l(3)malignant brain tumor and Three Novel Genes Are Required for Drosophila Germ-Cell Formation

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ABSTRACT

To identify genes involved in the process of germ-cell formation in Drosophila, a maternal-effect screen using the FLP/FRT-*ovo*^{D} method was performed on chromosome 3R. In addition to expected mutations in the germ-cell determinant *oskar* and in other genes known to be involved in the process, several novel mutations caused defects in germ-cell formation. Mutations in any of three genes [*l(3)malignant brain tumor*, *shackleton*, and *out of sync*] affect the synchronous mitotic divisions and nuclear migration of the early embryo. The defects in nuclear migration or mitotic synchrony result in a reduction in germ-cell formation. Mutations in another gene identified in this screen, *bebra*, do not cause mitotic defects, but appear to act upstream of the localization of *oskar*. Analysis of our mutants demonstrates that two unique and independent processes must occur to form germ cells—germ-plasm formation and nuclear division/migration.

CELL type specification is a crucial process in the development of multicellular organisms. In many organisms, one of the first cell types specified in the developing embryo are the primordial germ cells (PGCs). These cells eventually develop as sperm and egg. One key step in the specification of PGCs in Drosophila is the establishment of a specialized cytoplasm at the posterior of the egg. This cytoplasm, called the pole plasm or germ plasm, consists of maternally supplied RNAs and proteins that are deposited and localized during oogenesis. A crucial component of the establishment of germ plasm is *oskar*—the *osk* mRNA is localized to the posterior and translated there, and OSK protein organizes and recruits the remaining germ-plasm components (Ephrussi *et al.* 1991; Kim-Ha *et al.* 1991). *osk* is both necessary and sufficient for germ-plasm assembly, as mislocalization of *osk* to the anterior of the embryo leads to ectopic germ-cell formation there (Ephrussi and Lehmann 1992). The germ plasm also has a second function—abdomen formation—which is accomplished through the recruitment of *nanos* (*nos*) RNA to the posterior by OSK.

PGCs form prior to any of the somatic cells of the embryo. In the early Drosophila embryo, the male and female pronuclei fuse and then undergo 13 rounds of synchronous mitoses without cell division to produce a syncytium (Zalokar and Erk 1976; Foe and Alberts 1983). Only after the fourteenth cycle does somatic cellularization occur. Germ cells, on the other hand, are formed at an earlier stage. During cycles 6–8, the dividing nuclei undergo an actin and myosin-dependent axial expansion in which they spread out internally along the A-P axis (Zalokar and Erk 1976; Hatanaka and Okada 1991; Wheatley *et al.* 1995; Royou *et al.* 2002). During cycles 8 and 9, the nuclei migrate in a microtubule-dependent process to the cortex of the syncytial embryo (Zalokar and Erk 1976; Baker *et al.* 1993). At the posterior, pole buds, the precursors to cellularized germ cells, are forming. At cycle 10, the posterior-most nuclei, along with the germ plasm surrounding them, are incorporated into the PGCs as the pole buds cellularize. These cells then break from the cycling synchrony of the rest of the nuclei, undergoing two to three additional, asynchronous rounds of cell division. The remaining nuclei continue their migration to the periphery of the embryo, completing the last four rounds of synchronous mitoses until somatic cellularization (Zalokar and Erk 1976; Foe and Alberts 1983).

Prior to the eighth mitotic cycle, the zygotic genome is transcriptionally silent (Pritchard and Schubiger 1996). During this time of transcriptional quiescence (and for some time afterward), the embryo depends on maternally provided proteins and RNAs. For example, the synchronous mitotic divisions are regulated by maternal stores of cyclins A, B, and B3 (Edgar *et al.* 1994; Jacobs *et al.* 1998; Stiffler *et al.* 1999). Also, initial patterning is dependent upon *nanos* and *bicoid* RNAs (among others) from the mother (St. Johnston and Nusslein-Volhard 1992). Those components of the germ plasm that are responsible for germ-cell formation

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are also maternal, especially given that transcriptional silence continues even longer in the newly formed PGCs then in the soma (Van Doren *et al.* 1998; Seydoux and Dunn 1997). Thus the maternal genotype is critical for PGC formation in the embryo.

Genetic screens have contributed much to what we know about how germ cells are formed in Drosophila. However, many of the genes implicated genetically in germ-cell formation were in fact discovered serendipitously as members of the posterior group genes. Genes in this group, *osk* among them, were initially identified on the basis of their posterior patterning defects and not on their defects in germ-cell formation (Nusslein-Volhard *et al.* 1987). These germ-plasm mutations were selected for their defect in the second function of the germ plasm, posterior axis determination in the embryo through the function of *nos*.

Here, we describe a screen to identify defects in germ-cell formation specifically. As gene products involved in this process are expected to be contributed maternally, germ-line clones were made in otherwise heterozygous animals so that potentially lethal mutations could be screened. We used the yeast FLP recombinase and its recognition site (FRT) in combination with the dominant female sterile mutation ovo^D (Chou and Perrimon 1996) to make germ-line clones of EMS mutations on the right arm of chromosome 3. Several mutants that affect germ-cell formation were identified. One gene is involved in the formation of the germ plasm, while three other genes are required for nuclear migration and cycling.

MATERIALS AND METHODS

Fly stocks: The following alleles were identified in the screen and used for phenotypic analysis: *osk^{GM52}*, *l(3)mbt^{GM76}*, *l(3)mbt^{GM79}*, *l(3)mbt^{GM161}*, *shkl^{GM45}*, *shkl^{GM130}*, *shkl^{GM163}*, *oosy^{GM73}*, *oosy^{GM47}*, *beb^{GM29}*, and *beb^{GM50}*. *Df(3R)mbt^{PE3}* (Wismar *et al.* 1995), *l(3)mbt^{E2}* (Löffler 1988), and *l(3)mbt^{ts1}* (Gateff 1978) were obtained from Jasmine Wismar. Transgenic flies used to create starting stocks for the screen, *osk^{166}* and *Df(3R)D605*, were obtained from the Bloomington Stock Center or were present in the laboratory. All flies were raised at 25° unless otherwise indicated.

Mutagenesis: See Figure 1 for a schematic of the screen. A recently isogenized line containing FRT sequences near the centromere on chromosome 3R (82B) with *e* as a marker and the *fat-facets-lacZ* (*P{faf-lacZ}*) transgene (Fischer-Vize *et al.* 1992) on the X was used for the mutagenesis strain. This line was selected for its low frequency of germ-cell migration loss. A total of 8550 *w P{faf-lacZ}; P{FRT}82B e* males were mutagenized with 25–35 mM EMS (Sigma, St. Louis) in 1% sucrose for 16–24 hr as described (Ashburner 1989a) with the modification that they were starved on a water-soaked Kimwipe for 6 hr prior to mutagenesis. These males were mated to ~8600 virgin females of the genotype *w P{faf-lacZ}; Pr Dr/TM3 Sb P{hs:hid}*. The crosses were kept at room temperature (22°) and the males were discarded after 5 days to avoid clonal mutations. Approximately 21,600 single virgin females from the F_1 generation with the genotype *w P{faf-lacZ}; P{FRT}82B e/TM3 Sb P{hs:hid}* were each mated to two or three males of the genotype *y w P{hs:flp}/Y ; P{FRT}82B P{ovo^D}/TM3 Sb P{hs:hid}*. The *hs:flp* transgene (*P{hs:flp}*) allows for expression of the yeast FLP recombinase upon heat induction (Golic and Lindquist 1989; Golic 1991), while the *ovo^D* (*P{ovo^D}*) transgene is a dominant female-sterile mutation that allows for positive selection of female germ-line clones (Chou and Perrimon 1996). These crosses were allowed to lay eggs for 4 days, when the parents were discarded. The vials were subjected to heat shock on day 6 by placing the vials in a 37° water bath for 2 hr. Heat-shock induction of the FLP recombinase produced mutant clones in the otherwise heterozygous flies, while all flies containing the TM3 balancer were killed by virtue of the *hs:hid* transgene (*P{hs:hid}*) (Grether *et al.* 1995; Moore *et al.* 1998). The F_2 progeny were transferred to a vial containing yeast for 2–3 days. Embryos from these crosses were collected and stained for β-galactosidase activity from the germ-line-specific *faf-lacZ* transgene as described (Moore *et al.* 1998). Balanced lines were established from the F_2 males.

Complementation testing, mapping, and allele sequencing: Mutant lines were tested for complementation by anti-Vasa immunostaining of embryos laid by *trans*-heterozygous females. Once complementation groups were established, representative lines were complementation tested against relevant deletion strains from the deficiency kit maintained by the Bloomington Stock Center or obtained from other laboratories. Candidate transposon insertions and mutant alleles were complementation tested as well. All deficiency and mutant lines used complemented the *shkl*, *oosy*, and *beb* alleles. For mapping, one allele of each complementation group was crossed to a *ru st cu sr e+ ca* mapping strain. *Trans*-heterozygous *P{FRT}82B e* allele^1/*ru h st cu sr e+ ca* females were crossed with *ru h st cu sr Pr e ca/TM6* males. Single males carrying the recombinant chromosome ("Rec") *in trans* to *ru h st cu sr Pr e ca* were crossed to *P{faf-lacZ}; P{FRT}82B e* allele^2/*TM3 Sb* females. In the next generation the *P{faf-lacZ}; P{FRT}82B e* allele^2/Rec female progeny was tested for the mutant germ-cell phenotype by staining for β-galactosidase activity from the PGC-specific *faf-lacZ* transgene. The map positions for *shkl*, *oosy*, and *beb* are listed in Table 1.

One complementation group with three members failed to complement deletion lines that uncover *l(3)mbt*. This group proved to be allelic to *l(3)mbt*. DNA from flies *trans*-heterozygous for each allele of *l(3)mbt* and a deletion of the *l(3)mbt* gene [*Df(3R)D605*] was prepared as described (Ashburner 1989b). Multiple independent PCR reactions were used to amplify each exon of the gene prior to pooling for sequencing in a ABI Prism 3700 machine (Rockefeller University DNA Sequencing Resource Center). SeqMan II (DNASTAR, Madison, WI) and EditView (Applied Biosystems, Foster City, CA) were used for analysis of sequencing data.

Whole-mount immunostaining and *in situs*: Embryos were fixed (after dechorionation in 50% bleach for 5 min) by gentle shaking for 20 min in 8 ml heptane, 0.25 ml 37% formaldehyde, 1.75 ml PBS, followed by devitellinization by addition of methanol. Following rehydration, primary antibody staining was carried out in 0.2% Tween in PBS overnight at 4°. Biotinylated secondary antibody (1:2000; Roche, Indianapolis) followed by Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine (DAB) detection were performed as described (Moore *et al.* 1998). Embryos were embedded in PolyBed812 (Polysciences, Niles, IL) and analyzed using a Zeiss Axiophot and a Sony digital camera with Adobe Photoshop software. Antibodies used were anti-Vasa at 1:5000 and anti-phospho-Histone (H3; Upstate Biotechnology, Lake Placid, NY) at 1:1000. For anti-Centrosomin (T. Kaufman) labeling, embryos were fixed by permeabilization in octane for 30 sec followed by fixation in methanol for 1–2 hr. After rehydration and primary antibody incubation (1:1000 over-

TABLE 1

Summary of mutant phenotypes

Genotype[a]	No. of alleles	Map position[b]	No. of PGCs[c]	*osk* localization (%)[d]	*osk-bcd* 3′-UTR; effect on PGCs[e]	Nuclear defect[f]
Wild type		—	27.9 ± 3.3	93	None	None
osk	5	—	0[g]	0	P only	None
l(3)mbt	3	—	9.6 ± 7.4	88	A and P	Synchronous divisions
shkl	3	*e-ca* (96.4)	6.3 ± 4.9	91	A and P	Axial expansion
oosy	2	*sr-e* (64.2)	8.7 ± 6.5	83	A and P	Cortical migration, synchronous divisions
beb	2	*e-ca* (92)	6.0 ± 6.3	31	P only	None

[a] Genotype of females that produce embryos with the described phenotypes.
[b] Only the interval and approximate map position for genes identified in this screen are given.
[c] Primordial germ cells (PGCs) were counted in embryos stained with an anti-VASA antibody at multiple embryonic stages (stages 5–13). Standard deviation (SD) is given; $n > 25$ for each genotype.
[d] The percentage localized number is the percentage of embryos at stages 1–3 that show *osk* RNA localization at the posterior via RNA *in situ*; $n > 40$ for each genotype.
[e] A, anterior; P, posterior. The pole(s) where the mutant genotype causes a germ-cell defect is indicated.
[f] Major nuclear defect is indicated.
[g] Since our screen identified mutations on the basis of the germ-cell phenotype we were able to identify novel alleles of *oskar* with a reduced number of germ cells.

night at 4°), a Cy3-conjugated secondary (Jackson ImmunoResearch Laboratories, West Grove, PA) was used at 1:500. DNA was stained with OliGreen (Molecular Probes, Eugene, OR) at 1:5000 with the addition of 5 μg/ml RNase A. Embryos were mounted in 50% glycerol, 2.5% DABCO, and PBS and analyzed using a Leica TCS/NT confocal microscope.

Whole-mount *in situ* hybridizations were performed as described (Lehmann and Tautz 1994). Embryos were hybridized at 55° for ~18 hr. Antisense digoxigenin-labeled RNA probes were synthesized with the Genius kit (Roche). The *oskar* (C. Rongo), *gcm* (T. Jongens), and *pgc* (S. Kobayashi) probes were made from pBluescript plasmids containing the respective cDNAs. The *gcm* probe was made from a pNB40-derived cDNA clone. Embryos were mounted and analyzed as described above for DAB labeling.

RESULTS

Isolation of mutants affecting germ-cell number: To identify genes involved in the formation of PGCs in Drosophila, we undertook a screen for recessive maternal mutations. We used the FLP-FRT/ovo^D system (Chou and Perrimon 1996), in which clones were generated by the yeast FLP recombinase in animals heterozygous for EMS-induced mutations. Homozygous mutant clones generated in the germ line were selected by loss of a dominant female-sterile mutation (ovo^D). Embryos laid by these females were screened for phenotypes caused by mutation or loss of a maternally supplied gene product (see Figure 1 and materials and methods). To identify defects in the germ cells, a transgene expressing a fusion of the Fat-facets protein and β-galactosidase enzyme was used (Fischer-Vize *et al.* 1992). This protein localizes to the germ cells and is an excellent marker for germ cells throughout embryogenesis.

Approximately 10,000 mutagenized lines were screened. Slightly more than one-quarter of these lines (2699) did not lay any eggs, suggesting that the germ-line clones caused oogenesis defects. These lines were further characterized in a separate oogenesis screen (Morris *et al.* 2003). Eggs were collected from the remaining lines (7346) and stained for β-galactosidase activity. Lines that had a change in the number of germ cells were kept and established as stable stocks.

Initially, 166 lines were maintained. These were rescreened using an antibody against the Vasa protein (which is maternally deposited and localized to the germ cells). Many lines either had an inconsistent germ-cell reduction phenotype or had significant patterning/developmental defects beyond the germ-cell phenotype. However, 14 lines had a consistent reduction in germ-cell number with minimal patterning or other defects and were therefore selected as specifically affecting germ-cell formation. These 14 lines were subjected to complementation analysis with each other, with genes on 3R known to affect germ-cell formation, and with the other lines from the screen that showed, as clones, inconsistent phenotypes or pleiotropic effects. Although some lines are homozygous lethal, all *trans*-heterozygous animals are viable. Lethal mutations could often be mapped to a second site via complementation with deficiencies. Alleles in three genes were previously known to affect specifically germ-cell formation and map to chromosome 3R. Five *oskar* alleles and one Tropomyosin I allele were identified. We did not identify new alleles of *barentsz* (Lehmann and Nusslein-Volhard 1986; Erdelyi *et al.* 1995; Van Eeden *et al.* 2001). This suggests that the scale of the screen approached saturation of 3R. Three alleles of a previously identified tumor

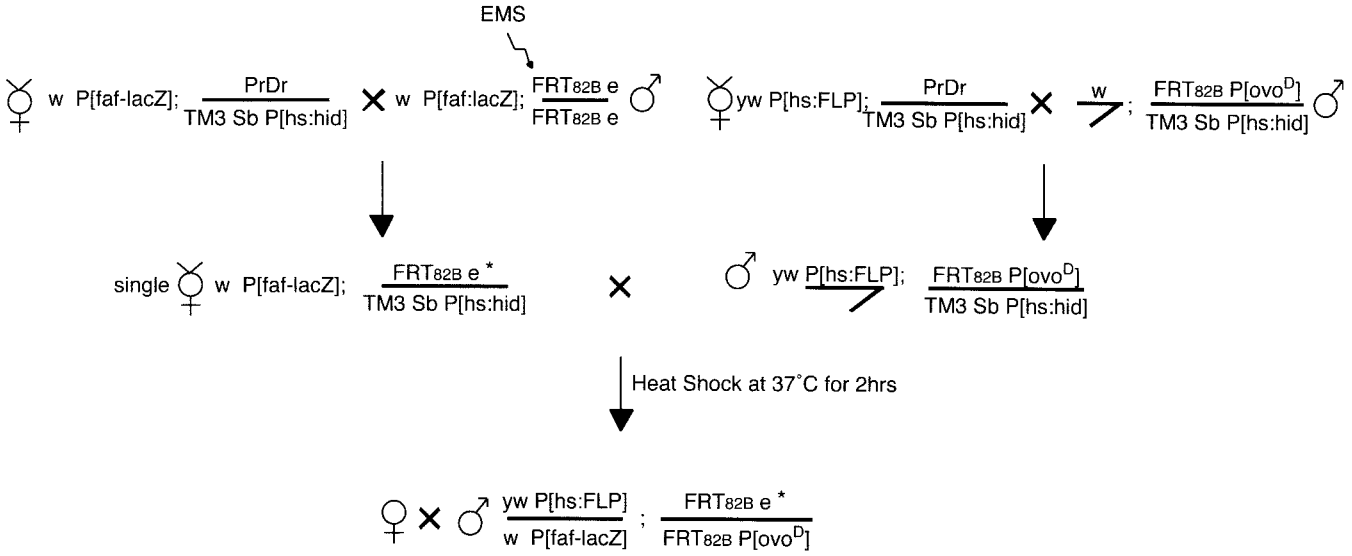

FIGURE 1.—Crossing scheme to establish females heterozygous for a mutagenized third chromosome and the dominant female-sterile mutation *ovo*^{D1}. Both third chromosomes contain FRT sequences near the centromere of the right arm. Heat shock as larvae induces the FLP recombinase and kills flies with the balancer. A faf-lacZ fusion gene is used as a marker for germ line. (For an explanation of stocks used, see MATERIALS AND METHODS). *, the mutagenized chromosome.

suppressor gene, *l(3)malignant brain tumor* [*l(3)mbt*], were shown to have germ-cell formation defects. Additionally, six novel complementation groups were also identified. Three of these were single alleles and are not discussed further. The other complementation groups are *shackleton* (*shkl*) with three alleles and *out of sync* (*oosy*) and *bebra* (*beb*), both with two alleles (see Table 1 for alleles and map position).

The maternal-effect phenotypes of these mutations [*l(3)mbt*, *shkl*, *oosy*, and *beb*] are shown in Figure 2. Embryos laid by females *trans*-heterozygous for two mutant alleles of a given gene were examined. For the remainder of this work, these are referred to as mutant embryos. These mutant embryos were stained with an antibody to the Vasa protein. The number of germ cells was greatly reduced in all four mutants (Table 1). In some embryos, no germ cells were present. This reduction results from failure to initially form germ cells, as opposed to formation and subsequent death, since the defect is visible at early stages (Figure 2, C, E, G, and I) as well as at later stages of embryogenesis (Figure 2, D, F, H, and J). In these four mutants, the number of germ cells formed varied within a collection of embryos, in that a range (from none to almost wild-type numbers) could occur. This is unlike most *osk* alleles, where the lack of germ cells is consistent and penetrant (LEHMANN and NUSSLEIN-VOLHARD 1986). Additionally, many of the mutant embryos from *l(3)mbt*, *shkl*, *oosy*, and *beb* complete embryogenesis, hatch, and produce viable, fertile adults.

Only *bebra* affects germ-plasm components: One of the requirements for germ-cell formation is assembly of the specialized cytoplasm, the germ plasm, at the posterior of the egg. Changing the amount of germ plasm causes a concomitant change in germ-cell number (EPHRUSSI and LEHMANN 1992; SMITH *et al.* 1992). Therefore, the integrity of the germ plasm was investigated by analyzing the localization of known germ-plasm components. Table 1 shows the percentage of mutant embryos that show localized *osk* RNA (from RNA *in situ* hybridization experiments). The *osk* mutants used do not show any localization of *osk* RNA. In *l(3)mbt*, *shkl*, and *oosy* embryos, *osk* RNA is localized similarly to wild-type embryos, as no decrease in *osk* localization is seen. Analysis of other germ-plasm components (*gcl* RNA and *pgc* RNA) showed similar results (data not shown). In *beb* mutant embryos, *osk* RNA localization is reduced, although 31% of embryos still show normal *osk* localization. This defect in *osk* RNA localization is also observed during oogenesis in *beb* mutant females (data not shown). Thus the deficiency in germ-cell formation in *beb* mutant embryos appears to be a result of germ-plasm defects.

***l(3)mbt*, *shkl*, and *oosy* act independently from or downstream of *oskar*:** The integrity of the germ plasm in *l(3)mbt*, *shkl*, and *oosy* mutant embryos suggests that the germ cell defect is downstream of or independent of *osk* function, while *beb*'s defect in *osk* localization could account for the loss of germ-cell formation. The sufficiency of *osk* for assembly of germ plasm and formation of germ cells was shown by localizing *osk* RNA to the anterior of embryos using the *bicoid* (*bcd*) 3′-untranslated region (UTR; EPHRUSSI and LEHMANN 1992). Since the *osk* coding region in this transgene is under different RNA localization and translational control, it can also be used to determine whether other genes in the germ-plasm assembly pathway are genetically upstream of or downstream/parallel to *osk* function.

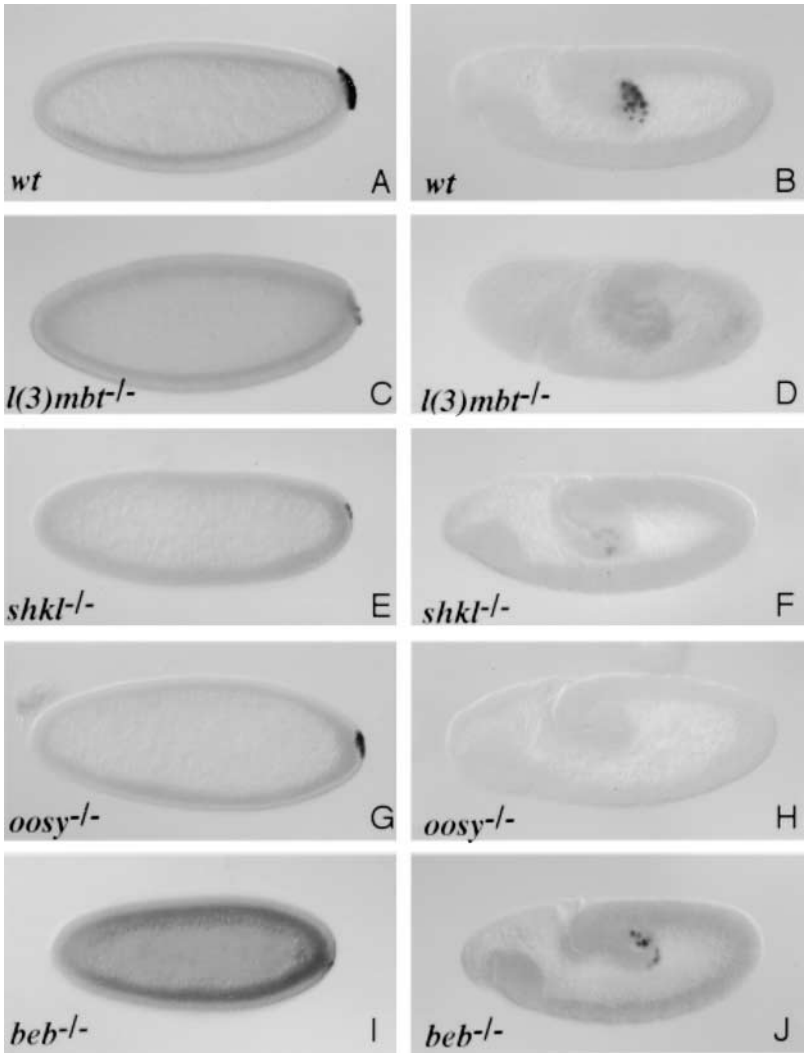

FIGURE 2.—Embryos laid by mutant females fail to form wild-type numbers of germ cells. Anterior is to the left. Germ cells are visualized with an anti-Vasa antibody. (A, C, E, G, and I) Stage 5 embryos. (B, D, F, H, and J) Stage 9–10 embryos. (A and B) wild type; (C and D) $l(3)mbt^{GM79/Df}$; (E and F) $shkl^{GM45/GM163}$; (G and H) $oosy^{GM47/GM73}$; (I and J) $beb^{GM29/GM50}$. The number of germ cells formed is severely reduced (in some cases to zero) in embryos laid from all mutant lines (C–J).

Therefore, the *osk-bcd* 3′-UTR transgene was crossed into the *l(3)mbt*, *shkl*, *oosy*, and *beb* backgrounds. Figure 3A shows the phenotype of the *osk-bcd* 3′-UTR transgene in an otherwise wild-type background. Germ cells form at the posterior due to endogenous *osk* localization, while germ cells at the anterior are a result of ectopic *osk* function. If the gene in question functions upstream of the localization of *osk* RNA, then only the endogenous, posterior germ cells should be affected. If the gene in question functions either downstream of or in parallel with *osk* localization, then both anterior and posterior germ cells should be affected. Mutations in *osk* in conjunction with this transgene (see Figure 3B) demonstrate the independence of the anterior germ cells from *oskar* function (or other upstream components). When the *osk-bcd* 3′-UTR transgene is crossed into either the *l(3)mbt* or *shkl* mutant embryos, germ cells fail to form at both posterior and anterior poles (Figure 3, C and D), suggesting that these genes act downstream of or in parallel with *osk* localization. The *oosy* mutant embryos with an *osk-bcd* 3′-UTR transgene show some reduction in germ-cell formation at both poles as well (Figure 3E). *beb* mutant embryos, on the other hand, still form germ cells at the anterior in the presence of the *osk-bcd* 3′-UTR transgene while failing to form germ cells posteriorly (Figure 3F). Thus *beb* appears to act upstream of *osk* localization.

***l(3)mbt*, *shkl*, and *oosy* cause defects in axial expansion, cortical migration, and nuclear division:** Beyond germ-plasm assembly, another critical step in germ-cell formation is the migration of nuclei into the germ plasm just prior to germ-cell formation. Two processes lead to the accumulation of nuclei at the cortex: during nuclear cycles 6–8 nuclei undergo an expansion along the longitudinal axis of the embryo and during cycles 8–9 nuclei migrate to the cortex. The status of the nuclei was investigated in *osk*, *l(3)mbt*, *shkl*, *oosy*, and *beb* alleles using the DNA marker Oligreen. *osk* mutant embryos fail to form germ plasm and thus neither form germ cells nor develop an abdomen. However, the synchronous nuclear divisions are not disrupted and nuclei at cycles 9–10 still migrate to the posterior of the embryos (Figure 4B), with no apparent difference relative to wild type (Figure 4A). In contrast, the synchronous mitotic divisions of

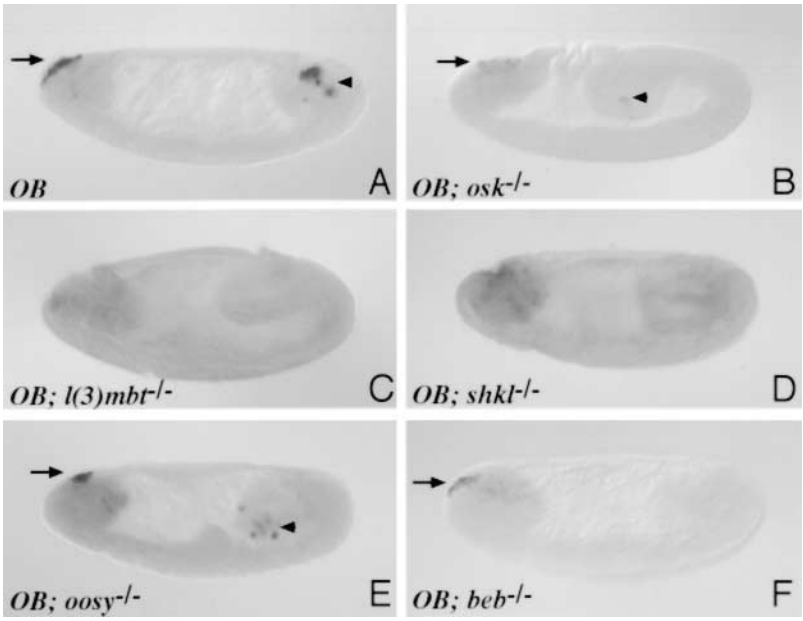

FIGURE 3.—Dependence of maternal germ-cell formation defects on *oskar* RNA localization. Anterior is to the left. Germ cells are visualized with an anti-Vasa antibody. Arrows point to ectopic anterior germ cells where present. Arrowheads point to endogenous posterior germ cells where present. (A–F) Embryos laid by females containing a *osk-bcd 3′ UTR* (OB) transgene, which causes ectopic *osk* RNA localization (and thus germ-cell formation) at the anterior. (A) wild type; (B) $osk^{GM52/166}$, note that the osk^{GM52} allele is weak and produces some germ cells (arrowhead); (C) $l(3)mbt^{GM76/GM79}$; (D) $shkl^{GM45/GM163}$; (E) $oosy^{GM47/GM73}$; (F) $beb^{GM29/GM50}$. In an otherwise wild-type background, the *osk-bcd 3′ UTR* transgene causes germ cells to form at the anterior (A). Mutations that disrupt germ-cell formation at the posterior without affecting the ectopic anterior germ cells include $osk^{-/-}$ and $beb^{-/-}$ (B and F). Mutations in *l(3)mbt*, *shkl*, and *oosy* cause similar germ-cell defects (within a given embryo) for both the endogenous posterior germ cells and the ectopic anterior germ cells (C–E).

the early embryo are disrupted in *l(3)mbt* embryos (Figure 4C). There are nuclei in almost all stages of mitosis within a single embryo at a given time point, a situation that never occurs in wild type. Gaps in the density of nuclei can sometimes be seen (data not shown), which could result from either migration defects or severe mitotic synchrony disruption. Nuclei in *shkl* mutant embryos fail to undergo proper axial expansion (Figures 4D and 5D), and as a consequence nuclei fail to reach the posterior of the embryo. *oosy* mutant embryos have a more extensive nuclear phenotype, in that both axial expansion and nuclear migration to the cortex seem disrupted and the synchrony of the divisions is disturbed (Figure 4E). There is no obvious defect in axial expansion, cortical migration, or synchrony of nuclear divisions in *beb* mutant embryos (Figure 4F), consistent with a role for *beb* upstream of *osk* localization rather than one affecting the nuclei.

To further understand the nuclear defect, mutant embryos were stained with an antibody against centrosomin, a component of the mitotic centrosome (LI *et al.* 1998). Additionally, an antibody that recognizes a phosphorylated form of histone H3 was used, which recognizes condensed chromosomes only during mito-

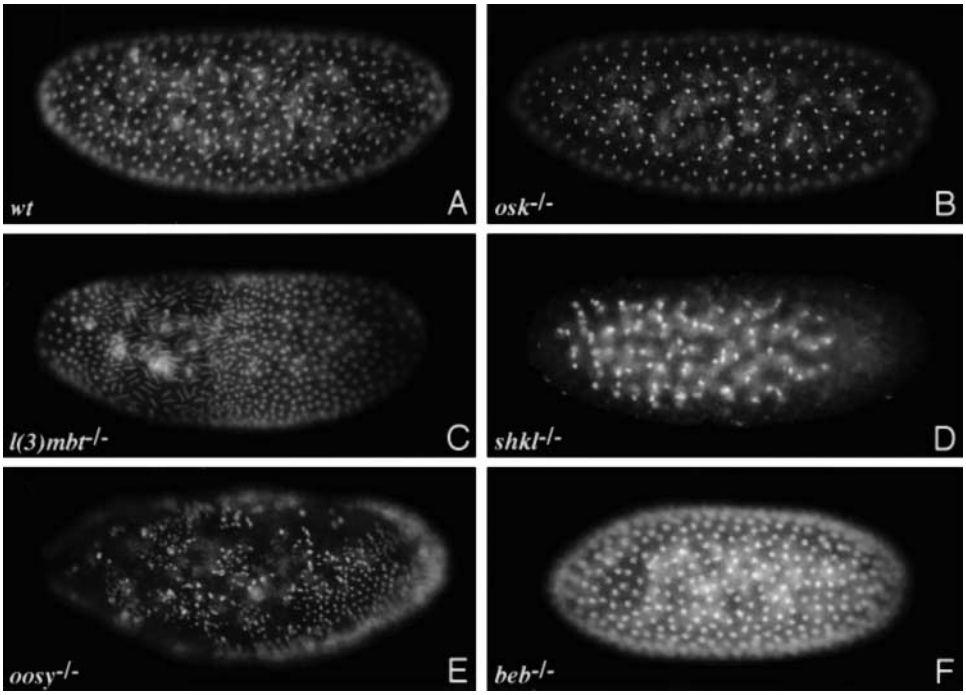

FIGURE 4.—Early nuclear divisions and migration in embryos laid by mutant females. Anterior is to the left. Nuclei were visualized with OliGreen. (A) wild type; (B) $osk^{-/-}$; (C) $l(3)mbt^{GM79/Df}$; (D) $shkl^{GM45/GM163}$; (E) $oosy^{GM47/GM73}$; (F) $beb^{GM29/GM50}$. There are no obvious nuclear division or migration defects caused by $osk^{-/-}$ or $beb^{-/-}$ mutations (B and F). Mutations in *l(3)mbt* disrupt the synchrony of the mitotic divisions (C). Nuclei fail to migrate to the termini, particularly the posterior, in $shkl^{-/-}$ mutants (D). *oosy* mutations cause mitotic synchrony and migration defects (E).

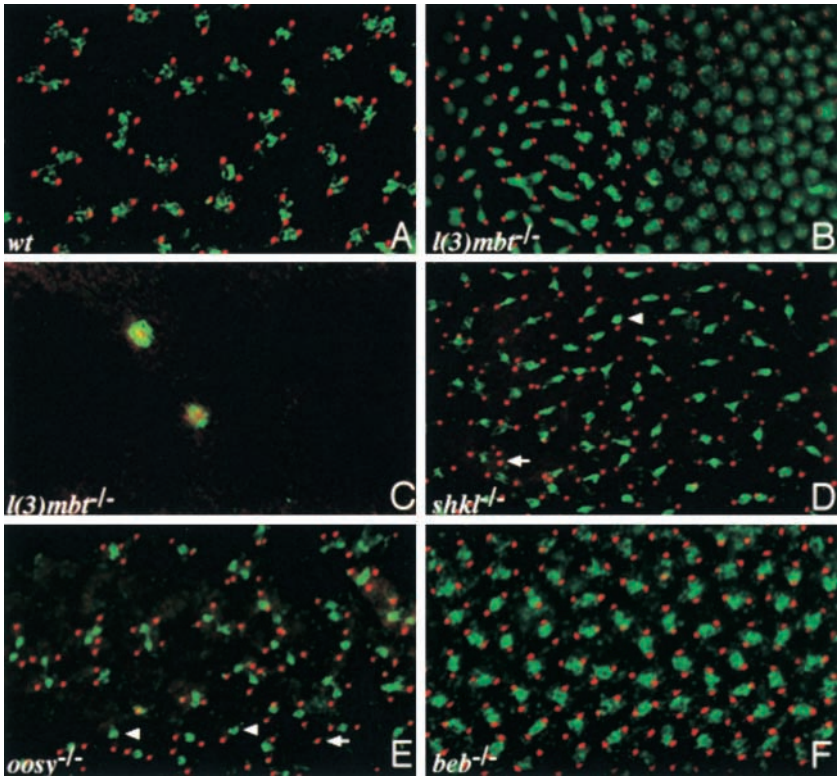

FIGURE 5.—Centrosomes in embryos laid by mutant females. DNA was visualized with OliGreen (green) while centrosomes were visualized with an anti-Centrosomin antibody (red). (A) wild type; (B) $l(3)mbt^{GM79/Df}$; (C) $l(3)mbt^{E2/Df}$; (D) $shkl^{GM45/GM163}$; (E) $oosy^{GM47/GM73}$; (F) $beb^{GM29/GM50}$. Free centrosomes without associated chromosomes are indicated by arrows, while acentrosomal DNA is indicated by arrowheads. The asynchrony caused by mutations in $l(3)mbt^{GM79}$ can be seen in B, but no centrosomal defects are evident. Only two nuclei are present in this $l(3)mbt^{E2}$ mutant embryo where mitotic divisions have stopped (C). Mutations in *shkl* sometimes cause centrosome loss, but mitotic synchrony appears undisturbed (D). Many centrosomal defects and gaps in nuclear density appear in *oosy* mutants (E). There are no obvious centrosomal defects in *beb* mutants (F).

sis (HENDZEL *et al.* 1997). As seen with DNA staining, *beb* mutant embryos show no defect in centrosomes (Figure 5F) or chromatin condensation (Figure 6E). Nuclei in *l(3)mbt* mutant embryos seem to have normal mitotic spindles, despite their disrupted mitotic synchrony (Figure 5B). Staining with anti-phospho-Histone H3 starkly reveals the asynchrony of mitotic divisions in *l(3)mbt* mutant embryos, as domains of nuclei with condensed

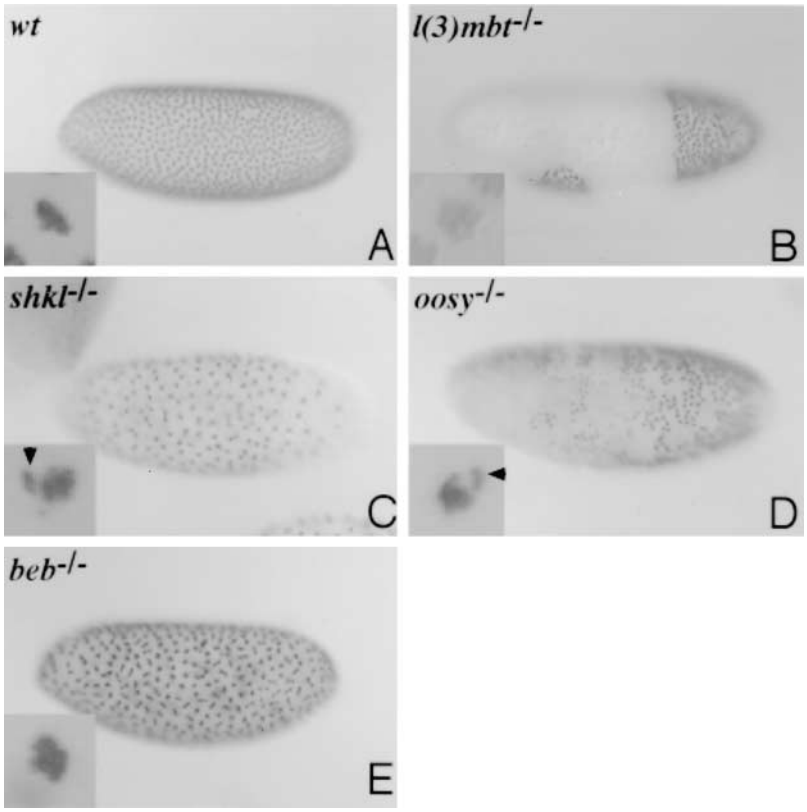

FIGURE 6.—Condensed chromatin in embryos laid by mutant females. Anterior is to the left. Chromatin was visualized with an anti-phospho-Histone antibody. (A) wild type; (B) $l(3)mbt^{GM79/Df}$; (C) $shkl^{GM45/GM163}$; (D) $oosy^{GM47/GM73}$; (E) $beb^{GM29/GM50}$. Insets show a close-up of an individual nucleus. The mitotic asynchrony seen in *l(3)mbt* mutants is evident by the patchiness of phospho-Histone staining. No consistent pattern of the domains of nuclei containing condensed chromatin is present in a collection of *l(3)mbt* mutant embryos. Individual nuclei appear normal (B). The pattern of nuclei with condensed chromatin in *shkl* mutants is consistent with a failure of nuclei to migrate to the posterior. Occasional chromosome loss is seen (arrowhead; C). Mutations in *oosy* cause frequent chromosome loss (arrowhead), asynchronous divisions, and defects in nuclear migration (D). There are no obvious chromosomal defects in *beb* mutants (E).

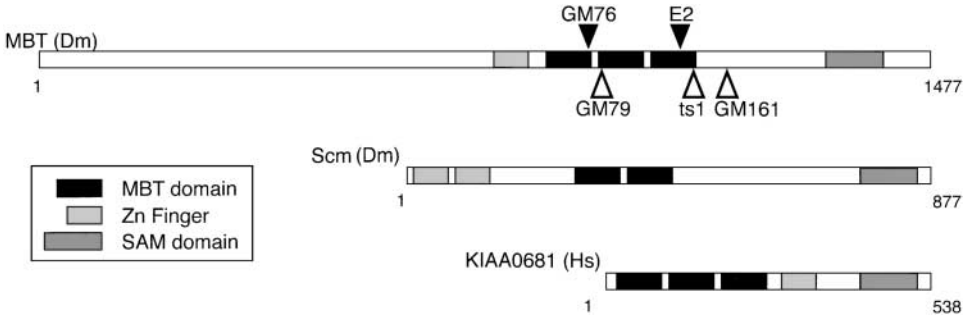

FIGURE 7.—Schematics of the Drosophila MBT protein and two related proteins from Drosophila (Scm) and human (KIAA0681). The MBT-repeat domains, putative zinc-fingers, and SAM domains are indicated as shaded blocks. Mutations present in the Drosophila *l(3)mbt* alleles are indicated by arrowheads (solid arrowheads for nonsense mutations, open arrowheads for missense mutations). Mutations cause amino acid changes as follows: GM76 (W928STOP), GM79 (M964K), E2 (W1099STOP), ts1 (P1130S), and GM161 (H1161Y). Percentage identity between domains is as follows (compared to the corresponding domain from MBT): Scm MBT1, 41%; Scm MBT2, 36%; Scm SAM, 30%; KIAA0681 MBT1, 55%; KIAA0681 MBT2, 45%; KIAA0681 MBT3, 49%; and KIAA0681 SAM, 29%.

chromatin are seen (Figure 6B). The pattern and size of these regions are not consistent from embryo to embryo, suggesting a stochastic nature for the asynchrony. However, the fact that phospho-Histone (and therefore condensed chromatin) is present in patches or domains suggests that some mechanism of synchronization is still present in these mutant embryos. Despite the asynchrony, the chromatin in individual nuclei appears normal (Figure 6B, inset). The *l(3)mbt*^{E2} allele shows a stronger phenotype (Figure 5C) in which the nuclei appear to stop dividing and most embryos have only 2–16 nuclei. *shkl* mutant embryos, beyond the retarded axial migration, show occasional loss of centrosomes (Figure 5D, arrows), and occasional chromosome loss (Figure 6C, inset). The abnormal nuclei seen in *oosy* mutant embryos are often dissociated from their centrosomes (Figure 5E) and show frequent chromosome loss (Figure 6D). The nuclear perturbations seen in *l(3)mbt*, *shkl*, and *oosy* embryos, coupled with their germ-cell formation defects, suggest that any disruption to the synchronous divisions and migration of the nuclei in the early embryo can have adverse effects on germ-cell formation.

***l(3)mbt* alleles define functional domains and reveal a temperature-sensitive process:** *l(3)mbt* was originally identified on the basis of a temperature-sensitive phenotype of malignant overgrowth of larval brain cells (GATEFF *et al.* 1993). Sequencing of genomic DNA from the *l(3)mbt* alleles identified the molecular lesions in both the alleles from this screen as well as those previously identified. Three of the alleles are missense mutations, while the other two are nonsense mutations (see Figure 7). The protein produced from *l(3)mbt* features several conserved domains (WISMAR *et al.* 1995) and shows homology with proteins in vertebrates (KOGA *et al.* 1999; USUI *et al.* 2000; WISMAR 2001; Figure 7). *Sex comb on midleg* (*Scm*), a member of the Polycomb group (PcG) of genes, is the closest Drosophila homolog of *l(3)mbt* (BORNEMANN *et al.* 1996). These proteins share putative Cys_2-Cys_2 zinc fingers whose spacing and sequence conservation define a unique subclass of zinc fingers. They also share malignant brain tumor (MBT) repeats, 74-amino-acid domains with 33–41% identity between the two proteins. The mutations in *l(3)mbt*^{GM79} and *l(3)mbt*^{ts1} alter conserved residues within the MBT domains, demonstrating the functional relevance of the MBT domains. Finally, there is a *s*terile *a*lpha *m*otif (SAM) domain (also called SPM) with 30% identity between MBT and Scm (Figure 7).

In trans to a deficiency that uncovers *l(3)mbt* [*Df(3R)D605* or *Df(3R)mbt*^{PE3}], the alleles form a phenotypic series (GM161 < ts1 < GM79 < E2 < GM76) that ranges from a weak version of the maternal-effect mitotic asynchrony described here to a more severe mitotic block (Figure 5C) to oogenesis defects in *trans*-heterozygous females (data not shown). Additionally, each of these phenotypes is temperature sensitive, in that any allele can be shifted up or down the phenotypic series on the basis of the temperature at which the flies are raised (Figure 8). At 18°, the GM161 and ts1 missense alleles have little or no embryonic defect, while the GM79 missense allele shows slight mitotic asynchrony. The E2 nonsense allele, which causes a mitotic stop at 25° (Figure 5C), completes mitotic cycles at 18°, although they still show significant asynchrony. Females carrying the GM76 nonsense allele at 18° lay embryos with only a few nuclei. These flies do not lay any eggs when raised at 25°. None of the *l(3)mbt* alleles produce

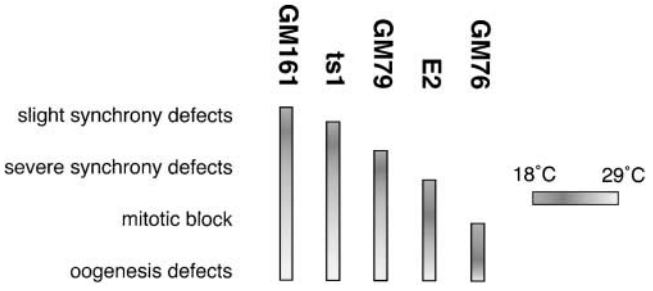

FIGURE 8.—Temperature sensitivity of *l(3)mbt* alleles. Alleles are listed across the top and phenotypic descriptions are listed at the left. The bars represent the predominant phenotype at a given temperature, tested at 18° (black), 25° (gray), and 29° (white).

eggs if the flies are raised at 29°, as all have oogenesis defects (data not shown). The larval tumorigenic phenotype originally described for the *ts1* allele at the restrictive temperature can also be seen in other allelic combinations (data not shown) with a temperature sensitivity profile similar to that described for *l(3)mbt*^{ts1} (Gateff *et al.* 1993). The fact that all of the *l(3)mbt* alleles show temperature sensitivity indicates that *mbt* mutations somehow render an underlying cellular process temperature sensitive rather than altering each mutant protein to temperature sensitivity.

DISCUSSION

Germ-cell formation requires two distinct steps: The process of germ-cell formation is essential for the reproductive success of the organism. In many organisms, the germ cells are determined early in embryogenesis; in Drosophila, they are the first cells formed. This process is controlled by germ-line-specific determinants, accumulating in a specialized cytoplasm called germ plasm. In Drosophila, the germ plasm is established during oogenesis, in a process that is dependent upon *oskar*. Failure to form an intact germ plasm, through mutations in the *osk* locus or by other means, results in a failure to form germ cells in the developing embryo (Williamson and Lehmann 1996).

A second process is also required for normal germ-cell formation in Drosophila. The early embryo is a nuclear syncytium, a single large cell that undergoes 13 rounds of synchronous mitoses without cell division. The migration of these nuclei to the periphery of the embryo begins at the eighth mitotic cycle, when some nuclei move toward the posterior and the germ plasm. The nuclei and germ plasm cellularize at the posterior, forming PGCs. Mutants defective in this migration of nuclei to the posterior of the embryo fail to form germ cells (Niki 1984).

These two processes, germ-plasm formation and nuclear migration, while both required for germ-cell formation, are parallel and independent pathways. In mutants defective in axial expansion, nuclei fail to reach the posterior at the appropriate time [*shkl*, reported here and *gs(1)N26* and *gs(1)N441* (Niki and Okada 1981)], the germ plasm is intact, but the germ-plasm components do not remain stable (Iida and Kobayashi 2000). This breakdown occurs after these components would normally be sequestered from cytoplasmic degradation pathways because of germ-cell cellularization. Germ-plasm integrity is not dependent upon the migration of nuclei until after the time when cellularization would have normally occurred. If cellularization fails and the germ plasm is not sequestered in the germ cells, the localized components of the germ plasm either are degraded or become delocalized (Iida and Kobayashi 2000). Additionally, the migration of nuclei to the posterior is not dependent upon proper germ-plasm formation. In *osk* mutant embryos, where germ plasm does not form (Lehmann and Nusslein-Volhard 1986), germ cells do not form. This is not due, however, to any defect in nuclear migration. At mitotic cycle nine, nuclei have moved to the posterior of *osk* embryos in a manner that is indistinguishable from wild-type embryos. *beb* mutant embryos are similar in that the nuclei divide and migrate normally although the germ plasm is compromised. Thus the germ plasm plays no role in axial expansion or cortical migration of the syncytial nuclei and the two processes required for germ-cell formation, namely germ-plasm formation and nuclear migration, act independently in the early embryo.

Germ-plasm components downstream of *oskar*: Mutations that affect the formation of the germ cells have generally been identified on the basis of a phenotype distinct from germ-cell formation, one affecting the second function of germ plasm: abdominal patterning. The only exception are the two *grandchildless(gs)*-like genes (Niki and Okada 1981), but those two mutants affect axial expansion and nuclear migration (as in *shkl*), not germ plasm. Mutations that affect formation of the germ plasm generally also have defects in abdominal patterning as both processes are dependent upon localization of other germ-plasm components through *osk* function. Failure to localize *nos* RNA in an intact germ plasm causes embryonic patterning defects (Wang and Lehmann 1991; Gavis and Lehmann 1992). Those germ-plasm components that are downstream of *oskar* and specific for germ-cell formation were identified through molecular or other nongenetic means. Mitochondrial large rRNA was identified through rescue (by injection of the RNA) of the germ-cell formation defect caused by UV irradiation (Kobayashi and Okada 1989). The *germ-cell-less* (*gcl*) gene was identified serendipitously via its RNA localization pattern (Jongens *et al.* 1992). The screen described here was designed to identify components of the germ plasm that are specific for germ-cell formation. While new alleles of some genes known to be involved in this process were identified, and *beb* mutants appear to affect germ-plasm localization and therefore germ-cell formation, no new germ-cell-specific genes downstream of *osk* function were identified. The screen covered ~20% of the genome (chromosome 3R). This does suggest that the number of germ-cell-specific genes that can be identified using our screening protocol has to be rather small. The pathway leading to PGC formation downstream of *osk* function currently includes the large ribosomal RNA from mitochondria (Iida and Kobayashi 1998) and a novel protein that localizes to the nucleoplasmic surface of the nuclear envelope (GCL; Jongens *et al.* 1994). It seems likely that additional components will be required, so it is somewhat surprising that none were uncovered here. Perhaps many of the RNAs and proteins required for germ-cell formation have additional roles in oogenesis or cellulari-

zation that would prohibit their discovery in this type of screen.

Perturbations in cell cycle or nuclear migration specifically affect germ-cell formation: The three genes identified in this screen [*l(3)mbt*, *shkl*, and *oosy*] affect the axial and cortical nuclear migration or the synchrony of the nuclear division cycles in the early embryo. Our mutant analysis shows that defects in these processes lead to a specific phenotype in germ-cell formation because of the precise temporal and spatial requirements placed on the nuclei in this process. The most prominent defect we observe in embryos from females mutant for *l(3)mbt* is a disruption in the synchronous mitotic cycles of the early embryo. The strongest allelic combinations of *l(3)mbt* alleles show defects in the divisions already at the onset of embryonic development. It is thus likely that nuclear division defects are the primary cause of the subsequent defects in synchrony of nuclear division, nuclear migration, and germ-cell formation in *l(3)mbt* mutants. The primary defect in *shkl* mutants seems to lie in a failure of nuclei to reach the posterior pole during axial expansion. This process has been shown to be actin dependent (Hatanaka and Okada 1991). Interestingly, mutations in *spaghetti squash* (*sqh*), the Drosophila myosin II, also exhibit defects in axial expansions and asymmetry of nuclear division, similar to those observed in *shkl* mutants (Wheatley *et al.* 1995). *oosy* mutant embryos exhibit defects in axial and cortical nuclear migration but also show severe defects in nuclear divisions with centrosome defects and chromosome loss. This suggests that in this mutant, similar to *l(3)mbt*, nuclear migration defects may be the consequence rather than the cause of defects in nuclear division.

l(3)mbt, *oosy*, and *shkl* mutant embryos generally recover from their somatic defects. Somatic cellularization still occurs, and most embryos go on to develop and hatch. When mitotic arrest or nuclear loss is induced in embryos by UV irradiation or DNA injection, a majority of the embryos still cellularize and form a cuticle (Yasuda *et al.* 1991). Despite the unusual plasticity of the early embryo and its ability to recover from dramatic nuclear defects, the ability to form germ cells is restricted to a specific time and place. The sensitivity of germ-cell formation to disruptions in nuclear migration and the early embryonic cell cycle may thus provide an efficient and reliable tool to uncover genes regulating the actin and microtubule network or the cell cycle in the early Drosophila embryo.

Despite the different types of nuclear defects caused by mutations described here, the numbers of germ cells that do form in each mutant line are similar (Table 1). Additionally, these germ cells are fully functional, as most embryos develop into fertile adults. While *l(3)mbt* and *shkl* mutants show little or no chromosomal or centrosomal aberrations (Figures 5, B and D, and 6, B and C), nuclei in *oosy* embryos frequently lose chromosomes or centrosomes (Figures 5E and 6D), but are still able to form germ cells. This is not too surprising since centrosomes alone have been proposed to be able to induce germ-cell formation (Raff and Glover 1989). However, the fact that the germ cells formed in *oosy* embryos are functional in the adult may suggest that the most critical factor in germ-cell formation is the timing and presence of nuclei and centrosomes and that some defects in mitosis may be tolerated.

The cyclins are known to regulate the early mitotic divisions, and perturbations in the levels of CyclinB have been shown to also affect the speed of nuclear migration via its effects on microtubule dynamics (Stiffler *et al.* 1999; Ji *et al.* 2002). Indeed, the phenotypes of embryos from mothers with six copies of a CyclinB transgene show nuclear asynchrony defects similar to those observed in *l(3)mbt, shkl*, and *oosy* mutant embryos (Ji *et al.* 2002). Preliminary experiments in which the level of Cyclin A, B, or B3 was changed (via hemizygous Cyclin mothers or maternal addition of transgenic genomic copies) in a *l(3)mbt* mutant showed no effect on the maternally determined asynchrony defect (data not shown). Additional investigations into the effects of these mutants on Cyclin levels and regulation, as well as potential interactions with Cyclins or the Cyclin regulatory machinery, may elucidate the molecular nature of the perturbations in the synchronous divisions and migration in the early embryo.

***l(3)mbt* as a model for tumorigenesis:** *l(3)mbt* was originally identified as a tumor suppressor (Gateff 1978). The temperature-sensitive allele *ts1* caused malignant overgrowth of the larval brain and imaginal disc overproliferation at the restrictive temperature (Gateff *et al.* 1993). Interestingly, all *l(3)mbt* alleles are temperature sensitive, including the nonsense alleles, which would produce a truncated and potentially unstable protein. This suggests that MBT protein regulates a temperature-sensitive process, rather than temperature sensitivity being specific to any allele. Progression of the cell cycle in yeast is known to be temperature sensitive at several stages, and heat-shock proteins have been shown to regulate Cyclin complexes (Zhu *et al.* 1997). Thus, MBT may target or protect one of these temperature-sensitive stages of the cell cycle.

Scm is the closest Drosophila homolog of MBT (Figure 7; Bornemann *et al.* 1996). Scm is a member of the PcG of genes. Embryos lacking both maternal and zygotic Scm product undergo a homeotic transformation of most segments to the eighth abdominal segment (Breen and Duncan 1986). *l(3)mbt* does not appear to be a PcG gene, as mutant alleles do not cause homeotic transformations. Analysis of the protein domains conserved between Scm and MBT protein may help to reveal the functional properties of MBT. The SAM domain of Scm has been shown to mediate heterotypic and homotypic protein-protein interactions with the *polyhomeotic* (*ph*) gene product, another PcG gene (Peterson

et al. 1997). Analysis of *Scm* mutant alleles with alterations in the MBT repeats suggests that, while these protein domains are not crucial for the PcG protein-protein interaction or targeting of the protein, they are crucial for the protein's biochemical function (Bornemann *et al.* 1998). While the function of MBT domains remains elusive, MBT repeats are found in vertebrate proteins. KIAA0681 is a human protein that contains three MBT repeats (45–55% identical to those in the Drosophila MBT protein), a putative Cys_2-Cys_2 zinc finger, and an SAM domain (29% identical to the SAM domain in Drosophila MBT; Figure 7). This protein associates with chromatin only during mitosis in human cultured cell lines, appearing scattered in interphase nuclei. Upon overexpression of the human *l(3)mbt* homolog in a human glioma cell line, nuclear segregation and cytokinesis are affected and multinucleated cells are formed (Koga *et al.* 1999). This protein could be a *l(3)mbt* ortholog, functioning in vertebrate cell-cycle control as it does in Drosophila. Given *l(3)mbt*'s role as a tumor suppressor in a model organism with powerful genetics, further study may lead to an understanding of the cell cycle's role in tumorigenesis that can be applied to human biology.

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