okra and spindle-B encode components of the RAD52 DNA repair pathway and affect meiosis and patterning in Drosophila oogenesis

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okra (okr), spindle-B (spnB), and spindle-D (spnD) are three members of a group of female sterile loci that produce defects in oocyte and egg morphology, including variable dorsal-ventral defects in the eggshell and embryo, anterior-posterior defects in the follicle cell epithelium and in the oocyte, and abnormalities in oocyte nuclear morphology. Many of these phenotypes reflect defects in grk-Egfr signaling processes, and can be accounted for by a failure to accumulate wild-type levels of Gurken and Fs(1)K10. We have cloned okr and spnB, and show that okr encodes the Drosophila homolog of the yeast DNA-repair protein Rad54, and spnB encodes a Rad51-like protein related to the meiosis-specific DMC1 gene. In functional tests of their role in DNA repair, we find that okr behaves like its yeast homolog in that it is required in both mitotic and meiotic cells. In contrast, spnB and spnD appear to be required only in meiosis. The fact that genes involved in meiotic DNA metabolism have specific effects on oocyte patterning implies that the progression of the meiotic cycle is coordinated with the regulation of certain developmental events during oogenesis.

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The anterior-posterior and dorsal-ventral axes of the Drosophila embryo are established during oogenesis by a series of intercellular signaling events that generate asymmetries in the developing egg chamber, which are subsequently transmitted to the egg. Early in oogenesis, signaling between the germ-line-derived oocyte and the overlying somatic follicle cells specifies posterior follicle cell fates. These cells, in turn, signal back to the oocyte, initiating a reorganization of the microtubule network that defines the anterior-posterior axis of the oocyte and embryo. In mid-oogenesis, signaling from oocyte to follicle cells specifies dorsal follicle cell fates, and this in turn restricts the activation of a second follicle cell to oocyte signaling process that defines the dorsal-ventral axis of the embryo (for review, see Ray and Schüpbach 1996).

Both of these oocyte to follicle cell signaling events are mediated by a single signaling pathway involving the Gurken (grk) and Epidermal growth factor receptor (Egfr) genes. grk and Egfr are a ligand/receptor pair: grk encodes a TGF-α-like protein that is expressed in the germ-line and localized to the oocyte (Neuman-Silberberg and Schüpbach 1993, 1996); Egfr encodes the Drosophila homolog of the EGFR (Livneh et al. 1985) and is expressed in the somatic follicle cells (Kammermeyer and Wadsworth 1987; Sapir et al. 1998) in which it acts as a receptor for grk in these signaling events. The mutant phenotypes of grk and Egfr reflect their roles in anterior-posterior and dorsal-ventral patterning during oogenesis. Eggs produced by grk or Egfr mutant females have a duplication of anterior chorion structures at their posterior ends, reflecting a defect in the specification of posterior follicle cell fates (Schüpbach 1987; González-Reyes et al. 1995; Roth et al. 1995). The eggs also lack dorsal appendage material, reflecting a defect in the specification of dorsal follicle cell fates (Schüpbach 1987). The polarization of the anterior-posterior axis of the oocyte and embryo, and the polarization of the dorsal-ventral axis of the embryo, are also defective in the mutants. In the oocyte, RNAs that are normally localized to the anterior cortex in wild type, like the bicoid (bcd) mRNA, are found at both the anterior and posterior poles, whereas RNAs localized to the posterior pole, like the oskar (osk) mRNA, are found mislocalized to the middle (González-Reyes et al. 1995; Roth et al. 1995). In the embryo there is an expansion of ventral pattern elements at the ex-
pense of more dorsal ones, reflecting a ventralization of the embryonic dorsal-ventral axis (Schüpbach 1987; Roth and Schüpbach 1994).

In addition to grk and Egfr, a number of other genes have been identified that are required either in the germline or the follicle cells to regulate or transmit the grk–Egfr signal. In the germline, several genes, in particular fs(1)K10 (K10), have been shown to be required for the proper localization of the grk mRNA within the oocyte, and females mutant for these genes give rise to dorsIALIZED eggshells and embryos that reflect this mislocalization of grk. Another germline required gene, cornichon (cni) has been shown to be involved in the secretion or activation of Grk. In the follicle cells, components of the Ras signaling pathway have been implicated in the transmission of the grk–Egfr signal from receptor to the nucleus (for review, see Ray and Schüpbach 1996).

To identify other genes involved in this signaling process, we focused on a group of female sterile loci on the second and third chromosomes that produce ventralized eggshells similar to those produced by mutants in the grk–Egfr pathway. These genes include okra (okr), deadlock (del), squash (squ), zucchini (zuc), aubergine (aub), and vasa (vas) on the second chromosome (Schüpbach and Wieschaus 1991), and the spindle genes (spnA, spnB, spnC, spnD, and spnE) on the third (Tearle and Nüsslein-Volhard 1987). Recent studies on aubergine (Wilson et al. 1996) and the spindle genes (González-Reyes et al. 1997) have provided evidence that mutations in these genes affect grk–Egfr signaling.

We have concentrated on three genes, okr, spnB, and spnD. We show here that mutations in these genes produce defects in the oocyte and embryo that are consistent with a role in regulating the grk–Egfr signaling process. Our results indicate that many of the patterning defects produced by these mutations are the result of a failure to accumulate wild-type levels of Grk and K10 protein in the oocyte. We have cloned okr and spnB, and find that the genes encode two components of the yeast RAD52 DNA repair pathway. In light of these homologies, we have investigated a requirement for the genes in mitotic and meiotic DNA repair, and find that okr is required for both mitotic DNA repair and meiotic recombination, whereas spnB and spnD are required only for recombination. These data provide evidence that the progression of meiotic events in the oocyte nucleus is providing cues to the cytoplasm that are necessary for the proper regulation or timing of developmental processes.

Results

okr, spnB, and spnD affect D/V patterning in the eggshell and the embryo

The predominant phenotype produced by females mutant for okr, spnB, and spnD is a ventralization of the eggshell, reflected in a loss of dorsal appendage material that is similar to the phenotype produced by mutations in the grk–Egfr pathway. However, unlike grk and Egfr alleles, which produce fairly discrete ventralized phenotypes, all alleles of okr, spnB, and spnD produce a broad spectrum of ventralization. In addition, the mutant females also produce eggshell phenotypes that are not observed in grk–Egfr mutants, including dorsIALIZED eggs with extra appendage material or multiple appendages as well as small eggs, although these phenotypes are comparatively rare. The majority of the eggs produced by okr, spnB, and spnD mutant females, including those that are only mildly ventralized, do not hatch and show no indication of embryonic development.

To quantify the ventralization observed in these mutants, eggshells were assigned to one of four phenotypic classes. Class 1 eggs resemble wild type with two normal dorsal appendages, class v2 eggs have two dorsal appendages that are fused at the base, class v3 eggs have a single dorsal appendage, and class v4 eggs have little or no dorsal appendage material (Fig. 1A–D, Table 1). Females mutant for alleles of okr, spnB, and spnD produce eggs in all four classes, and this variability is not caused by differential expressivity, as a single female produces the full range of phenotypes. Although all three genes give rise to the same range of phenotypes, differences in the distribution of eggs among the various classes reflect the severity of a particular genotype (Table 1). We have observed that the spnB and spnD eggshell phenotypes become more severe over time. Newly eclosed spnB and spnD mutant females produce 90%–95% class 1 eggs in the first day after mating, and some of these eggs hatch and give rise to viable progeny. By the fourth day, however, the mutant females produce only 10%–20% class 1 eggs, and there is a corresponding increase in the percent of class 4 eggs. We do not observe a change in the severity of the okr eggshell phenotype with time.

A characteristic of mutations in the grk–Egfr signaling pathway is that they affect patterning in both the eggshell and embryo. The embryos that develop within the ventralized eggshells produced by grk and Egfr mutant females are also ventralized, and show an expansion of the mesodermal anlage (Schüpbach 1987; Roth and Schüpbach 1994). To determine if okr, spnB, and spnD affect embryonic patterning as well as eggshell patterning, we examined the expression of Twist (Twi), a mesodermal marker (Thissè et al. 1988), in the mutant embryos. Even though only a small percentage of the mutant embryos develop to the cellular blastoderm stage, we find that those that do show a variable expansion of the mesoderm (Fig. 1E–G), ranging from cases in which the mesoderm is fairly normal to cases in which it encompasses most of the blastoderm. Notably, this expansion is always more severe at the posterior than the anterior. In addition to these phenotypes, we also see cases in which the mesoderm is normal at the anterior end of the embryo, but posteriorly splits into two independent domains that run up the lateral sides of the embryo and meet at the dorsal midline (Fig. 1G). Apart from the difference in ventralization along the anterior-posterior axis, these ventralized phenotypes are similar to those that have been observed in grk and Egfr mutant embryos,
indicating that the follicle cells have adopted an anterior fate. 

The posterior pole of the egg (micropyles are indicated by arrows) and suggest that okr affects anterior–posterior patterning via an effect on grk–Egfr signaling.

and suggest that okr, spnB, and spnD affect dorsal–ventral patterning via an effect on grk–Egfr signaling.

okr affects anterior–posterior patterning in the egg chamber

In addition to the dorsal–ventral patterning defects described above, okr mutants share another phenotype with mutants in the grk–Egfr signaling pathway: They produce eggs that often have a second micropyle at the posterior end (Fig. 1H). This phenotype appears in ~2% of the eggs laid by females homozygous for amorphic okr alleles, and in 42% of the eggs laid by females mutant for the more severe antimorphic alleles. This follicle cell defect can also be visualized with molecular markers:

We find that 77% of the egg chambers from strong okr mutations show dpp expression at both the anterior and posterior poles instead of the normal restricted expression in anterior follicle cells (Twombly et al. 1996). In these mutant ovaries, we also observed a defect in bcd RNA localization: We find that 5% of the egg chambers show localization of bcd to both the anterior and posterior poles of the oocyte, indicating that the anterior–posterior polarity of the oocyte is also affected. These data are consistent with the hypothesis that okr affects both the early (anterior–posterior) and late (dorsal–ventral) grk–Egfr signaling processes. However, we have found that the appearance of second micropyles on okr mutant eggs does not necessarily reflect the severity of the dorsal–ventral defect: Second micropyles are sometimes observed on eggs with normal dorsal–ventral polarity (see Fig. 1H), and strongly ventralized eggs do not necessarily have a second micropyle. This uncoupling of the two phenotypes implies that okr can affect the early grk signaling process independently from the later one. In spnB and spnD mutant eggs, we do not observe a significant number of second micropyles, nor have we seen duplication of dpp or mislocalization of bcd in the mutant ovaries.

okr, spnB, and spnD affect grk RNA localization and protein accumulation

To more precisely establish the role of okr, spnB, and spnD in grk–Egfr signaling, we have looked more directly at their effects on the signaling process. Specifically, because the three genes are required in the germline (our observations, for spnB and spnD see also González-Reyes et al. 1997), we have examined the effect of the mutants on the expression and localization of grk RNA and protein. In wild-type ovaries, grk RNA is localized to the oocyte during the early stages of oogenesis, and then, in mid-oogenesis, it is localized within the oocyte, first transiently in an anterior–cortical ring (stage 8), and then to a dorsal–anterior patch overlying the oocyte nucleus (stages 9 and 10). In okr mutant ovaries, grk RNA is correctly localized to the oocyte in early stages. In mid-oogenesis, however, we find instances of persistent localization of the RNA in an anterior–cortical ring. The spnB and spnD mutant phenotypes are more severe. In stage 9, 85% of the mutant egg chambers show persistent localization of grk RNA in the anterior–cortical ring (see also, González-Reyes et al. 1997). In stage 10, the spnB and spnD mutant egg chambers show a range of phenotypes including cases in which the RNA is normally localized, others in which it is only partially localized, others in which it is still present in an anterior cortical ring, and others in which the level of RNA is reduced or undetectable (data not shown).

In addition to their effects on grk mRNA localization in the oocyte, okr, spnB, and spnD also affect the accumulation of grk protein. In wild-type egg chambers, Grk is restricted to the oocyte, and in mid-oogenesis, it is localized to a dorsal–anterior patch (Neman-Silberberg and Schüpbach 1996; Fig. 2A,B). In okr mutant ovaries,
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Table 1. Phenotypes produced by okr, spnB, and spnD mutant females

A. Eggshell phenotypes of representative alleles as homozygotes and hemizygotes

| Genotype                  | N  | 1  | v2 | v3 | v4 |
|---------------------------|----|----|----|----|----|
| okr<sup>R1</sup>/okr<sup>RU</sup> | 960 | 43 | 30 | 22 | 5  |
| okr<sup>R1</sup>/Df(2L)Js17    | 866 | 65 | 27 | 6  | 2  |
| okr<sup>R8</sup>/okr<sup>R8</sup> | 479 | 10 | 45 | 43 | 2  |
| okr<sup>R4</sup>/Df(2L)Js17    | 497 | 79 | 14 | 4  | 3  |
| spn<sup>B153</sup>/spn<sup>B153</sup> | 543 | 29 | 32 | 24 | 15 |
| spn<sup>B153</sup>/Df(3R)trxE12 | 888 | 38 | 32 | 29 | 1  |
| spn<sup>B</sup>U/<sup>B</sup>U   | 828 | 19 | 11 | 10 | 60 |
| spn<sup>B</sup>U/Df(3R)trxE12  | 929 | 48 | 19 | 23 | 10 |
| spn<sup>D150</sup>/spn<sup>D150</sup> | 383 | 14 | 7  | 15 | 64 |
| spn<sup>D150</sup>/Df(3R)trxE12 | 903 | 8  | 4  | 9  | 79 |

B. Eggshell phenotypes in double mutant combinations with fs(1)K10

| Genotype                  | N  | d2<sup>b</sup> | d2<sup>h</sup> | 1  | v2 | v3 | v4 |
|---------------------------|----|----------------|----------------|----|----|----|----|
| K10                       | 374 | 99              | 1              | 1  |    |    |    |
| K10; grk<sup>H1</sup>+/   | 511 | 61              | 28             | 9  | 1  |    |    |
| K10; spn<sup>B153</sup>+  | 150 | 92              | 8              |    |    |    |    |
| K10; spn<sup>B153</sup>/spn<sup>B</sup>566 | 218 | 39              | 31             | 22 | 6  | 2  |
| K10;+; spn<sup>B153</sup>/spn<sup>B</sup>566 | 325 | —              | —              | 9  | 8  | 41 | 42 |
| K10; okr<sup>R4</sup>/Df(2L)Js17 | 581 | 75              | 13             | 1  | 3  | 6  | 2  |

<sup>a</sup>N (N) Total number of eggs scored that fall into the four classes shown in the table. Dorsalized and small eggs, which account for <5% of the total, were not scored in this experiment. For spnB and spnD, collections were made after at least 5 days at 25°C.

<sup>b</sup>(d2, d3) Dorsalized eggshells were classified as follows: (d2) intermediate dorsalization resulting in eggs with two dorsal appendages spaced far apart, i.e., shifted laterally; and (d3) strongly dorsalized eggs with dorsal appendage material extending around the lateral and ventral side of the egg (Wieschaus et al. 1978).

have analyzed the phenotypes produced by double mutants with K10. For all three double-mutant combinations, we find that rather than producing all ventralized or all dorsalized eggs, the mutant females produce a broad spectrum of phenotypes ranging from completely dorsalized to completely ventralized (Table 1). Given that these experiments do not reveal a simple epistatic relationship between okr, spnB, spnD, and K10, the three genes must affect Grk activity by a pathway that is at least partially independent of K10. Significantly, as this same spectrum of phenotypes is produced by K10 mutant females that have only one wild-type copy of grk (Table 1; see also Forlani et al. 1993), these results are consistent with a role for these genes in directly affecting the accumulation of Grk in the oocyte.

Given that the mislocalization of grk mRNA that is observed in okr, spnB, and spnD mutant egg chambers is similar to that observed in K10 mutant egg chambers (Neuman-Silberberg and Schüpbach 1993; Roth and Schüpbach 1994), we have also looked for a defect in the accumulation of K10 protein in okr, spnB, and spnD ovaries. We find that in all three mutant genotypes, there is a reduction in the level of K10 in the oocyte (Table 1; see also Forlani et al. 1993), these results are consistent with a role for these genes in directly affecting the accumulation of Grk in the oocyte.

Relationship between okr, spnB, and spnD and the dorsalizing mutant K10

The effects of okr, spnB, and spnD on Grk accumulation in the oocyte place these genes upstream of grk in the genetic hierarchy controlling dorsal–ventral patterning in the egg chamber. To assess the relationship between okr, spnB, and spnD and a different class of genes in the patterning hierarchy that are required for the localization of grk RNA and produce dorsalizing phenotypes, we

levels of Grk are variably reduced throughout oogenesis. Within a single ovariole, egg chambers expressing Grk can alternate with egg chambers that do not (Fig. 2C,D). In spnB and spnD mutant egg chambers, the early accumulation of Grk in the oocyte is normal (Fig. 2E). By mid-oogenesis, however, the level of Grk in the oocytes is reduced and is often undetectable (Fig. 2F).

The failure to accumulate wild-type levels of Grk leads to ventralization of the eggshell and embryo. Whereas the failure to accumulate wild-type levels of K10 leads to the mislocalization of grk mRNA in mid-oogenesis, whereas the failure to accumulate wild-type levels of Grk leads to ventralization of the eggshell and embryo. The former defect may also account for the production of rare dorsalized eggs by okr, spnB, and spnD mutant females. Moreover, the fact that we observe both ventralized and dorsalized eggs suggests that the two effects are to some degree independent: Ventralized eggs arise from cases in which grk levels are reduced and K10 levels are normal or reduced, whereas dorsalized eggs arise from cases in which Grk levels are fairly normal and K10 levels are reduced.

Oocytes from okr, spnB, and spnD ovaries show defects in the morphology of the oocyte nucleus

Oocytes from okr, spnB, and spnD mutants also have defects in nuclear morphology. Studies on chromosome behavior in wild-type ovaries have shown that during stage 3, the DNA in the oocyte nucleus condenses into a compact spherical structure, the karyosome, which is maintained through the later stages in oogenesis until the onset of metaphase I (Spradling 1993). In ovaries stained with a DNA dye, this structure appears as a bright spot within which there is a spot of greater intensity (Fig. 2I). In okr, spnB, and spnD mutant egg chambers, this condensation is aberrant and a variety of defective structures are observed. In some cases, the DNA assumes an ellipsoidal shape that is larger than the normal spot (Fig. 2J), and in others, the DNA is present in clumps that line the inside of the nuclear membrane (Fig. 2K). As this defect is not seen in grk or K10 mutant egg chambers (data not shown), it does not arise from a
threadlike and fragmented (KspnD, spnB, or a number of other genes (including aub, del, squ, vas, zuc, spnA, spnC, and spnE), the DNA is more diffuse (I) or threadlike and fragmented (K).

Figure 2. (A–F) Expression of Grk in okr, spnB, and spnD mutant ovaries. Ovarioles (A, C, E) and stage 10 egg chambers (B, D, F) with Grk in green and cortical actin detected with Phalloidin in red. In wild-type ovaries, Grk is detected in the oocyte throughout oogenesis (A), and becomes localized to the presumptive dorsal–anterior corner in stages 9 and 10 (B). In okr mutant ovaries, Grk expression is reduced or undetectable in many egg chambers, and these are interspersed among egg chambers that have apparently normal Grk expression (C, D). In spnB and spnD mutant ovaries, Grk expression is apparently normal in the early stages, but less and less protein is detectable in the oocyte as oogenesis proceeds (E). The majority of stage 10 spnB and spnD mutant egg chambers have no detectable Grk (F). (G, H) Expression of fs(1)K10 in okr, spnB, and spnD mutant ovaries. Triple stainings of egg chambers with K10 shown in red and cortical actin and DNA shown in green. In wild type (G), K10 protein is observed in the oocyte nucleus, and is particularly concentrated around the karyosome. In the mutant egg chambers (H) K10 protein is reduced or absent. (I–K) Defects in oocyte nuclear morphology in okr, spnB, and spnD. Stage 8 egg chambers stained for cortical actin (red) and DNA (green). In wild type (H), the DNA in the oocyte nucleus is condensed into a tight sphere. In ovaries mutant for okr, spnB, spnD, or a number of other genes (including aub, del, squ, vas, zuc, spnA, spnC, and spnE), the DNA is more diffuse (I) or threadlike and fragmented (K).

defect in grk-Egfr signaling. Our findings corroborate those of a previous study on the spindle genes (González-Reyes et al. 1997), and more recent studies on vas have shown that mutations in this gene have a similar nuclear defect (Styhler et al. 1998; Tomancak et al. 1998). We have examined ovaries from females mutant for the remaining loci in this group, and find that del, squ, zuc, and aub produce the phenotype as well. Thus, the nuclear defect appears to be a phenotype common to all the genes in this class.

Molecular analysis of okr and spnB

The okr locus was cloned from a genomic walk spanning the 23C interval to which the gene was localized (see Materials and Methods; Fig. 3). Within this region, a 4.7-kb genomic fragment was found to rescue the okr mutant phenotype. Northern analysis of ovarian poly(A)^+ RNA identified three transcripts of 4.5, 2.7, and 1.6 kb, of which only the 2.7-kb transcription unit is completely contained within the rescuing fragment. A cDNA corresponding to this RNA was isolated, sequenced, and found to be identical to the previously characterized Drosophila gene DmRad54 (Kooistra et al. 1997). Thus, okr is the Drosophila homolog of the yeast RAD54 gene, a DNA helicase of the RAD52 epistasis group that is required for double-strand break (DSB) repair. In situ hybridization to wild-type ovaries by use of the okr cDNA as a probe indicates that the RNA is widely expressed at all stages of oogenesis (data not shown). The sequence of the seven okr alleles was determined, and all showed single nucleotide changes in the coding region of the 2.7-kb transcript that resulted either in missense or nonsense mutations (Table 2). Two of the alleles, okr^AA and okr^AC, appear to be molecular nulls. In okr^AA, the ninth codon is mutated to a stop codon, thus truncating the protein after the eighth amino acid, and in okr^AC, the initial methionine is mutated to an isoleucine. The other five alleles were found to contain lesions in regions that are conserved among all members of the Snf2 DNA helicase family of proteins (Fig. 3C).

The spnB locus was cloned from an existing genomic walk in the 88B region (see Materials and Methods, Fig. 4, and legend). The spnB mutant phenotype was rescued by a 5.9-kb genomic fragment that was found to hybridize to at least four transcripts (Fig. 4B). Smaller rescue constructs specific for the 550-nucleotide and 1.35-kb RNAs were tested, and it was found that only constructs containing the complete 1.35-kb transcription unit were able to rescue spnB mutants. Multiple cDNAs were isolated corresponding to the 550-nucleotide and the 1.35-kb transcripts, and cDNAs for each gene, as well as the entire genomic region were sequenced (see Materials and Methods). In situ hybridization to wild-type ovaries with both cDNAs revealed that, like okr, the genes are uniformly expressed throughout oogenesis (data not shown). The spnB cDNA was found to encode a protein of 341 amino acids with an apparent molecular weight of 38
characterized genes, XbaI sites indicated. Three previously characterized genes, Rbp9, Rrp1, and γTub23C are shown as open boxes with arrows indicating the direction of transcription. (Hatched boxes) Unmapped ovarian transcripts defined by Northern analysis of poly(A)+ RNA using corresponding genomic fragments. Approximate sizes are shown below each box. The rescue construct, pRRa54E4.7w+, which rescues the okr mutant phenotype, is shown beneath the 2.7-kb transcription unit which it includes. (B) Restriction map of the 4.7-kb EcoRI fragment included in the rescue construct shown in A. The structure of the okr gene was derived from the existing sequences for DmRad54 (Kooistra et al. 1997), and our genomic and cDNA sequences (see Materials and Methods). The 4.3-kb cDNA was identified by Northern analysis by use of either the entire rescue fragment or the okr cDNA, and the overlap was verified by sequencing the 3' ends of the EST corresponding to this gene. (C) Protein structure of Okr. (Light shading) Six domains of homology shared with other members of the Snf2 family of DNA helicases (Bork and Koonin 1993). (Black bars) Positions of mutation in the alleles indicated (Table 2).

Table 2. Molecular lesions associated with alleles of okr and spnB

| Allele   | Residue | Alteration   |
|----------|---------|--------------|
| okrAA    | 9       | Q → ochre    |
| okrAB    | 325     | S → F        |
| okrAG    | 1       | M → I        |
| okrAK    | 619     | C → opal     |
| okrAO    | 601     | A → V        |
| okrAU    | 391     | Q → amber    |
| okrAS    | 579     | G → D        |
| spnB<sub>56</sub> | 113 | G → R |
| spnB<sub>153</sub> | 102 | G → R |
| spnB<sub>C</sub> | 234 | R → opal |
| spnB<sub>BU</sub> | 107 | G → E |
| spnB<sub>CN</sub> | 107 | G → E |

Effects on mitotic and meiotic DSB repair

In yeast, components of the RAD52 epistasis group are required for the recombinational repair of DSBs in both mitotic and meiotic cells. In mitotic cells, mutations in these genes interfere with the cell's ability to repair...
DNA damage, whereas in meiotic cells, they block genetic recombination resulting from the failure to repair DSBs associated with crossing over (Resnick 1987; Petes et al. 1991). In light of the homology of okr and spnB to genes in this epistasis group, we have determined whether mutations in okr, spnB, and spnD affect mitotic and meiotic DSB repair. To look for a requirement in mitotic DSB repair, we have tested various mutant genotypes for sensitivity to DNA damage. To look for a requirement in meiotic DSB repair, we have tested mutant genotypes for a reduction in meiotic exchange.

To test for sensitivity to DNA damage, crosses producing okr, spnB, and spnD mutant larvae were fed a solution of 0.08% methylmethanesulfonate (MMS), a chemical mutagen that induces DSBs. The survival of MMS-treated larvae was compared with that of mutant larvae from an untreated control cross (Fig. 5). We find that okr mutants are sensitive to MMS, showing a significant reduction in survival in MMS-treated crosses relative to control crosses. spnB and spnD, on the other hand, are not sensitive, showing equal percentages of expected progeny in both crosses. The MMS sensitivity of Dmrad54 mutations has been shown previously (Kooistra et al. 1997), and our data on okr alleles corroborate this finding. The fact that spnB and spnD mutants do not show MMS sensitivity suggests that they may not be required for mitotic DSB repair. To test the effect of okr, spnB, and spnD mutations on meiotic DSB repair, we have measured the effects of

![Figure 4. Molecular characterization of spnB.](image-url)
these mutants on meiotic exchange as reflected in the frequency of recombination and X-chromosome nondisjunction. For these experiments, we took advantage of the fact that females mutant for even the strongest spnB and spnD alleles produce escaper progeny in the first days after mating. In the case of okr, it was not possible to use the strongest alleles because they are almost completely sterile. Instead, we used a weak allele, okrA/O, which is fertile as a homozygote and hemizygote (25% and 50% hatching, respectively). In spnB or spnD mutant females heterozygous for X chromosomal markers, the frequency of recombination is 10%–25% of normal levels, whereas for the weak okr allele the frequency of recombination is at 50% of normal levels (Table 3). In crosses that allowed us to score the exceptional progeny 3. Some of the new mutant lines were used to score the exceptional progeny in two intervals between the markers y–v and v–f was a second event in the recombination is 10%–25% of normal levels (Table 3). In crosses that allowed the sex marker to be scored independently; thus, the y–v column includes all recombinant progeny which is fertile as a homozygote and hemizygote (25% and 50% hatching, respectively). In spnB or spnD mutant females heterozygous for X chromosomal markers, the frequency of recombination is 10%–25% of normal levels, whereas for the weak okr allele the frequency of recombination is at 50% of normal levels (Table 3). In crosses that allowed us to score the exceptional progeny allowed us to score the exceptional progeny allowed us to score the exceptional progeny in two intervals between the markers y–v and v–f was a second event in the recombination is 10%–25% of normal levels (Table 3). In crosses that allowed us to score the exceptional progeny in two intervals between the markers y–v and v–f was a second event in the recombination is 10%–25% of normal levels (Table 3). In crosses that allowed us to score the exceptional progeny.}
siblings. Similarly, in females, normal disjunction gives rise to +/Y (XY) males; nondisjunction gives rise to exceptional progeny that can be distinguished from their phenotypically wild-type siblings.

Progeny were scored from a cross of yvf/Y males to wild type or +/+; spn/spn females. Normal disjunction of the X chromosome in females gives rise to +Y (XY) males; nondisjunction gives rise to exceptional yvf/O (XO) males that can be distinguished from their phenotypically wild-type siblings.

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\text{Percent nondisjunction} = \left( \frac{2 \times [\text{XO males}]}{N} \right) \times 100.
\]

The sum of XO males and XXY females was multiplied by 2 to account for the YO products.

Mutations in okr, spnB, and spnD affect developmental patterning

Mutations in okr, spnB, and spnD cause a number of specific patterning defects. We have shown that many of the observed phenotypes reflect defects in the regulation or expression of grk. grk is affected at two levels: The mRNA is not always properly localized, presumably as a consequence of reduced levels of K10 within the oocyte, and the accumulation of Grk protein itself is reduced. The failure to accumulate wild-type levels of Grk and K10 in okr, spnB, and spnD mutants could reflect a defect in the translation or stability of these proteins. The effects of these mutations on protein accumulation, however, do not appear to be caused by a general defect in the translation or stability of all oocyte-specific proteins, as, for instance, levels of Orb protein do not appear to be altered (A. Ghahrial and T. Schüpbach, unpubl.).

The relationship between the meiotic and developmental phenotypes of okr, spnB, and spnD

We show here that the regulation of meiotic processes occurring within the oocyte nucleus affects accumulation of the nuclear protein K10, and in addition, also affects accumulation of Grk protein within the cyto-

### Table 4. X-chromosome nondisjunction in mutant females

| Maternal genotype | N       | XY | XO | % nondisjunction |
|-------------------|---------|----|----|-----------------|
| Oregon-R          | 11,413  | 11,407 | 6  | 0.1             |
| spnBбу/spnBбу     | 366     | 343 | 23 | 13              |
| spnBбу/spnB153    | 167     | 160 | 7  | 8               |
| spnD349/spnD349   | 395     | 374 | 21 | 11              |

**A. X-chromosome nondisjunction in spB and spD mutant backgrounds**

| Maternal genotype | N       | XY | XX | XO | XXY | % nondisjunction |
|-------------------|---------|----|----|----|-----|-----------------|
| b pr cn bw okrA0/okrA0 | 1,270 | 642 | 615 | 9  | 4   | 0.09            |

**B. X-chromosome nondisjunction in okr mutant backgrounds**

- **Percent nondisjunction** calculated as \( \left( \frac{2 \times [\text{XO males}]}{N} \right) \times 100 \). Only male progeny was counted in this experiment, and the number of XO males was multiplied by 2 to account for the YO products.

- Progeny were scored from a cross of yw/B⁵-Y males to b pr cn bw or okr females. Normal disjunction of the X chromosome in females gives rise to +Y (XY) males; nondisjunction gives rise to exceptional yw/O (XO) males that can be distinguished from their Bar-Stone siblings. Similarly, in females, normal disjunction gives rise to yw/+ (XX) females; non-disjunction gives rise to +/+ (XY) females that can be distinguished from their phenotypically wild-type siblings.

- Percent nondisjunction calculated as \( \left( \frac{2 \times [\text{XO males} + \text{XYY females}]}{N} \right) \times 100 \). The sum of XO males and XXY females was multiplied by 2 to account for YO and XXX products.

- Maternal genotype for loss of okr function. Redundancy might also be present among the RAD51-like genes. For instance, the yeast RAD51 and DMC1 genes appear to have partially overlapping functions in meiotic DSB repair (A. Shinohara et al. 1997).

- Maternal genotype for loss of okr function. Redundancy might also be present among the RAD51-like genes. For instance, the yeast RAD51 and DMC1 genes appear to have partially overlapping functions in meiotic DSB repair (A. Shinohara et al. 1997).
plasm. These findings establish the existence of a connection between the regulation of meiotic progression in the oocyte nucleus, and the regulation of specific patterning processes in the oocyte cytoplasm.

One way of accounting for the patterning defects caused by mutations in okr, spnB, and spnD is that defects in DSB repair would lead to a general disorganization of the oocyte nucleus that would affect the organization of the oocyte as a whole. However, we did not observe general defects in the mutant egg chambers, characteristic of a global misorganization of the cytoskeleton.

Alternatively, a failure to repair DSBs could result in checkpoint-mediated arrest of meiotic progression, which, in turn, would block certain regulated processes in the cytoplasm. In yeast, mutations in the DSB repair genes, RAD51 and DMC1, lead to a checkpoint-mediated arrest in pachytene (Bishop et al. 1992; Lydall et al. 1996; Xu et al. 1997). We have not investigated the nature of the nuclear defects in okr, spnB, and spnD, but, as discussed above, it is possible that these defects could be explained by an early arrest in meiosis. In yeast and multicellular eukaryotes, it is well established that mitotic and meiotic checkpoint proteins, in addition to their effect on genes involved in DNA metabolism, also regulate various cytoplasmic processes such as spindle assembly and nuclear envelope breakdown (for review, see Murray and Hunt 1993). It is therefore possible that in Drosophila the same factors that regulate meiotic cell cycle targets might also be used in parallel to regulate specific developmental targets. Such targets could include proteins that control translation of developmentally important proteins like Grk and K10.

Effectors for this kind of regulation might be found among the genes described above that produce mutant phenotypes similar to those of okr, spnB, and spnD. Such effectors could act downstream of the DSB-repair checkpoint and regulate translation in response to a signal from the oocyte nucleus. Whereas only two of these genes, vasa and spnE, have been cloned, they both encode RNA helicases and are implicated in the translational regulation of grk (Lasko and Ashburner 1988; Gillespie and Berg 1995; González-Reyes et al. 1997; Styhler et al. 1998; Tomancak et al. 1998). Regulation of genes required for the translation of developmentally important proteins, such as Grk, in response to the status of the oocyte nucleus, could serve to coordinate the timing of progression through meiosis with the developmental program. However, because vasa and spnE also produce nuclear defects, the pathway cannot be unidirectional. Thus, information from the cytoplasm (e.g., factors required for chromosome condensation or karyosome formation) contributes to nuclear processes as well.

Meiotic prophase in Drosophila oogenesis occurs over an extended time period during which many different developmental events take place. The pachytene stage of meiotic prophase is believed to be achieved as early as region 2a of the germarium (for review, see Spradling 1993). Although the repair of DSBs in wild-type ovaries presumably occurs during pachytene, nevertheless, we find that most of the events of oogenesis appear to proceed normally in these mutants, and only a few specific processes appear to be severely affected. This suggests that for Drosophila, only a subset of the processes occurring within the oocyte cytoplasm are dependent on normal meiotic progression within the oocyte nucleus. Perhaps the processes that are linked to progression through meiosis are those for which precise temporal regulation is of particular importance. It will be interesting to see whether similar effects will be associated with meiotic mutants in other developmental systems.

**Materials and methods**

Drosophila strains and manipulations

okra alleles Two EMS-induced alleles of okr, okrAB and okrWS, were identified previously (Schüpbach and Wieschaus 1991). We have mapped the gene to 5.6 cM on 2L. The locus is uncovered by Df(2L)S7 (23C1-2; 23E1-2), and falls between the proximal and distal breakpoints of Df(2L)C144 (23A1-2; 23C3-5) and Df(2L)S7 (23C3-5; 23D3), respectively, placing it in 23C. To generate more okr alleles, we performed a standard F3 female sterile mutagenesis. Thirty-eight hundred EMS-mutagenized chromosomes were screened, and five new alleles, okrAA, okrAB, okrAS, okrAS, and okrWS, were isolated on the basis of their failure to complement okrWS.

spnB and spnD alleles Two EMS-induced alleles of spnB, spnB153, and spnB153, were identified previously (Spradling and Hunt 1993). We have not investigated the nature of the nuclear defects in okr, spnB, and spnD, but, as discussed above, it is possible that these defects could be explained by an early arrest in meiosis. In yeast and multicellular eukaryotes, it is well established that mitotic and meiotic checkpoint proteins, in addition to their effect on genes involved in DNA metabolism, also regulate various cytoplasmic processes such as spindle assembly and nuclear envelope breakdown (for review, see Murray and Hunt 1993). It is therefore possible that in Drosophila the same factors that regulate meiotic cell cycle targets might also be used in parallel to regulate specific developmental targets. Such targets could include proteins that control translation of developmentally important proteins like Grk and K10.

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recessive antimorphs. We have not been able to assess the phenotype of spnB556 homoyzgyous because despite repeated outcrossing, we have not been able to recover a vigorous homozygous viable chromosome. spnB153 is unique in showing extraordinary sensitivity to genetic background, making assessment of its phenotype difficult. spnD350 and spnD360 both behave as loss-of-function mutations in genetic tests, however, the fecundity of the homozygous females when compared with the hemzygous females is significantly reduced. All alleles of spnB and spnD are viable in trans to deficiency, thus, these genes are also not essential for viability. Even though many of the alleles test as recessive antimorphs, even the most severe antimorphic alleles do not produce qualitatively different phenotypes than the more straightforward loss-of-function alleles, rather, the antimorphic character merely affects the frequency with which certain phenotypes are observed.

Antibody stainings and in situ hybridizations

Antibody staining in oocytes Immunolocalization of grp protein was performed as described previously (Neuman-Silberberg and Schüpbach 1996). The secondary antibody, biotin-anti-Rat (Vector), was used at a dilution of 1:1000 in a 1 hr incubation at room temperature, and this was followed by a tertiary detection step using Cy3-conjugated Streptavidin (Cy3-SA, Jackson) at a dilution of 1:1000 also for 1 hr at room temperature. The same protocol for fixation and labeling was used for immunolocalization of K10 protein with the Rat-anti-K10 antibody (Cohen and Serano 1995), with the addition of a 1 hr permeabilization step in PBS + 0.3% Triton-X100 (Sigma) prior to blocking. The K10 antibody was used at a dilution of 1:1600. Cell outlines were visualized by staining cortical actin with OregonGreen488- or Rhodamine-conjugated phalloidin (Molecular probes), as per manufacturer’s recommendation. To visualize nuclei, ovaries were incubated in a 1:5000 dilution of OliGreen (Molecular Probes) and 20 µg/ml of RNaseA for 1 hr at room temperature. Fluorescent images were examined with a Bio-Rad MRC 600 confocal microscope.

Antibody staining of embryos Eggs were collected in 4 hr intervals, fixed in 4% paraformaldehyde in PBS for 20 min, and devitellinized in methanol according to standard protocols. Fixed embryos were transferred to a silanized glass slide and cellular blastodermic cells were hand selected under a dissecting microscope. The selected embryos were blocked in PBS +10% BSA +0.1% TWEEN 20 (Sigma), and the primary rabbit-anti-Twist antibody (a gift of S. Roth, Max-Planck Institute for Developmental Biology, Tübingen, Germany) was used at a dilution of 1:2000 in PBS +1% BSA +0.1% TWEEN 20 in an overnight incubation at 4°C. A secondary antibody, biotin-anti-rabbit (Vector) was used at a dilution of 1:1000 in a 1 hr incubation at room temperature, and this was followed by detection with the horseradish peroxidase-streptavidin (HRP–SA) ABC kit (Vector) according to the supplier’s recommendation.

In situ hybridizations RNA in situ hybridizations on oocytes and embryos were done according to standard procedures (Roth and Schüpbach 1994) with minor modifications. Antisense RNA probes were made by use of linearized cDNA templates with the Genius RNA Labeling Kit (Boehringer) according to the manufacturer’s protocol. Hybridizations were performed at 55°C.

Molecular cloning

okra The locus falls in the region between the proximal breakpoint of Df(2L)C144 and the distal breakpoint of Df(2L)J517 which contains three previously characterized genes, RNA binding protein 9 (Rbp9) (Kim and Baker 1993), Recombination repair protein 1 (Rpr1) (Sander et al. 1991), and a ⋅tubulin isoform (γ Tub23C) (Sunkel et al. 1995). Southern mapping of the deficiencies in the region indicated that the Rbp9 gene spanned the distal breakpoint of Df(2L)C144, and we used this as an entry point to initiate a genomic walk in the region. Phage were isolated from a dp on bw .DASH genomic library (R. Padgett, unpubl.) that spanned the interval between Rbp9 and the distal break of Df(2L)J57. Subclones from this walk were used to probe Northern blots of ovarian poly(A)+ RNA to screen for candidate transcripts in the region. In addition to the transcripts corresponding to Rpr1 and γ Tub23C, four other fragments were found to hybridize to ovarian RNAs. A single copy of the rescue construct pRRaS4E4.7w+, which includes a complete 2.7-kb ovarian transcription unit (see Fig. 3), rescues the okr mutant phenotype, indicating that the 2.7-kb RNA corresponds to the okr gene. A single cDNA corresponding to the 2.7-kb RNA was isolated from a poly(dt) primed ovarian cDNA library (Stroumbakis et al. 1994), and this cDNA and the entire 4.7-kb genomic fragment were sequenced. The other two transcription units in the fragment were identified as expressed sequence tags (ESTs) in the Berkeley Drosophila Genome Project Database, the 4.3-kb transcript corresponding to sequences LD23852 and LD24692, and the 1.4-kb transcript corresponding to GM04879. The cDNA corresponding to the latter EST was sequenced (GenBank accession no. AF069781) and it was found to overlap with the last exon of the 2.7-kb transcript, reading off the opposite strand (see Fig. 4). The GenBank accession number for the okr genomic rescue fragment is: AF069779, the accession number for the partial okr cDNA is AF069780.

spindleB spnB was mapped to the 88B interval on 3R based on its inclusion in Df(3R)redP93 and not in Df(3R)redP52. This region was included in an existing genomic walk in the region (kindly provided by R. Kelley, Baylor College of Medicine, Houston, TX), and the interval between the distal breakpoints of the two deficiencies included 12 kb of DNA that was contained in a single cosmid (cos144) of this walk. Subclones of this cosmid were used to probe Northern blots of ovarian poly(A)+ RNA to identify candidate transcripts in the region. Four transcripts were identified, and subclones including each of these transcripts were cloned into Casper4 to generate rescue constructs, pRR144Xb14w+, pR1444N5.9w+, pR1445E5.9w+, and pR5E9BE3.5w+ (see Fig. 7). Of these, only the last two rescue the spnB mutant phenotype, indicating that the 1.35-kb transcript corresponded to the spnB gene. Multiple cDNAs corresponding to the 550-nucleotide (GenBank accession no. AF069530) and 1.35-kb transcripts were isolated from a poly(dt) primed ovarian cDNA library (Stroumbakis et al. 1994), and two cDNAs for each gene, and the entire genomic region, were sequenced. The GenBank accession number for the spnB cDNA is AF069531.

Sequencing of mutant alleles Genomic DNA was prepared from flies of the genotypes okr*/okr or spnB+/Df(3R)trxE12 according to standard procedures (Sambrook et al. 1989). By use of primers flanking the coding region of the gene in question, the mutaion locus was amplified by PCR with the KlenTaq high fidelity polymerase (Clonetech) by the two-step cycle program recommended by the manufacturer. The PCR product was verified by gel electrophoresis, purified by Wizard PCR Prep (Promega), and sequenced with a Perkin-Elmer ABI Prism 377 DNA Sequencer. Sequences were assembled with the AssemblyLign
program (Kodak/IBI) and compared with the wild-type genomic sequence by use of the MacVector (Kodak/IBI) or Genetics Computer Group (Devereux et al. 1984) programs. In all cases, a single unique nucleotide change was found to be associated with each mutant chromosome.

Tests of DSB repair in mitosis and meiosis

MMS sensitivity To test for a requirement in mitotic double-strand break repair, okr, spnB, and spnD homozygotes were exposed during larval development to the mutagen MMS (Sigma). Crosses of appropriate genotypes were made in pairs, one of each pair to be treated with the mutagen, and the other to serve as a control. For okr, okrA/+; spnD/cyO females, and the crosses were treated as described above. To determine the percent of expected progeny, the number of spnB*; spnD153 progeny was compared with the number of spnB*/ry females treated as described above. The percent of expected was determined as the number of spnD153/ry females divided by the number of spnD+/TM3, 150/ry progeny.

Recombination frequency To determine the recombination frequency in spnB mutants, females of the genotypes yvf/++, spnB153/+ or yvf/++; spnB153 were mated to yvf/Y males and the progeny were scored for recombination events in the y-v and f-l intervals independently. A cross of yvf/+++ females by yvf/Y males was used as a control. For okr; the recombination frequency was determined in females of the genotypes yvf/++; okrA/+ or yvf/++; okrA and yvf/++; okrA or yvf/++; okrA. These females were mated to yvf/Y males and the progeny scored for recombination events in the w-f interval. A cross of yvf/++; okrA/+ females by yvf/Y males was used as a control.

Nondisjunction To assess the frequency of nondisjunction in spnB mutant females, y v f/Y males were crossed to +/++; spnB153/+ or +/++; spnB153 females, and male progeny were scored for exceptional XO males of the genotype yvf/O that can be distinguished from their +/+Y brothers. For okr, yw/B-Y males were crossed to +/okrA/+ or +/okrA females, and both male and female progeny were scored. Exceptional X0 male progeny of the genotype yw/o can be distinguished from their +/+Y brothers, and exceptional XXY female progeny of the genotype +/+B-Y can be distinguished from their yw/+ sisters.

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