG* 2280
A N N A N S N S N A N P N N A N H S P S 502

*TCATAGTTA*CTTCGTCGGCGACACCTGGAAGATAAAGGTGCTGCAGAACACCACGGTGAT 2340
H S Y F V G D T W K I K V L Q N T T V I 522
< AAG

AGCGAATGTCAAGCAGTCGGTGTTTGTGACCGACATCA*TACTGAAGTATGCGGCCA*AGAA 2400
A N V K Q S V F V T D I I L K Y A A K N 542
AAO >

CGAGAGCATAGTGGTCTCCCTGGACTGCGAGGGCATTAACCTGGGCCTGAAGGGCGAGAT 2460
E S I V V S L D C E G I N L G L K G E I 562

CACCCTGATTGAGATTGGCACAACGCGGGGCGAAGCCTTCTTGTTCGACGTGCAGTCCTG 2520
T L I E I G T T R G E A F L F D V Q S C 582

CCCGGCGATGGTAACCGATG*GCGGATT**GAAGACCGTGTTGGAGCA*CGACCAGGTGATCAA 2580
P A M V T D G G L K T V L E H D Q V I K 602
AAW > < AAP

GGTTATACACGACTGCCGCAACGACGCTGCCAACCTT*TATCTGCAATTCGGCATCC*TGCT 2640
V I H D C R N D A A N L Y L Q F G I L L 622
< AAH

GCGGAATGTGTTCGACACCCAGGCCGCACATGCCATCTTGCAGTATCAGGAGAGCGGCAA 2700
R N V F D T Q A A H A I L Q Y Q E S G K 642

GCAGGTCTACAAGGCCAAGTACATATCGCTAAACTCGCTGTGCGAGCAGTACAACGCCCC 2760
Q V Y K A K Y I S L N S L C E Q Y N A P 662

CTGCAACCCGATCAAG*GATCAGTTGAAGCAGATCTA*C*CGCAGGGACCAGAAGTTCTG*GGC 2820
C N P I K D Q L K Q I Y R R D Q K F W A 682
< AAI2 AAU >
(AAI seq: 5' TAG ATC TGC TTC AAC TGA 3'; AAI2 adds TC at 3' end.)

GAAGAGACCCCTCACACGCGAAATGATGCTGTACGCAGCGGGCGATGTCCTGGTGCTCAT 2880
K R P L T R E M M L Y A A G D V L V L I 702

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CCACGATCAGCTATTCGGCAACCTGGCGCGGCAGATCAAGCCAGAGAATCGGGCTCTCTT 2940
  H  D  Q  L  F  G  N  L  A  R  Q  I  K  P  E  N  R  A  L  F  722
                          < AH    AG >

CTCAGAGCTGTGCACCGAGCAAATACTCATGCAGATCAAACCCAACGAGGTCAAGATACG 3000
  S  E  L  C  T  E  Q  I  L  M  Q  I  K  P  N  E  V  K  I  R  742

CAAGAAGCAGCGCAAGGTCAGCACCGAGGTGTCCGATCTCAAACAGAAGCTGGCCCAGAC 3060
  K  K  Q  R  K  V  S  T  E  V  S  D  L  K  Q  K  L  A  Q  T  762

CAGCAAGAGCATTGTGCTCTCCAATCGCGAGATACGCTTGCTGCGGTATATGGATCTGAC 3120
  S  K  S  I  V  L  S  N  R  E  I  R  L  L  R  Y  M  D  L  T  782
        AAJ >  AAV >                        (GG Intron #4; 61 nt)
                          (WU50 TGCGgta...agGTA -> TGCGgtt...aaGTA)
                    (frameshift->     L  L  R  I  W  I  .)

AGAGGACGAGAAAGAGCGCCTCAAGGGCTACTACAAGGTGGCGAAGAAGCTGGAGAAGAT 3180
  E  D  E  K  E  R  L  K  G  Y  Y  K  V  A  K  K  L  E  K  M  802

GGAGTCCGCCGGCAATCCCAGCAAGTGGGTTCTATCGATTTCAAATCAAATAGCTATCCA 3240
  E  S  A  G  N  P  S  K  W  V  L  S  I  S  N  Q  I  A  I  Q  822

ACTGACAACTCTATTTGTACTTCCAGAGATCAAAGTGACTCCGAGGATGAACAAGAGCCG 3300
  L  T  T  L  F  V  L  P  E  I  K  V  T  P  R  M  N  K  S  R  842
   < AC                                                 AV >

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ACATTTTCGCCACGCTTCAGTTCAGAGCCACCCAGCCTGACTGAATCCATGCAAATGCTG 3420
  H  F  R  H  A  S  V  Q  S  H  P  A                          874

GAAGAGATTCTCCAGAACAAGTCAATGGATCGCATAGCCCGTATTGACAAGCTGGAGGCC 3480
                                              < AAT

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     AS >                          (AA Intron #5; 59 nt)

GAGCAATTAGGTTCGAGCATTGCGACCACCGAGAATCTACAGATTATTCGCGAGAAATCC 3600
           < AR

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         AG >         < AQ

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GAACGACTGACAAGCGTGTGGTGAAGCTCGTGGACGCCGAATCGCAGACACTGAGCACCG 3780

GCGACGTGGTCATCACCAAGATCTT*CTTCCAGGACGAGCACGA*GCGAGCCAAGGAGGCGG 3840
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CCCTGCTGAGCAACTCGCCCGCGAAGCGTGTGTCTCCCACATAAACATTTCGACCTTC*GT* 3900
AAR >

*ACTATGATGATTCCTA*ACAAAAAAAAAGCGCAGACACACTACGTATTATTATTTTGTTTT 3960
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ACAGTTGAGGGGCTTGGCCTTGGGTCCCTCGACGCTTAACGACTTTGCAATAACTTTCAT 4020

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AM >

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ATGGTACGATTATAAATTATATATCTAATTG**AATAAA**CATGTATATACCAACATT 4555

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