Oocyte determination and the origin of polarity in *Drosophila*: the role of the spindle genes

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SUMMARY

The two main body axes in *Drosophila* become polarised as a result of a series of symmetry-breaking steps during oogenesis. Two of the sixteen germline cells in each egg chamber develop as pro-oocytes, and the first asymmetry arises when one of these cells is selected to become the oocyte. Anterior-posterior polarity originates when the oocyte then comes to lie posterior to the nurse cells and signals through the Gurken/Egfr pathway to induce the adjacent follicle cells to adopt a posterior fate. This directs the movement of the germinal vesicle and associated *gurken* mRNA from the posterior to an anterior corner of the oocyte, where Gurken protein signals for a second time to induce the dorsal follicle cells, thereby polarising the dorsal-ventral axis. Here we describe a group of five genes, the spindle loci, which are required for each of these polarising events. *spindle* mutants inhibit the induction of both the posterior and dorsal follicle cells by disrupting the localisation and translation of *gurken* mRNA. Moreover, the oocyte often fails to reach the posterior of mutant egg chambers and differentiates abnormally. Finally, double mutants cause both pro-oocytes to develop as oocytes, by delaying the choice between these two cells. Thus, these mutants reveal a novel link between oocyte selection, oocyte positioning and axis formation in *Drosophila*, leading us to propose that the *spindle* genes act in a process that is common to several of these events.

Key words: oogenesis, *spindle* genes, oocyte determination, axis formation, *Drosophila*, polarity, germline, Gurken

INTRODUCTION

The two main body axes of *Drosophila* are determined by the targeting of three specific mRNAs to distinct positions within the oocyte. The polarity of the anterior-posterior (AP) axis is defined by the localisation of *bicoid* mRNA to the anterior of the oocyte, where it is translated after fertilisation to specify the patterning of the head and thorax of the embryo (Driever, 1993), and the localisation of *oskar* mRNA to the posterior, where it specifies the site of pole plasm formation and hence where the abdomen and germline will form (Ephrussi and Lehmann, 1992; St Johnston, 1993). At the same time, *gurken* mRNA accumulates above the germinal vesicle (the oocyte nucleus) in the dorsal-anterior corner of the oocyte, where it specifies the site of pole plasm formation and hence where the abdomen and germline will form (Ephrussi and Lehmann, 1992; St Johnston, 1993). At the same time, *gurken* mRNA accumulates above the germinal vesicle (the oocyte nucleus) in the dorsal-anterior corner of the oocyte, where it is translated to produce a TGFα-like protein which is thought to bind to the *Drosophila* EGF receptor homologue, Egfr, in the adjacent somatic follicle cells (Schüpbach, 1987; Price et al., 1989; Schejter and Shilo, 1989; Neuman-Silberberg and Schüpbach, 1993; Neuman-Silberberg and Schüpbach, 1996). This activates a signal transduction cascade that induces these cells to adopt a dorsal rather than a ventral fate. Since the ventral follicle cells eventually secrete the asymmetric signal that establishes the morphogen gradient of dorsal protein in the blastoderm nuclei, this polarisation of the dorsal-ventral (DV) axis in the follicle cell layer defines the corresponding axis in the embryo (Chasan and Anderson, 1993). The correct positioning of these three transcripts, and thus the formation of both embryonic axes, depends on a series of steps during early oogenesis which generate the asymmetries within the oocyte that direct the localisation of each mRNA.

At the beginning of oogenesis, a germline cystoblast divides four times with incomplete cytokinesis to produce a cyst of sixteen cells that remain connected to each other by cytoplasmic bridges called ring canals (Spradling, 1993). These divisions are oriented by the association of one pole of each mitotic spindle with a germline-specific structure called the fusome, and the resulting cyst therefore has a stereotypic pattern of intercellular connections (Lin et al., 1994; Lin and Spradling, 1995). Two of the sixteen cells in the cyst contain four ring canals and enter meiotic prophase to become pro-oocytes; their chromosomes condense and the bivalents align to form synaptonemal complexes (Koch et al., 1967; Rasmussen, 1974; Carpenter, 1975; Schmekel et al., 1993). Although the two pro-oocytes initially appear equivalent, the first asymmetry in development arises when one of these cells is selected to differentiate as the oocyte. This cell remains in meiosis and condenses its chromatin into a hollow sphere called the karyosome, whereas the second pro-oocyte exits meiosis and starts to endoreplicate its DNA to become a polyploid nurse cell, along with the fourteen other cells of the cyst (King, 1970). The mechanism of oocyte determination is unknown, but the first sign that one pro-oocyte is different from the other is the accumulation in one of the two pro-oocytes of a number of proteins, such as Bicaudal-D (Bic-D), Egalitarian...
(Egl), Cup and Orb (Wharton and Struhl, 1989; Suter and Steward, 1991; Lantz et al., 1994; Keyes and Spradling, 1997; Mach and Lehmann, 1997). Bic-D and Egl seem to play a central role in this process, since they are required for the determination of the oocyte, and for the formation within this cell of an active microtubule-organising centre (MTOC) (Mohler and Wieschaus, 1986; Suter et al., 1989; Wharton and Struhl, 1989; Schüpbach and Wieschaus, 1991; Theurkauf et al., 1993). The MTOC assembles a microtubule network that extends into the other fifteen cells of the cyst and this polarised cytoskeleton is then thought to direct the accumulation within the oocyte of specific transcripts such as oskar mRNA (Ephrussi et al., 1991; Neuman-Silberberg and Schüpbach, 1993; Theurkauf et al., 1993; Pokrywka and Stephenson, 1995).

At about the same time that the oocyte is beginning to differentiate, a monolayer of somatic follicle cells surrounds the cyst of germline cells and the resulting egg chamber then buds off from the germarium. The first AP asymmetry in development becomes apparent at this stage, as the oocyte always lies posterior to the nurse cells, a position that is maintained during the rest of oogenesis. Once the oocyte has reached the posterior, it generates AP polarity in the somatic follicle cell layer by inducing the adjacent terminal follicle cells to adopt a posterior, rather than an anterior fate (González-Reyes and St Johnston, 1994). At this stage of oogenesis, gurken mRNA is localised next to the germinal vesicle at the posterior of the oocyte and Gurken protein becomes concentrated in oocyte plasma membrane on this side of the cell (Neuman-Silberberg and Schüpbach, 1996). Since the determination of the posterior follicle cells requires gurken activity in the germline and the putative Gurken receptor Egfr in the soma, Gurken is thought to signal from the oocyte to activate the Egfr in the follicle cells to induce them to adopt a posterior fate (González-Reyes et al., 1995; Roth et al., 1995).

The AP polarity of the follicle cell layer is transmitted back to the germline later in oogenesis when the posterior follicle cells send an unknown signal that induces the disassembly of the original MTOC at the posterior of the oocyte (Ruohola et al., 1993; Theurkauf et al., 1992). At the same time, a new diffuse MTOC appears at the anterior margin of the oocyte, which organises an anterior-posterior gradient of microtubules, in which the minus ends are believed to lie at the anterior of the oocyte and the plus ends at the posterior (Theurkauf et al., 1992; Clark et al., 1994, 1997). This polarised microtubule array defines the AP axis of the embryo by directing the microtubule-dependent localisation of bicoid and oskar mRNAs to the anterior and posterior poles, respectively, and also directs the microtubule-dependent movement of the germinal vesicle from the posterior of the oocyte to a point at the anterior margin (Koch and Spitzer, 1983; Pokrywka and Stephenson, 1991, 1995; Clark et al., 1994). Gurken mRNA re-localises to an anterior corner of the oocyte with the germinal vesicle, and Gurken protein then signals for a second time to polarise the DV axis.

The five spindle (spn) loci, spn-A, spn-B, spn-C, spn-D and spn-E were originally identified in a screen for maternal-effect mutants on the third chromosome because homozygous mutant females lay ventralised eggs (Tearle and Nüsslein-Volhard, 1987). Only two of these loci have previously been analysed in any detail. spn-C mutations have been shown to disrupt the positioning of the oocyte posterior to the nurse cells, resulting in the development of bipolar egg chambers in which bicoid mRNA localises to both poles of the oocyte and oskar mRNA to the centre (González-Reyes and St Johnston, 1994). Mutations in spn-E (also known as fs(3) homeless) give a very similar oocyte misplacement phenotype, but also affect the oocyte cytoskeleton and mRNA localisation even when the oocyte is at the posterior (Gillespie and Berg, 1995). Here we present a detailed analysis of the phenotypes produced by mutants in all five spindle loci, which reveals that this group of genes is required for each of the symmetry-breaking steps, that generate polarity during Drosophila axis formation.

### MATERIALS AND METHODS

#### List of mutant alleles and deficiencies used in this study

<table>
<thead>
<tr>
<th>Allele</th>
<th>Meiotic position</th>
<th>Uncovered by</th>
<th>Type of allele</th>
</tr>
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<tbody>
<tr>
<td>spn-C</td>
<td>094</td>
<td>3-23</td>
<td>Df(3L)spnC127</td>
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<td></td>
<td>422</td>
<td></td>
<td>65F3; 66B3-9</td>
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<tr>
<td></td>
<td>660</td>
<td></td>
<td></td>
</tr>
<tr>
<td>spn-B</td>
<td>056</td>
<td>3-54</td>
<td>Df(3R)spnB121</td>
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<tr>
<td></td>
<td>153</td>
<td></td>
<td>87F2-14; 88C1-3</td>
</tr>
<tr>
<td>spn-E*</td>
<td>616</td>
<td>3-62</td>
<td>Df(3R)spnE108</td>
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<tr>
<td></td>
<td>653</td>
<td></td>
<td>88F9-89A1; 89B9-10</td>
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<td></td>
<td></td>
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<td>hlsA125</td>
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<td>hlsA157</td>
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<tr>
<td>spn-D</td>
<td>150</td>
<td>3-91</td>
<td>Df(3R)spnD19</td>
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<td></td>
<td>225†</td>
<td></td>
<td>97A; 98A1-2</td>
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<tr>
<td></td>
<td>349</td>
<td></td>
<td></td>
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<tr>
<td>spn-A</td>
<td>003</td>
<td>3-96</td>
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<td>050</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>057</td>
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</table>

*The spn-E locus has also been called fs(3) homeless (hls) (Gillespie and Berg, 1995). We prefer to retain the original nomenclature for the locus and to refer to the homeless alleles mentioned in this study as hlsA125, hlsA157 and hls3987 as spn-EbicsA125, spn-EbicsA157 and spn-Ebics3987.*

†During the course of this work fs(3) 225-19 (Tearle and Nüsslein-Volhard, 1987) was found to be allelic to spn-D. We have therefore renamed it spn-D225.

#### Immunohistochemistry and in situ hybridisation

Antibody stainings and in situ hybridisations were performed as described (St Johnston et al., 1991). The antibodies were used at the following dilutions: 1/10 for α-BicD, 1/30 for α-Orb and 1/2500 for α-Gurken. Rhodamine-phalloidin, X-gal and Hoechst stainings were performed as described (González-Reyes and St Johnston, 1994). Images from ovaries stained with fluorescent antibodies and rhodamine-phalloidin were collected using an MRC1024 confocal microscope (Biorad).

Germline clones were generated using the DFS technique (Chou et al., 1993).

#### Egg shell phenotype

To visualise the chorion under the microscope, eggs were collected...
from apple juice agar plates, washed with water and 0.1% Triton X-100 and mounted in Hoyer’s medium. Egg shells were photographed using pseudo-Nomarski optics.

RESULTS

Lack of spindle function affects the polarisation of the DV and AP axes

Wild-type eggs possess a clear polarisation of the egg shell along the DV axis. The dorsal side is shorter and less curved than the ventral side and a pair of specialised chorion appendages develop from the dorsal anterior end (Fig. 1A). In contrast, a proportion of the eggs laid by females mutant for any of the *spn* genes lack dorsal structures and develop into ventralised embryos on the rare occasions when they are fertilised. We have divided the phenotypes observed in *spn* egg shells into two classes: weakly ventralised eggs display fused dorsal appendages (Fig. 1B), whereas strongly ventralised eggs are longer than wild type and are completely symmetric along the DV axis (Fig. 1C). Although the penetrance of the phenotype is somewhat variable, the majority of the eggs produced by females mutant for any *spn* gene are ventralised (Table 1).

Since all other mutants that produce a similar ventralisation of the chorion and the embryo disrupt the Gurken signalling pathway that induces dorsal follicle cell fate, we examined the expression of *gurken* mRNA in *spn* mutant egg chambers. In wild-type egg chambers, *gurken* mRNA localises to the anterior-dorsal corner of wild-type stage 9 and (C) stage 10 oocytes. (B) In the majority of *spn* stage 9 oocytes, *gurken* mRNA is present as an anterior ring. (D) Stage 10 egg chambers of the same genotypes have a wild-type localisation of *gurken* mRNA. (E) Gurken protein is expressed in the anterior-dorsal membrane of a wild-type stage 9 oocyte. (F) The majority of *spn* stage 9 egg chambers show a strong reduction or complete lack of Gurken protein. (G) Wild-type stage 9 egg chamber showing *sibo*-directed X-Gal staining in the border cells. (H) *spn* stage 9 egg chambers show a duplication of border cells at the posterior pole. (B,D) *spn-D*349, (F) *spn-B*656, (H) *spn-C*94.

Table 1. Phenotypes shared among spindle mutant eggs and egg chambers

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Ventrilised eggs:</th>
<th>Misplaced oocytes (n)</th>
<th>Lack of karyosome*</th>
<th>Mutant grk mRNA localisation (n)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>weak  strong (n)</td>
<td>24 (229)</td>
<td>97 (92)</td>
<td>83 (24)</td>
</tr>
<tr>
<td><em>spn</em>-C94</td>
<td>0 100 2 (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>spn</em>-B056/Df(3R)red31</td>
<td>54 27 (584)</td>
<td>52 (203)</td>
<td>92 (111)</td>
<td>84 (70)</td>
</tr>
<tr>
<td><em>spn</em>-E516/Df(3R)sbd105</td>
<td>0 100 2 (2)</td>
<td>&lt;2 (188)</td>
<td>94 (83)</td>
<td>n.d.</td>
</tr>
<tr>
<td><em>spn-D</em>349/Df(3R)5B-RXP</td>
<td>50.5 23.5 (924)</td>
<td>&lt;2 (335)</td>
<td>100 (120)</td>
<td>83 (95)</td>
</tr>
<tr>
<td><em>spn-A</em>505</td>
<td>30 35 (467)</td>
<td>5 (132)</td>
<td>100 (176)</td>
<td>n.d.</td>
</tr>
<tr>
<td><em>spn-A</em>505/spn-A*357</td>
<td>32 48 (219)</td>
<td>10 (313)</td>
<td>46 (126)</td>
<td>50 (87)</td>
</tr>
</tbody>
</table>

*Data referring to stage 4, 5, 6 or 7 egg chambers only.
†Only stage 9 egg chambers were scored.
‡Females of these genotypes lay very few eggs.
 n.d.: not determined.
delayed. In the vast majority of stage 9 mutant egg chambers, the mRNA is found in a ring around the whole anterior margin of the oocyte rather than above the nucleus (Fig. 2B; Table 1). By stage 10b, however, gurken mRNA shows a normal localisation above the oocyte nucleus in all cases (Fig. 2D). Gillespie and Berg (1995) have also recently reported that gurken mRNA localisation is defective in 30% of spn-E3987 stage 9 and 10 egg chambers. To rule out the possibility that the spn mutants also affect gurken mRNA levels, we simultaneously hybridised both wild-type and mutant ovaries with a gurken probe and a probe for oskar mRNA as an internal control, and observed no detectable difference in the relative signal in the mutants.

The transient mislocalisation of gurken mRNA to an anterior ring in spn mutant stage 9 egg chambers is very similar to the mislocalisation of gurken mRNA that is observed in K10 mutants (Neuman-Silberberg and Schüpbach, 1993). However, K10 mutations produce a dorsalisation of the egg chamber rather than a ventralisation, because the mislocalised gurken mRNA directs Gurken signalling to the follicle cells on all sides of the oocyte (Wieschaus et al., 1978; Serano et al., 1995; Neuman-Silberberg and Schüpbach, 1996). Since the comparison with K10 indicates that the ventralisation produced by the spn mutants cannot be accounted for by the mislocalisation of gurken mRNA alone, we also examined the distribution of Gurken protein in these egg chambers. In wild-type egg chambers, Gurken protein accumulates in the plasma membrane above the germinal vesicle from stage 9 onwards (Fig. 2E). In contrast, approximately 19% of spn-A (19/101), 85% of spn-B (22/26), 87% of spn-C (47/54), 58% of spn-D (15/26) and 100% of spn-E (18/18) egg chambers show a strong reduction or a complete absence of Gurken protein in the oocyte membrane (Fig. 2F). The penetrance of this phenotype correlates well with the proportion of ventralised eggs produced by each mutant, indicating that the reduction in Gurken protein levels is primarily responsible for the dorsal-ventral patterning defects observed in spn eggs.

Since K10 and spn mutants cause a similar mislocalisation of gurken mRNA but have opposite effects on Gurken protein expression, we examined the phenotype produced by females that were homozygous for both K101 and mutations in spn-B, spn-C or spn-D. In all three double mutant combinations, Gurken protein is expressed in a ring around the entire anterior margin of the oocyte, as it is in K10 mutants alone, and the resulting eggs are completely dorsalisated (Fig. 3A-D). This result indicates that the spn genes are no longer required for normal levels of Gurken protein expression in the absence of K10 activity.

In addition to its role in the induction of the dorsal follicle cells, Gurken also signals earlier in oogenesis to induce the follicle cells at the posterior of the egg chamber to adopt a posterior rather than an anterior fate (González-Reyes et al., 1995; Roth et al., 1995). We therefore examined the expression of an enhancer trap line that specifically labels anterior follicle cells to determine whether the spn mutants also disrupt this Gurken signalling event. In wild-type egg chambers, an enhancer trap insertion in the slbo gene is expressed in the anteriormost follicle cells, the border cells, as they migrate between the nurse cells towards the anterior margin of the oocyte (Montell et al., 1992) (Fig. 2G). In all of the spn mutant combinations that we have tested (spn-A050/spn-A057, spn-B056, spn-C094, spn-D349 and spn-E616), a number of follicle cells at the posterior of the egg chamber also express slbo, indicating that these cells have not been induced to become posterior and have adopted the default anterior fate (Fig. 2H). Thus, the spn genes inhibit Gurken signalling to polarise both the AP and DV axes. This phenotype shows only partial expressivity, since a few follicle cells are normally induced to be posterior, and these are sufficient to signal back to the oocyte to polarise the microtubule cytoskeleton (data not shown).

The spindle genes are required to position the oocyte at the anterior of the egg chamber

It has previously been shown that mutants in both spn-C and spn-E sometimes block the polarisation of the AP axis at an even earlier stage of oogenesis, by preventing the oocyte from reaching its normal position at the posterior end of the egg chamber (González-Reyes and St Johnston, 1994; Gillespie and Berg, 1995). To determine whether this is a general property of mutants in the spn genes, we have examined the arrangement of the nurse cells and the oocyte in mutant egg chambers by labelling with rhodamine-phalloidin to reveal the distribution of F-actin. The oocyte can be unambiguously identified in these stainings because it always possesses four actin-rich ring canals and its membrane cytoskeleton contains more F-actin than that of the nurse cells. In contrast to wild type where the oocyte always lies posterior to the nurse cells (Fig. 4A), mutants in all five spn genes cause a partially penetrant disruption in the positioning of the oocyte, which can be located anywhere in the egg chamber (Fig. 4B-F; Table 1).

In the spn-C mutant egg chambers in which the oocyte is correctly positioned at the posterior, K10 and bicoid mRNAs...
localise to the anterior of the oocyte and oskar mRNA to the posterior, whereas the localisation of all three transcripts is defective in spn-E mutants, most probably as a result of the formation of an abnormally dense microtubule network within the oocyte (González-Reyes and St Johnston, 1994; Gillespie and Berg, 1995). We therefore examined the localisation of these mRNAs in spn-A, spn-B and spn-D mutants. As is the case for spn-C, all three transcripts are correctly localised in the mutant egg chambers in which the oocyte lies at the posterior, whereas K10 and bicoid mRNA localise to both poles of the oocyte and oskar mRNA to the centre in the bipolar egg chambers (data not shown). Thus, spn-E is the only gene in this group that is required for the localisation of K10, bicoid and oskar mRNAs, although the other four genes play an indirect role in this process through their function in the polarisation of the AP axis.

**spn oocytes fail to form a proper karyosome**

After the oocyte leaves the germarium, the dispersed chromosomes of the synaptonemal complex condense to form a hollow sphere of highly packed chromatin called the karyosome, which is clearly visible in egg chambers stained with the DNA dye Hoechst (Fig. 5A) (King, 1970; Rasmussen, 1974; Spradling, 1993). While examining the positioning of the oocyte in spn egg chambers, we noticed that all of the spn mutants share another more penetrant phenotype; almost all of the oocytes lack a karyosome and the oocyte chromosomes are arranged instead in thread-like figures with irregular shape (Fig. 5B; Table 1). The appearance of these chromosomes resembles that seen in the pro-oocytes before one cell is selected to differentiate as the oocyte (A. Carpenter, personal communication).

**spn gene activity is required in the germline**

To determine whether the function of the spn gene products is required in the germline cells of the egg chamber, the nurse cells and the oocyte, we generated germline clones of the strongest available alleles of each spn gene using the DFS technique (Chou et al., 1993). Chimeric egg chambers composed of wild-type follicle cells, and spn-A050, spn-A057,
spn-B\textsuperscript{056}, spn-C\textsuperscript{094} and spn-D\textsuperscript{349} mutant nurse cells and oocytes give rise to ventralised eggs and oocytes that lack a karyosome. We also observed chimaeric egg chambers in which the oocyte is misplaced in spn-A, spn-C and spn-D germline clones but not in spn-B clones, although this is most probably due to the low penetrance of this phenotype in spn-B homozygotes. Furthermore, gurken mRNA shows the same delay in its accumulation above the oocyte nucleus that is seen in the non-mosaic mutant ovaries. We did not obtain any visible clones for either of the two lethal spn-E alleles used, spn-\( \text{E}^{\text{blb}\Delta 125} \) or spn-\( \text{E}^{\text{blb}\Delta 137} \), although both chromosomes have a functional FRT and bear a mutation at the spn-E locus. As these two alleles are used as putative nulls (Gillespie and Berg, 1995), we presume that the complete lack of spn-E function blocks the development of the germline before the stage at which we can examine the chimaeric egg chambers. Nevertheless, it is likely that the later requirements for spn-E in oogenesis are also germline dependent, since the transcripts from the gene are expressed in the nurse cells and oocyte from the gerarium onwards (Gillespie and Berg, 1995). Thus, all of the spn gene functions are likely to occur in the germline.

Genetic interactions between spn mutants

Several of the phenotypes produced by spn mutants are incompletely penetrant (Table 1). The simplest explanation for this result is that the mutations that we are examining are not null, and this is certainly the case for spn-E where the two putative null alleles are homozygous lethal. The strongest alleles of spn-B and spn-D behave as nulls by genetic criteria, however, because the phenotypes that they produce as homozygotes are as strong as those seen in trans-heterozygous combinations with deficiencies. Since mutations in the five spn loci share a number of common phenotypes, an alternative explanation for the incomplete penetrance is that these genes act in a common process and are partially redundant with each other. This model leads to the prediction that females that are mutant for two spn genes simultaneously should show stronger phenotypes than those produced by either single mutant alone. To test this possibility, we generated nine of the ten possible double mutant combinations, and observed a significant enhancement of the phenotype in almost every case. For example, single mutants in spn-B, spn-D and spn-E very rarely lead to the mispositioning of the oocyte, but the frequency of this phenotype rises to 25% (\( n=167 \)) in spn-B spn-D and to 22% (\( n=250 \)) in spn-E spn-D double mutants. Since the spn-B and spn-D alleles used are putative nulls, this result strongly indicates that these genes play partially redundant roles in this process.

Reduction in spn gene function delays the choice between the two pro-oocytes

The analysis of the double mutant ovaries also revealed an unexpected phenotype. Five spn double mutant combinations (CB, CE, CA, EA and DA) give rise to young egg chambers that contain two cells that appear to be developing oocytes by the following criteria. Firstly, both of these cells are smaller than the adjacent nurse cells, and are similar in size to a wild-type oocyte of the same age (compare Fig. 6A with D). Secondly, like wild-type oocytes, both cells show high levels of F-actin staining in their membrane cytoskeleton (Fig. 6A,D). Thirdly, neither cell follows the nurse cell pathway of development by endoreplicating its DNA to become polyploid, and both fail to form a karyosome, a phenotype that is characteristic of the oocyte in spn mutants (Fig. 6E).

During wild-type oogenesis, the two cells in each germline cyst that have four ring canals initially appear equivalent, as both cells enter meiosis to become pro-oocytes in region 2a of

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**Fig. 6.** Several spn double mutant combinations give rise to egg chambers that contain 14 nurse cells and 2 oocytes. (A) Wild-type egg chambers have a single oocyte that possesses four ring canals, as visualised with rhodamine-phalloidin staining. (B) The oocyte accumulates BicD protein and (C) oskar mRNA. (D) In several mutant combinations (see below), a proportion of the egg chambers contain two oocytes. Two cells clearly show stronger staining with rhodamine-phalloidin, are smaller than their sister nurse cells and possess four ring canals each (they share one; in yellow in the drawing in D'). (E) Both of these cells accumulate BicD protein and fail to form a karyosome (arrowheads in E'). (E,E') Correspond to the same egg chamber. (F) Both cells also accumulate oskar mRNA. The two oocytes in this egg chamber are misplaced, as is often the case in these double mutant combinations (F). The mutant combinations that show the two-oocyte phenotype are the following: CB (42%, \( n=52 \)), CE (14%, \( n=70 \)), CA (13%, \( n=116 \)), BA (1%, \( n=172 \)), ED (1%, \( n=250 \)), EA (3%, \( n=132 \)), DA (7%, \( n=164 \)). The alleles used to make the double mutant chromosomes are A\textsuperscript{057}, B\textsuperscript{056}, C\textsuperscript{094}, D\textsuperscript{349} and E\textsuperscript{516}.
the germarium. In region 2b, one of these two cells is selected to develop as the oocyte and remains in meiosis, while the other exits meiosis and reverts to the nurse cell pathway of development. One way that a cyst with two oocytes can arise is if both pro-oocytes develop as oocytes, as has been observed in cyclinE01672 mutant ovaries, where both cells remain in meiosis, giving rise to two cells that resemble oocytes at least with respect to their nuclear morphology (Lilly and Spradling, 1996). Egg chambers with two oocytes also occur, however, in mutants that cause fusions between adjacent cysts such as brainiac, leading to the formation of egg chambers with extra germinal cells and more than one oocyte (Goode et al., 1992). To determine whether the spn double mutants cause the fusion of adjacent cysts or the transformation of one nurse cell into an oocyte, we examined the arrangement of the germine cells and ring canals in these egg chambers. In all cases, the cysts contain the normal number of germine cells and each oocyte possesses four ring canals. The first ring canal to form during cyst morphogenesis becomes the largest and brightest ring canal in the 16-cell cyst, and connects the two cells with four ring canals (Koch and King, 1969). In the mutant cysts, this ring canal always connects the two oocytes (Fig. 6D,D'). Thus, the two-oocyte phenotype in the spn double mutant egg chambers arises because the second pro-oocyte develops as an oocyte rather than a nurse cell.

The results above suggest that the spn mutants either inhibit the choice between the two pro-oocytes, or prevent the ‘losing’ pro-oocyte from reverting to the nurse cell pathway of development. One of the earliest steps in the determination of the oocyte is the accumulation of Orb and Bic-D proteins in a single cell of the cyst (Wharton and Struhl, 1989; Suter and Steward, 1991; Lantz et al., 1994). In the spn double mutants, these proteins often become concentrated in two cells rather than one (Fig. 6B,E and data not shown). Furthermore, oskar mRNA, which localises to the presumptive oocyte in region 2b of the germarium, also becomes concentrated in two cells in these mutants (Fig. 6C,F). In contrast, we have never observed Bic-D or Orb accumulation in more than one cell in cyclinE01672 ovaries (data not shown). These results suggest that the spn double mutant combinations impair or delay the choice between the two pro-oocytes and, as a consequence, both cells develop as oocytes. cyclinE01672, in contrast, acts at a later step, perhaps by preventing the losing pro-oocyte from replicating its DNA to become a nurse cell.

Although the choice between the two pro-oocytes does not occur in a significant proportion of the young spn double mutant egg chambers, one of the two oocytes always reverts to the nurse cell fate later in oogenesis. In the majority (19/25) of stage 4 to
7 spn-D^{499} spn-A^{057} egg chambers with 14 nurse cells and 2 oocytes (as shown by rhodamine-phalloidin and Hoechst stainings), BicD protein is concentrated in a single cell (Fig. 7A, A'). Furthermore, one of the two cells with four ring canals often contains more DNA than the other, although it is still has much less than a normal nurse cell (Fig. 7B). This most probably reflects the fact that one of the two cells is beginning to differentiate as a nurse cell, but because it started the process of becoming polyploid significantly later than the adjacent nurse cells, it still has a much lower DNA content. By stage 8, all of the double mutant egg chambers contain 15 nurse cells and one oocyte, as indicated by the pattern of expression of BicD and Orb proteins and nuclear morphology. These phenotypes have also been observed in the other combinations tested (EA, CB and CA). Thus, the strong reduction of spn activity in the double mutants delays but does not block the decision between the two pro-oocytes, as one of the two cells always becomes a nurse cell eventually, albeit much later in oogenesis than normal.

DISCUSSION

Although the spn genes were initially identified because homozygous mutant females produce ventralised eggs, our results reveal that this group of genes are required for each of the symmetry-breaking steps that lead to the polarisation of the two main body axes of Drosophila (Fig. 8). Several of the double mutant combinations delay the first of these events, the choice between the two pro-oocytes to select one cell to become the oocyte and the other to become a nurse cell. All of the single mutants disrupt the positioning of the oocyte at the posterior of the cyst to generate the first AP polarity. Finally, all of the mutants inhibit Gurken signalling to the follicle cell layer to polarise first the AP axis and then DV axis.

The role of the spindle genes in oocyte determination

Perhaps the most surprising of the spn phenotypes is the formation in five different double mutant combinations of germline cysts that contain fourteen nurse cells and two oocytes. Soon after the 16-cell cyst forms in region 2a of the gerarium, the two cells with four ring canals, the pro-oocytes, become different from the other 14 cells of the cyst in several respects. (1) Both of these cells enter meiosis and always form complete synaptonemal complexes. (2) A polarised nutrient stream causes a flow of cytoplasm and organelles from the nurse cells into the pro-oocytes. (3) The centrioles migrate from the nurse cells to the pro-oocytes, accumulating on both sides of their common ring canal (Mahowald and Strassheim, 1970; Rasmussen, 1974, 1975; Carpenter, 1979; Schmekel et al., 1993). This symmetric behaviour of the two pro-oocytes in young region 2 cysts is broken when the cyst progresses to germarial region 2b, where one of the pro-oocytes exits meiosis and enters the nurse cell developmental pathway, whereas the sister four-ring-canal cell carries on meiosis and becomes an oocyte (Mahowald and Kambyssellis, 1980). At about the same time, the centrioles migrate through the ring canal connecting the two pro-oocytes to accumulate within the oocyte (Mahowald and Strassheim, 1970; Carpenter, 1975, 1994).

Thus, these classical EM studies indicate that the process of oocyte determination occurs in two steps: first the two cells with four ring canals are selected to differentiate as pro-oocytes, and then one of these cells is chosen to become the oocyte, while the other reverts to being a nurse cell. The phenotype of the spn double mutant combinations strongly supports this two-step model, as both pro-oocytes follow the oocyte pathway of development for a much longer period of time than normal, and both cells accumulate several oocyte-specific markers, such as Orb and Bic-D proteins and oskar mRNA from germarial stages onwards. These results also imply that the spn genes are specifically required for the second step in this process, in which one of the pro-oocytes is selected to become the oocyte.

How the asymmetry between the two pro-oocytes arises is unknown, but it has been proposed that it could be generated during the first division of the cystoblast to give rise to a two-cell cyst (Theurkauf, 1994; Lin and Spradling, 1995). During this division, a vesicular structure called the spectrosome associates with one pole of the mitotic spindle and is asymmetrically partitioned between the two daughter cells (Lin et al., 1994; Lin and Spradling, 1995; McKearin and Ohlstein, 1995; de Cuevas et al., 1996). Since each of these cells gives rise to one pro-oocyte and seven nurse cells, this asymmetry might determine which pro-oocyte is fated to become the oocyte. Whatever mechanism generates the initial asymmetry, it seems that the key step in the selection of the oocyte is the accumulation of Bic-D and Egl proteins in a single cell. Null mutants in either gene block the localisation of the protein encoded by the other to the presumptive oocyte and prevent all other known steps in oocyte differentiation, such as the formation of an active MTOC in this single cell and the subsequent microtubule-dependent localisation of oocyte-specific transcripts such as oskar mRNA (Ephrussi et al., 1991; Theurkauf et al., 1993; Ran et al., 1994; Mach and Lehmann, 1997). Although it is unclear at what point in the pathway of oocyte selection the spn genes act, they must function upstream of the localisation of Bic-D (and presumably Egl) to a single cell, and we consider it most likely that they are directly involved in this process. Firstly, the spn double mutant combinations delay but do not block the choice between the two pro-oocytes, suggesting that they do not remove the initial asymmetry, but slow down its expression. Secondly, a reduction in Bic-D or Egl activity later in oogenesis leads to the same ventralised phenotype that is produced by the single spn mutations (Swan and Suter, 1996; Mach and Lehmann, 1997). This raises the possibility that the spn gene products interact with Bic-D and Egl at two different stages of oogenesis, first to select the oocyte and then to regulate Gurken expression once the oocyte has formed.

The relationship between the different spindle phenotypes

The pleiotropic effects of the spn mutations indicate that this group of genes is required for a number of apparently unrelated events during oogenesis. This requirement need not be direct, however, as it is possible that some of the later phenotypes arise as a consequence of the defects earlier in oogenesis. In egl mutant ovaries, all sixteen cells in the germline cyst briefly enter meiosis before reverting to the nurse cell fate (Carpenter, 1994). This observation has led to the model that at least some of the oocyte determinants are initially present in all sixteen cells, and that Egl activity is required to concentrate these
factors in a single cell in order to specify it as the oocyte. Thus, the two ‘oocytes’ in the spindledoubledouble mutants should contain only half the normal amount of these factors. This level of oocyte determinants is sufficient to induce the accumulation of oocyte-specific markers in both cells, but does not irreversibly commit both cells to the oocyte fate since one cell always becomes a nurse cell eventually. If the single mutants cause a similar but weaker delay in oocyte selection, the oocyte might not receive enough of these factors at the right time to induce all aspects of oocyte differentiation. The meiotic chromosomes in the oocyte of the single spn mutants and the two ‘oocytes’ of the double mutants fail to condense into a karyosome. Since these chromosomes resemble those seen in the pro-oocytes during normal development, the incomplete determination of the oocyte may arrest progression through meiosis at the pro-oocyte stage. Although the mechanism that leads to the placement of the oocyte posterior to the nurse cells is unknown, this process occurs in the germarium soon after the oocyte is specified, and it is easy to imagine that a small delay in oocyte selection could also lead to the failure of this cell to reach its correct location.

Although the karyosome- and oocyte-positioning phenotypes can perhaps be explained as secondary effects of a delay in oocyte determination, this is less likely to be the case for the defects in Gurken signalling caused by the spn mutants. The former two phenotypes arise in the germarium and are therefore likely to be very sensitive to a delay in oocyte selection, whereas Gurken signalling begins later in oogenesis. Indeed, the second Gurken signal, which induces the dorsal follicle cells, occurs long after Bic-D has accumulated in a single cell, even in the spn double mutants. Furthermore, there is a precedent for genes that are required for both oocyte selection and Gurken signalling, since both Bic-D and egl mutants disrupt both processes and in the case of Bic-D, these two functions have been shown to be temporally distinct (Mohler and Wieschaus, 1986; Schüpbach and Wieschaus, 1991; Ran et al., 1994; Swan and Suter, 1996; Mach and Lehmann, 1997).

The role of the spn genes in Gurken signalling

The five spn loci join the growing list of genes that are required for Gurken signalling (Neuman-Silberberg and Schüpbach, 1993; Christerson and McKearin, 1994; González-Reyes et al., 1995; Roth et al., 1995; Wilson et al., 1996). One reason why so many genes might be involved in this process is that the localisation and translation of gurken mRNA need to be tightly regulated to ensure that the same cell can signal in two perpendicular directions at different stages of oogenesis. Our analysis of the spn phenotypes suggests that these genes are likely to be involved in the localisation and/or translation of gurken mRNA. The mutants cause a delay in the localisation of gurken mRNA without having any discernible effect on its level, but dramatically reduce the amount of Gurken protein that is produced. K10 mutants cause a similar mislocalisation of gurken mRNA without significantly affecting protein expression (Serano et al., 1995). As the altered distribution of the mRNA in spn mutants cannot account for the lack of Gurken protein, we assume that the spn mutants are primarily required for the efficient translation of gurken mRNA. This raises the question of why these mutants also delay the localisation of gurken mRNA. One possibility is that the Spn gene products are directly involved in both processes, and perhaps play a role in coupling translation to localisation.

There are a number of similarities between the regulation of Gurken expression and the co-ordinated control of oskar mRNA localisation and translation, and the two processes even share a number of common components. capu, spire and orb are required for the localisation of both gurken and oskar mRNAs, while aubergine is necessary for the efficient translation of each transcript (Neuman-Silberberg and Schüpbach, 1993; Christerson and McKearin, 1994; Wilson et al., 1996). The translation of oskar mRNA is tightly coupled to its posterior localisation because the Bruno protein binds to the oskar 3'UTR to prevent the translation of unlocalised RNA and this protein has also been reported to bind to gurken mRNA (Kim-Ha et al., 1995). This observation and the other similarities between oskar and gurken regulation strongly suggest that the translation of gurken mRNA will also depend on its correct localisation. K10 mutants seem to uncouple these two processes, as the mRNA is translated all around the circumference of the anterior of the oocyte (Serano et al., 1995; Neuman-Silberberg and Schüpbach, 1996). Thus it is reasonable to suppose that wild-type K10 activity is required to repress the translation of unlocalised gurken mRNA. Although spn mutants seem to block almost all Gurken translation, they have no effect on Gurken expression in a K10 mutant background. One way that this surprising observation could be explained is if the spn gene products act to relieve the K10-dependent translational repression once gurken mRNA has been localised above the oocyte nucleus. In the absence of K10, the spn genes would no longer be required to activate Gurken translation, since it would not have been repressed in the first place. The proposal that the Spn gene products are involved in the translational activation of gurken mRNA is supported by the recent discovery that spn-E encodes a DEAD box protein that is likely to function as an RNA helicase (Gillespie and Berg, 1995). Several members of this protein family such as elf4A are involved in translation initiation, and one, Vasa protein, has been implicated in the translational derepression of localised nanos mRNA (Schmid and Linder, 1992; Gavis et al., 1996).

Mutants in the five spindled double mutants should contain one of these genes would no longer be required to activate Gurken translation, since it would not have been repressed in the first place. The proposal that these five loci constitute a novel group of genes that all act in a common process. As only one of these genes has so far been cloned, we can only speculate about the nature of the molecular process in which the spn gene products participate. Nevertheless, this group of genes provides a novel link between oocyte selection, oocyte positioning and axis formation, and the further molecular analysis of these gene products may reveal how the asymmetries that generate polarity in Drosophila arise.

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