Identification and Analysis of Mutations in *bob*, *Doa* and Eight New Genes Required for Oocyte Specification and Development in *Drosophila melanogaster*

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ABSTRACT

The Drosophila oocyte develops from a cluster of 16 interconnected cells that derive from a common progenitor. One of these cells, the oocyte, arrests in meiosis. The other cells endoreplicate their DNA and produce mRNAs and proteins that they traffic to the oocyte along a polarized microtubule cytoskeleton shared by the entire cyst. Therefore, Drosophila oogenesis is an attractive system for the study of cell cycle control and cell polarity. We carried out a clonal screen on the right arm of chromosome 3 for female sterile mutations using the *FLP-FRT-ovo*^{D} system to identify new genes required for early oogenesis. We identified alleles of *oo18* RNA *binding protein* (*orb*) and *Darkener of apricot* (*Doa*), which had previously been shown to exhibit oogenesis defects. We also identified several lethal alleles of the male sterile mutant, *bobble* (*bob*). In addition, we identified eight new lethal complementation groups that exhibit early oogenesis phenotypes. We analyzed mutant clones to determine the aspects of oogenesis disrupted by each complementation group. We assayed for the production and development of egg chambers, localization of ORB to and within the oocyte, and proper execution of the nurse cell cycle (endoreplication of DNA) and the oocyte cell cycle (karyosome formation). Here we discuss the identification, mapping, and phenotypic characterization of these new genes: *omelet, soft boiled, hard boiled, poached, fried, over easy, sunny side up,* and *benedict.*

THE development of the germ cells during Drosophila oogenesis poses an unusual challenge to the organism because within a single cyst of 16 cells, two radically different fates must be specified and executed (Figure 1). The 16 germ cells of each egg chamber are produced by four synchronous, incomplete cell divisions of a progenitor cell, the cystoblast (Spradling 1993). After each division, the daughter cells remain connected by apertures in their cell membranes called ring canals. As a result, after the fourth division, two cells will have four ring canals, two will have three ring canals, four will have two ring canals, and eight cells will have one ring canal. With each cell division, a membranous, cytoskeletal-rich organelle called the fusome extends through the newly formed ring canals so that every cell in the cyst contains part of this increasingly branched structure. The fusome is thought to play a role in synchronizing and determining the pattern of the cyst divisions. The fusome is not distributed equally, however; after each division the daughter cell with more ring canals contains more fusome material than the newly formed daughter cell does (de Cuevas and Spradling 1998). It has been posited that this asymmetry influences the different cell fates in the cyst and that the oocyte will develop from the cell that contains the most fusome material. After the final cystoblast division, the germ cells begin to differentiate and the cyst is surrounded by somatic follicle cells. The cells of the cyst establish a shared polarized microtubule cytoskeleton whose minus ends are localized to one of the two cells that share ring canal connections with four neighbors, the pro-oocytes (Theurkauf *et al.* 1993). Oocyte-specific factors, including BICAUDAL-D (BICD), EGALITARIAN (EGL), and ORB then become restricted to the pro-oocytes in a microtubule-dependent process (Suter and Steward 1991; Lantz *et al.* 1994; Mach and Lehmann 1997). At first, the cells with three ring canals, as well as the pro-oocytes, exhibit synaptonemal complex, the protein assemblage that mediates recombination, but synaptonemal complex is soon restricted to the pro-oocytes (Carpenter 1979). At the same time, the centrioles of the cyst migrate along the fusome to the pro-oocytes (Mahowald and Strassheim 1970; Grieder *et al.* 2000). Synaptonemal complex restriction and centriole trafficking are microtubule independent (Huynh and St. Johnston 2000; Bolivar *et al.* 2001). Cell fate determination is completed when one of the pro-oocytes exits meiosis and traffics the oocyte-specific factors it has accumulated to the remaining pro-oocyte. That pro-oocyte will maintain meiotic arrest and develop as an oocyte while the other 15 cells in the cyst will develop as nurse cells.

Once the fates in the cyst have been specified, the

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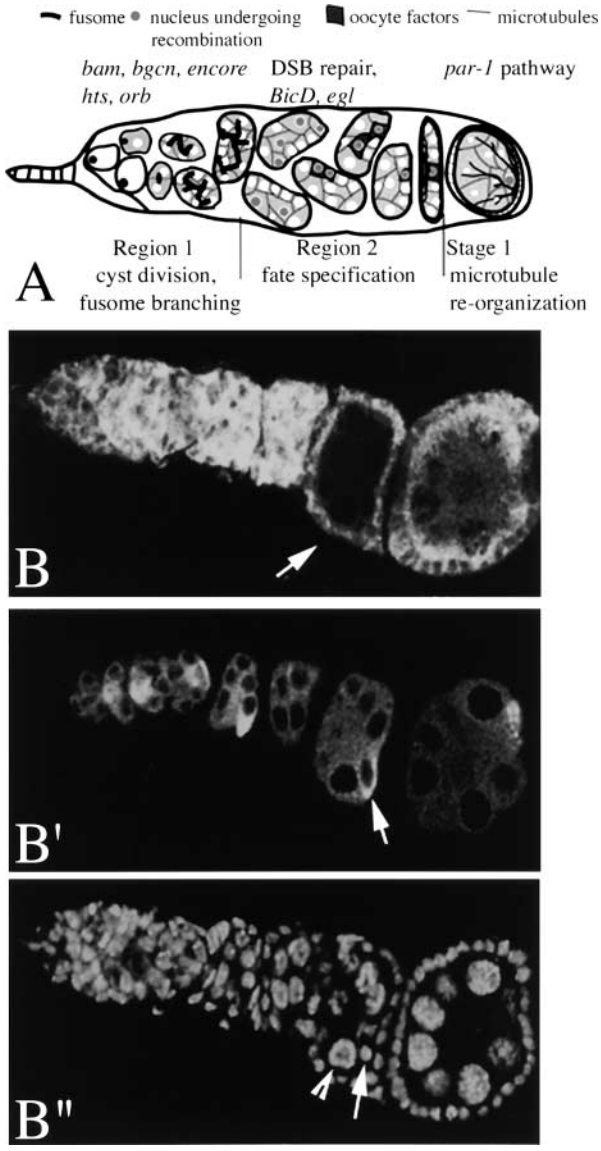

FIGURE 1.—Wild-type oogenesis. Anterior is left. (A) A cartoon of the earliest stages of oogenesis. (B, B′, and B′′) Confocal images of a wild-type ovariole. (B) Anti-β-galactosidase staining permits the identification of β-galactosidase negative germline clones (arrow) surrounded by β-galactosidase-positive follicle cells. (B′) Anti-ORB staining shows that ORB is localized to the posterior of the oocyte (arrow). (B′′) DAPI staining shows the endoreplicated nurse cells (arrowhead) and the condensed oocyte karyosome (arrow).

appropriate developmental programs must be properly carried out. The oocyte condenses its DNA into a compact karyosome and remains arrested in meiotic prophase I until late in oogenesis (stage 10; SPRADLING 1993). Karyosome formation depends on the activity of several genes, including the double-stranded break repair genes *spindle* (*spn*) *B* and *D* and *okra*; the ATP-dependent helicase genes, *vasa* and *spnE*; and genes of unknown function, such as *encore*, *BicD*, and *spnA* (LILLY and SPRADLING 1996; GONZALEZ-REYES *et al.* 1997; GHABRIAL *et al.* 1998; STYHLER *et al.* 1998; TOMANCAK *et al.* 1998; VAN BUSKIRK *et al.* 2000; OH and STEWARD 2001). The oocyte is thought to be nearly transcriptionally silent. The nurse cells meanwhile endoreplicate their DNA approximately once per stage of oogenesis to become highly polyploid cells that transcribe and translate very actively (SPRADLING 1993; DEJ and SPRADLING 1999). Many of the mRNAs and proteins produced by the nurse cells are trafficked to the oocyte along the microtubule network. Many factors trafficked to the oocyte exhibit a dynamic pattern of subcellular localization (JOHNSTONE and LASKO 2001). The centrioles and a number of oocyte-specific proteins, including ORB, are restricted to the anterior of the oocyte by region 2b of the germarium. At stage 1 of oogenesis (region 3 of the germarium), these factors begin relocalizing to the posterior of the oocyte nucleus. By stage 3, the relocalization is complete, and these factors will remain at the posterior of the oocyte until the microtubule cytoskeleton is reorganized during stage 8 (THEURKAUF *et al.* 1992; DE CUEVAS and SPRADLING 1998; HUYNH and ST. JOHNSTON 2000; BOLIVAR *et al.* 2001). Just before oogenesis is completed, the nurse cells dump most of their cytoplasm, including their mitochondria and most other organelles, into the oocyte. The nurse cells then undergo programmed cell death while the oocyte completes its maturation (SPRADLING 1993).

The genes that have been shown to disrupt the development of germ cells into early egg chambers fall into three classes: the "spindle" or "karyosome" class, the 16-nurse-cell class, and the cystoblast division class. The loss-of-function phenotypes of the double-stranded break repair genes and related genes (commonly referred to as the spindle group) suggested that activation of a meiotic checkpoint can delay restriction of the oocyte fate to one of the pro-oocytes (HUYNH and ST. JOHNSTON 2000). Mutants in the spindle class are characterized by a stringy or fragmented karyosome while double mutants in this class permit both pro-oocytes to develop as oocytes that each exhibit defective karyosomes (GONZALEZ-REYES *et al.* 1997; GHABRIAL *et al.* 1998). Genetic evidence suggests that the karyosome phenotypes observed in *vasa* and *encore* mutants are due to roles for these genes downstream or independent of the meiotic checkpoint (GHABRIAL and SCHUPBACH 1999; VAN BUSKIRK *et al.* 2000). Loss-of-function mutations of *BicD*, *egl*, *dynein heavy chain* (*Dhc*), *Dystroglycan*, or genes in the *par-1* pathway, as well as experiments with colchicine, have demonstrated that failure to establish or maintain the polarized microtubule network or microtubule-dependent transport in the cyst results in all 16 cells developing as nurse cells (KOCH and SPITZER 1983; SUTER and STEWARD 1991; MACH and LEHMANN 1997; HUYNH *et al.* 2001; DENG *et al.* 2003). Mutations in a third class of genes often disrupt the regulation of cystoblast divisions and cyst formation in addition to oocyte and nurse cell fate specification. Loss of function of *bag of marbles* (*bam*), *benign gonial neoplasm* (*bgcn*), *hu li tai shao*

(*hts*), and *orb* suggest that proper regulation of the cell cycle within the dividing cyst may be important for fate specification, but untangling the different phenotypes that result from defective cystoblast division is complex (Lantz *et al.* 1992; Lin *et al.* 1994; McKearin and Ohlstein 1995; Ohlstein *et al.* 2000).

Clearly, the few genes that are known to be required to specify and execute the germ cell fates in the ovary represent only a subset of all genes needed to execute this process. Most of these genes were identified in genetic screens designed to isolate viable female sterile mutants (Schupbach and Wieschaus 1989). We thought it likely that many genes required for oogenesis would be essential for viability, so we carried out a clonal screen using the *Flp-FRT-ovo^{D}* system (Chou and Perrimon 1996). We analyzed 9992 mutant lines and isolated 38 mutations that exhibited early oogenesis defects and 208 mutations that exhibited weaker, less penetrant, or later defects. As expected, we isolated several alleles of the oocyte specification gene *orb*, although we did not isolate any alleles of the known female sterile mutant, *bam*. We also identified weak alleles of *Darkener of apricot* (*Doa*), a gene previously reported to be required for cell viability and for ovary development and alleles of *bob*, a gene previously reported to be required for male fertility (Castrillon *et al.* 1993; Yun *et al.* 2000). Finally, we report here the identification, mapping, and characterization of eight new lethal complementation groups that exhibit phenotypes in oocyte specification or early oogenesis. We named these genes after some of the unfortunate fates commonly met by eggs: *omelet, soft boiled, hard boiled, poached, fried, over easy, sunny side up,* and *benedict*.

MATERIALS AND METHODS

Fly stocks: The following genes were newly identified in the screen and used for phenotypic analysis: *omelet, soft boiled, hard boiled, poached, fried, over easy, sunny side up,* and *benedict*. (For details on alleles used and map positions, refer to Table 1.) *Doa$^{\gamma 3B}$* and *Doa^{HD}* were obtained from Leonard Rabinow. Transgenic flies used to create starting stocks for the screen, *bob^{C871}*, the Bloomington 3R deficiency kit, and candidate mutants for complementation testing were obtained from the Bloomington Stock Center or were present in the laboratory. All flies were raised at 25° unless otherwise indicated.

Mutagenesis and screening: See Figure 2 for a schematic of the screen. Mutagenesis was carried out as described by C. Yohn and R. Lehmann (unpublished data). All mutations were induced on an isogenic *FRT-82B, e* chromosome. We used several *heat shock-FLP* (*hs-FLP*) insertion lines and found that *hs-FLP(22)* induced clones somewhat more efficiently than *hs-FLP(122)*. Our general heat-shock protocol was to place vials or bottles with larvae (3–5 days) into 37° conditions for 2 hr on 2 successive days. To optimize clone frequency, we also experimented with shorter and longer heat shocks, heat-shocking adults, and heat-shocking for 1 day instead of 2, without significantly increasing clone frequency. The cell lethality assay was performed as follows. *hs-FLP/R1*; *FRT e*/ovo^{D}* males were crossed to virgin females carrying an *eyeless-FLP* transgene (*w, ey-FLP*; *FRT, arm-lacZ, w+/TM6B* females). The "R1" denotes a reporter chromosome used by other participants in the screen. Male *w, ey-FLP*; *FRT e*/FRT arm-lacZ, w+* progeny were scored for white eye clones in an orange background. Males that generated eye clones were then balanced by crossing to *hs-hid/TM3, Sb* virgin females. The β-galactosidase, ORB, 4′,6-diamidino-2-phenylindole (DAPI) assay was carried out by crossing *FRT e*/TM3, Sb* to *hs-FLP*; *FRT arm-lacZ, w+/TM3,Sb*. Progeny were heat-shocked as larvae and *hs-FLP/+*; *FRT e*/FRT arm-lacZ, w+* females were then scored for clone formation, DNA morphology, and ORB localization as described below. In most cases, many more than 10 clones were assayed. We note in the text the few instances where, despite extensive repetitions, we observed <5 clones.

Dissection, fixation, staining, and microscopy: Ovaries were dissected and stained in 18-well dishes made of Plexiglas and mesh to permit transferring the dishes from solution to solution without losing any ovarioles. Ovaries were dissected in 1× PBS and fixed in 6% formaldehyde in 1× PBT (1× PBS + 0.1% Triton X-100) for 20 min at room temperature. Ovaries were then washed in 1× PBTB (1× PBS + 0.1% Triton X-100 + 1% BSA) several times for a total of 45 min. After blocking in PBTB + 5% normal donkey serum for 30 min, ovaries were incubated in primary antibody overnight at 4°. For ORB staining, 6H4 antiserum, 4H8 antiserum (Schedl, Developmental Studies Hybridoma Bank), and 1× PBTB were used at 1:1:8. β-Galactosidase antiserum (Cappel Laboratories, Malvern, PA) was used at 1:2000. The next day ovaries were washed four times for a total of 1 hr in PBTB blocked in PBTB + 5% normal donkey serum for 30 min and then incubated in secondary antibody for 2 hr at room temperature. Ovaries were then washed four times in PBT for a total of 1 hr and mounted in 50% glycerol, 2.5% DABCO (1,4-diazabicyclo [2.2.2] octane) in PBS or in aqua PolyMount (PolySciences, Warrington, PA). Staining was visualized using a Leica TCS-NT confocal microscope as noted in the figure legends.

Complementation, cloning, and allele sequencing: Within the group of the 38 most interesting mutants, lines with similar map positions based on deficiency mapping and lines that were not uncovered by any deficiency were tested for complementation against each other (see results). Lines that complemented all other candidates within this group were complementation tested for lethality against the 208 weaker mutants isolated in the screen (see results). In one of our complementation groups, *soft boiled*, allele *6-57* exhibited a consistent 16-nurse-cell phenotype while allele *30-1* generated mostly normal clones in our rescreen. The initial identification of *30-1* as an oogenesis mutant was based on two abnormal clones and was tentative. It is possible that these alleles fail to complement because they share a lethal mutation that maps to 91C7-91D1. The *soft boiled* 16-nurse-cell phenotype might therefore be unmapped. Although *sunny side up$^{133\text{-}46}$* carried several lethal mutations that mapped to different places on 3R, it was also lethal *in trans* to *114-11*, which complemented all the deficiencies in the deficiency kit. We meiotically mapped *114-11* by crossing it to *ru st cu sr ca* flies and allowing the *ru st cu sr/e** females to recombine and mate with *hs-hid/TM3* males. Balanced males were complementation tested against *ru st cu sr e Pr ca* and against *133-46* so that we could correlate the map position of the *114-11* mutation with the map positions of *ru st cu sr e* and *ca*.

Once mutants were mapped to a region, they were complementation tested against candidate sterile and lethal mutants from that region as annotated by the Berkeley *Drosophila* Genome Project and FlyBase. In this way, we identified our only sterile complementation group as new *orb* alleles. The molecular nature of our two *Doa* mutations was identified by using the Expand Hi Fidelity PCR kit (Roche, Indianapolis) to amplify the coding regions and splice sites of *Doa* from genomic

DNA isolated from the mutant lines that failed to complement *Doa* alleles. Multiple independent PCR reactions were used to amplify each exon of the gene prior to pooling for sequencing in an 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. Sequencing and analysis of *bob* candidates were performed in the same manner.

RESULTS

Generating mutants and primary screen: To identify oogenesis mutants, we participated in a large-scale mutagenesis and primary screen with other groups in our lab who were interested in genes required for germ cell formation, oocyte patterning, and germ cell migration (C. Yohn, A. Forbes, M. Samuels, E. Staeva-Vieira, J. Stein, H. Zinsner and R. Lehmann, unpublished observations). We induced clones in heterozygous females using the *hs-FLP-FRT/ovo^D* system (Chou and Perrimon 1996). Because the females carried the dominant female sterile mutation, *ovo^D*, *in trans* to the mutations we were studying, only females that produced clones lacking *ovo^D* and carrying two copies of the mutagenized chromosome arm would be fertile. A failure to produce eggs indicated either a failure to produce clones or production of clones in a line carrying a female sterile mutation on 3R. We tested 9992 independent lines for fertility and found that 7293 of these lines laid eggs. These lines were tested for embryonic phenotypes by other members of the lab. We kept the 2699 remaining lines to determine why they failed to produce eggs (Figure 2).

Isolating early oogenesis mutants: We were interested in only those lines that were sterile, because these lines might carry mutations that caused defects in early oogenesis. Before characterizing the oogenesis phenotype of these mutants in detail, we performed several tests. Repeating the sterility test permitted us to discard lines that simply failed to produce clones in the primary screen (Figure 2, recombination option ii). We also induced clones homozygous for each of our lines in the eye imaginal disc using the efficient *ey-FLP* system. We discarded lines that failed to generate viable clones in the eye to select for genes that specifically affect oogenesis, but are not required for general viability in other tissues such as the eye. To screen for early oogenesis phenotypes, we initially analyzed the ovaries of some of our sterile lines after inducing clones *in trans* to the *ovo^D* mutation. Since we were unable to distinguish early oogenesis defects from defects caused by *ovo^D* in heterozygous egg chambers (Figure 2, recombination options ii and iii), we proceeded to analyze the oogenesis phenotypes of all our lines after crossing the mutant lines to flies carrying an *FRT* and *arm-lacZ* insertion on 3R. We detected clones homozygous for the mutagenized FRT chromosome by the absence of β-galactosidase and characterized mutant phenotypes by staining with antibodies raised against the oocyte-specific marker, ORB, and the DNA marker DAPI. We divided the lines into three categories. Thirty-eight lines generated clones that arrested before stage 5 and/or showed ORB localization defects. A total of 208 lines failed to generate clones, suggesting a very early role, generated clones that did not develop beyond stage 8, or had phenotypes similar to those we observed in the set of 38 mutants, but at lower penetrance. We discarded lines that did not show an early oogenesis phenotype or that developed beyond stage 8.

Mapping and complementation: To determine how many different genes were represented among the 38 most interesting mutants, we placed these lines into complementation groups. We tested the 38 lines *in trans* to the Bloomington 3R deficiency kit for lethality and sterility. We then performed complementation tests among those lines that failed to complement the same deficiency and between lines that complemented the entire deficiency kit. Eighteen of these 38 lines complemented all of the other most interesting lines and also did not map to a well-defined region of chromosome 3R. In some cases, these lines complemented the entire deficiency collection, while in other cases the mutant line failed to complement several deficiencies in different regions of 3R. We did not include these mutants in our further study.

We isolated three alleles, *11-50, 36-53*, and *116-8*, that were not uncovered by any of the deficiencies tested and were sterile *in trans* to each other. These mutants displayed a range of phenotypes including early oogenesis arrest and oocyte-specification defects that were reminiscent of class II *orb* alleles (Lantz *et al.* 1994) and, indeed, our alleles failed to complement strong *orb* alleles. Because *orb*'s role in oogenesis has been previously described in detail we did not include these alleles in our further study. Within the 38 most interesting lines, we found three multiallelic lethal complementation groups. One lethal complementation group (*114-11* and *133-46*) complemented the deficiency kit and was mapped meiotically on the basis of lethality (Table 1). We named this group *sunny side up*.

The alleles of the next complementation group (*8-37, 38-2*, and *43-21*) were lethal *in trans* to each other and also *in trans* to deficiencies that limited their map position to the 82D3–82D8 interval. We complementation tested these lines against candidate lethal and sterile mutants that had been mapped to this region. One candidate, *bob*, yielded a very interesting result. *bob* has two previously reported mutant alleles: a lethal EMS-induced allele and a male sterile *P*-element allele (Castrillon *et al.* 1993). We found that the EMS allele of *bob* was lethal *in trans* to *8-37, 38-2*, and *43-21* while the *P* allele was viable and fertile. We sequenced candidate transcripts flanking either side of the transposon insertion. We found no changes in the predicted coding regions of these candidates in our alleles. Subsequent

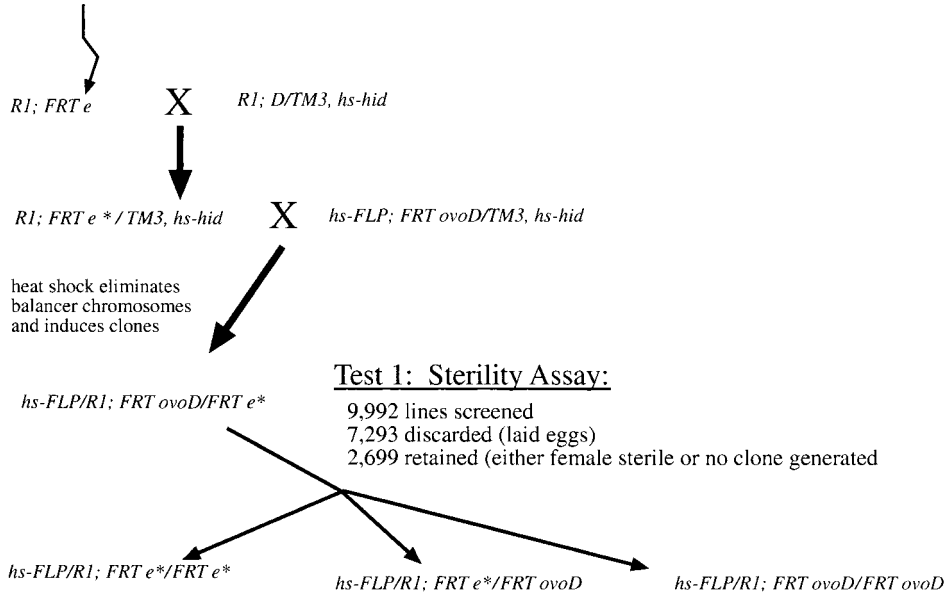

FIGURE 2.—Screen overview. *R1* is an X chromosome reporter used by other groups that took part in the primary screen (C. YOHN and R. LEHMANN, unpublished observations). *D* is a dominant marker selected against in order to balance mutagenized males. Only relevant markers are shown. See MATERIALS AND METHODS for details on mutagenesis and the subsequent assays. *, mutagenized chromosome.

Test 2: Dissection Assay
819/2,699 lines dissected (1880 directly to Test 3)
92 exhibited late development (discarded)
64 exhibited early defects distinguishible from *ovoD* (to Test 5)
663 either did not generate clones or arrested early (to Test 3)

Test 3: Cell Lethality Assay
553 lines not scored (lost in assay)
1,990 lines scored
729 did not generate eye clones (discarded)
135 lost in balancing
1,126 generated eye clones and were balanced (To Test 4 or directly to Test 5)

Test 4: Repeat Sterility Assay
809 lines scored (317 directly to Test 5)
530 generated fertile clones (discarded)
279 repeated sterility (to Test 5)

Test 5: β-Galactosidase, ORB, DAPI Assay
660 lines scored
414 did not exhibit interesting phenotypes (discarded)
38 exhibited defects before stage 6 and/or ORB localization defects (retained: most interesting class)
208 exhibited less penetrant or later defects (retained)

testing revealed that the transposon allele in the Bloomington stock collection is no longer male or female sterile *in trans* to any of our alleles or to the original *bob* EMS allele. Taken together, our data suggest that our alleles are lethal alleles of *bob* and that the *P* allele has been lost.

The third group included two alleles, *12-44* and *198-14*, that were lethal *in trans* to each other as well as to *Df(3R)3450*, which uncovers 98E3; 99A6-8. The alleles were also lethal *in trans* to a number of deficiencies that did not uncover the same region of 3R but that had in common an additional breakpoint in 98F1-2. We showed that *12-44* and *198-14* are lethal *in trans* to *Doa*, a candidate lethal mutation that mapped to 98F1-2. *Doa* was previously identified as a dual specificity, serine-threonine-tyrosine kinase (YUN *et al.* 1994). Strong *Doa* alleles are cell lethal in all tissues tested and were reported to produce clones indistinguishable from ovo^D in ovaries. Weaker *Doa* alleles have phenotypes in the nervous system and imaginal discs and are embryonic lethal. Sequencing of *198-14* and *12-44* showed that each carried a missense mutation in the nonconserved N-terminal region of DOA. The *12-44* lesion encodes a predicted R4L change and the *198-14* lesion encodes a predicted A41V change. To test whether the oogenesis phenotype we observed was specific to the new alleles or reflected a germline-specific function for *Doa*, we recombined a previously described partial loss-of-function *Doa* mutation (Doa^{HD}) onto the *FRT-82B* chromosome. Germline clones homozygous for this mutation exhibited a phenotype similar to that of *12-44* and *198-14*. We also generated clones of the strong allele, $Doa^{\gamma 3B}$,

TABLE 1

Map positions of oogenesis mutants

Gene name	Alleles	Map position or gene no.
bob	*8-37*, *38-2*, *43-21*	82D3-D8
poached	*35-22*, *38-6*	85E1-10
fried	*212-11*, *150-66*, *63-500*	88B-C3
hard boiled	*32-3*, *46-6*, *52-22*	91C7-91D1
soft boiled	*6-57*, *30-1*	91C7-91D1
benedict	*50-40*, *112-38*, *145-31*	91D1-F2
omelet	*146-34*, *40-17*, *36-63*	94A8-D13
orb	116-8, 11-50, 36-53	*CG10868*
over easy	*18-12*, *71-36*, *63-5*	95A5-7 or C10-D7
Doa	*12-44*, *198-14*	*CG1658*
sunny side up	*114-11*, *133-46*	Between *e* and *ca*
	194-26: single allele	
	20-51: single allele	
	27-25: single allele	

Allele names underlined were initially classed in the group of the 38 most interesting mutations. The other allele names were initially classed with the 208 less-well-characterized mutant lines and were reclassified after complementation testing. Map positions refer to cytological locations except for the cloned genes, *orb* and *Doa*, which have been assigned CG numbers by the Berkeley *Drosophila* Genome Project. The lines listed as single alleles were grouped with the most interesting mutations. They complemented all the other alleles generated in the screen. The single alleles mapped to only one region of 3R by lethal complementation with the Bloomington deficiency kit, but it is not certain that the lethal mutation is also the cause of the sterility we observed in these lines. Eighteen lines classed among the most interesting group that did not map to a defined region of 3R are not listed in the table.

in trans to a *FRT-82B, arm-lacZ* marker chromosome and found that homozygous $Doa^{\gamma 3B}$ germ cell clones died shortly after encapsulation by follicle cells, leading to budded egg chambers of healthy follicle cells in which germ cells were absent or dying. Taken together, our data demonstrate that *12-44* and *198-14* are weak alleles of *Doa* and that wild-type DOA activity is required for germ cell survival. Further phenotypic analysis indicates a role for *Doa* in early oogenesis as well (see below).

Ten of the remaining lines each mapped unambiguously to a single region of 3R on the basis of deficiency mapping, but these lines complemented the other candidates from the group of most interesting mutants. We therefore performed complementation tests with these lines against the 208 lines that had uncharacterized, later, or less penetrant defects. Consequently, in addition to *orb*, *Doa*, *bob*, and *sunny side up*, we were able to define seven additional multiallelic groups of mutations that produced early oogenesis defects: *poached*, *fried*, *soft boiled*, *hard boiled*, *benedict*, *omelet*, and *over easy*, which mapped to small regions of chromosome 3R (Table 1). Three lines from our screen, *20-51*, *27-25*, and *194-26*, mapped to defined regions of 3R on the basis of deficiency complementation data but complemented all other mutants found in our screen or candidate genes in the region uncovered by the relevant deficiencies. We did not analyze these single mutant lines further.

Phenotypic analysis: We studied the phenotypes of the 10 multiallelic, mapped complementation groups (excluding *orb*) by generating clones *in trans* to a *FRT-82B, arm-lacZ* marker chromosome and examining the pattern of ORB and DAPI staining with fluorescent and confocal microscopy. We characterized the mutants according to the following criteria: (1) nurse cell/oocyte fate specification; (2) stage of developmental arrest; (3) pattern and distribution of ORB protein; and (4) the morphology of the nurse cell and oocyte chromosomes (Table 2). Using these markers we were able to assess the extent to which nurse cell and oocyte fates were specified, whether egg chambers were capable of development or arrested at certain stages, the extent to which ORB was properly localized to the oocyte and within the oocyte, and the state of the oocyte nucleus and the nurse cell nuclei. From this analysis we concluded that the mutants fell into three general phenotypic classes.

Common to mutations in all complementation groups is an arrest early in oogenesis (Figure 3, A, A′, and A′′), usually before stage 6 and sometimes as early as stage 3, and defects in localizing ORB to the oocyte (Figure 3, A′ and B′). In addition and presumably as a consequence of the developmental arrest, many chambers degenerated, usually after they arrested. In degenerating chambers, DAPI staining showed condensed, fragmented chromosomes. ORB staining in these chambers was absent or punctate (Figure 3, C, C′, and C″). Phenotypic class I exhibited all the above defects, and mutants in this class show normal chromosomal morphology in the oocyte and nurse cells. This phenotypic class includes mutations in the genes *hard boiled*, *over easy*, *omelet*, *sunny side up*, and *poached* (Figure 3; Table 2). Phenotypic class II exhibited the defects observed in class I and in addition exhibited defects in the chromosomal morphology of the nurse cells and/or oocyte (Figure 4; Table 2). This class included *Doa*, *bob, benedict*, and *fried*. Phenotypic class III exhibited a 16-nurse-cell defect. This class included *soft boiled* (Figure 5; Table 2).

Class I: We isolated three alleles of *hard boiled*: *32-3*, *46-6*, and *52-22*. All three alleles produce arrested chambers, degenerating chambers filled with dying cells, and chambers that specify an oocyte that forms a karyosome and accumulates some ORB; but in many cases, total ORB levels in the egg chamber seemed reduced and ORB failed to accumulate strongly in the oocyte. The karyosome in *hard boiled* mutants did not always appear perfectly condensed, but it is difficult to determine if that is a specific chromosomal defect or the beginning of degeneration.

In addition to arresting at stage 4 or 5, $omelet^{146\text{-}34}$ chambers exhibited weak accumulation of ORB to the oocyte. Sometimes ORB was undetectable or very faint and evenly distributed within the oocyte although the karyo-

TABLE 2

Phenotypes of oogenesis mutants

Mutation	Stage of arrest	ORB localized to oocyte	Nurse cell endoreplication	Karyosome	ORB localized	16-nurse-cell phenotype
			Class I			
poached	3-6	Defective	Normal	Normal	Normal	No
hard boiled	3-6	Defective	Normal	Normal	Normal	No
omelette	4-5	Defective	Normal	Normal	Normal	No
over easy	3	Defective	Normal	Normal	Normal	No
sunny side up	3-6	Defective	Normal	Normal	Normal	No
			Class II			
Doa	2-8	Defective	Defective	Defective	Normal	Rarely
bob	3-6	Defective	Normal	Defective	Defective	Rarely
benedict	3-6	Defective	Defective	Defective	Defective	No
fried	No	Defective	Defective	Defective	Normal	No
			Class III			
soft boiled	NA	Defective	Normal	NA	NA	Yes

Stages listed in arrest column are the stages at which most arrested chambers were observed. No *fried* chambers were observed posterior to older chambers. *soft boiled* is marked NA because 16-nurse-cell chambers cannot be well staged and do not traffic ORB or condense karyosomes. Normal, the mutant clones showed wild-type pattern for that aspect of their phenotype; defective, mutants were defective in a process; rarely, 16-nurse-cell egg chambers were observed at low penetrance; NA, not available.

some appeared normal. The *36-63* allele generated only two clones out of hundreds of ovaries examined. One stage 10 clone appeared normal. The other degenerated at stage 8.

The three alleles of *over easy* that we isolated exhibited different degrees of defects (Figure 3, B, B′, and B″). *18-12* chambers always arrested at stage 3 and sometimes degenerated. ORB was barely enriched in one of the cells of the chamber. *over easy*$^{63\text{-}5}$ produced very few clones, several clones with wild-type morphology, one as late as stage 10, one clone that arrested at stage 6, and a clone that degenerated at stage 8. *71-36* failed to complement *over easy*$^{18\text{-}12}$ and *over easy*$^{63\text{-}5}$ for lethality, but we were unable to analyze clones homozygous for *71-36* although we examined hundreds of ovaries and tried several heat-shock regimens (see MATERIALS AND METHODS). It is possible that strong alleles of *over easy* cause cell lethality.

poached$^{35\text{-}22}$ egg chambers usually arrested between stage 3 and 6 (Figure 3, C, C′, and C″). ORB rarely accumulated in one cell of these egg chambers, and if so only barely. *poached*$^{38\text{-}6}$ chambers show a similar arrest

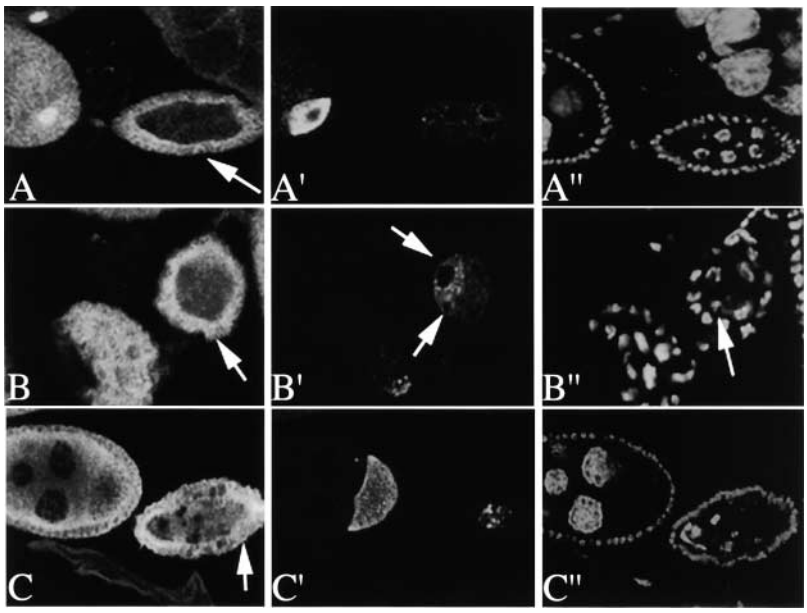

FIGURE 3.—Phenotypes common to class I, II, and III mutants. Anterior is left. (A, B, and C) Lack of anti-β-galactosidase staining identifies mutant egg chambers. (A′, B′, and C′) Anti-ORB. (A″, B″, and C″) DAPI. A, A′, and A″ show an arrested clone (A, arrow) of *sunny side up*$^{114\text{-}1}$. The mutant egg chamber is smaller and thus presumably arrested in comparison to the younger (anteriorly located) wild-type egg chamber. B, B′, and B″ show an *over easy*$^{18\text{-}12}$ clone exhibiting a defect in ORB localization. ORB is not fully restricted to the oocyte (B′, arrows) although the oocyte has condensed its DNA into a karyosome (B″, arrow). C, C′, and C″ show an arrested, degenerating *poached*$^{35\text{-}22}$ clone (C, arrow). In dying chambers, β-galactosidase sometimes "flows" into the center of the egg chamber from the follicle cells (C), ORB is quite punctate in the oocyte (C′), and the DNA is condensed and fragmented (C″).

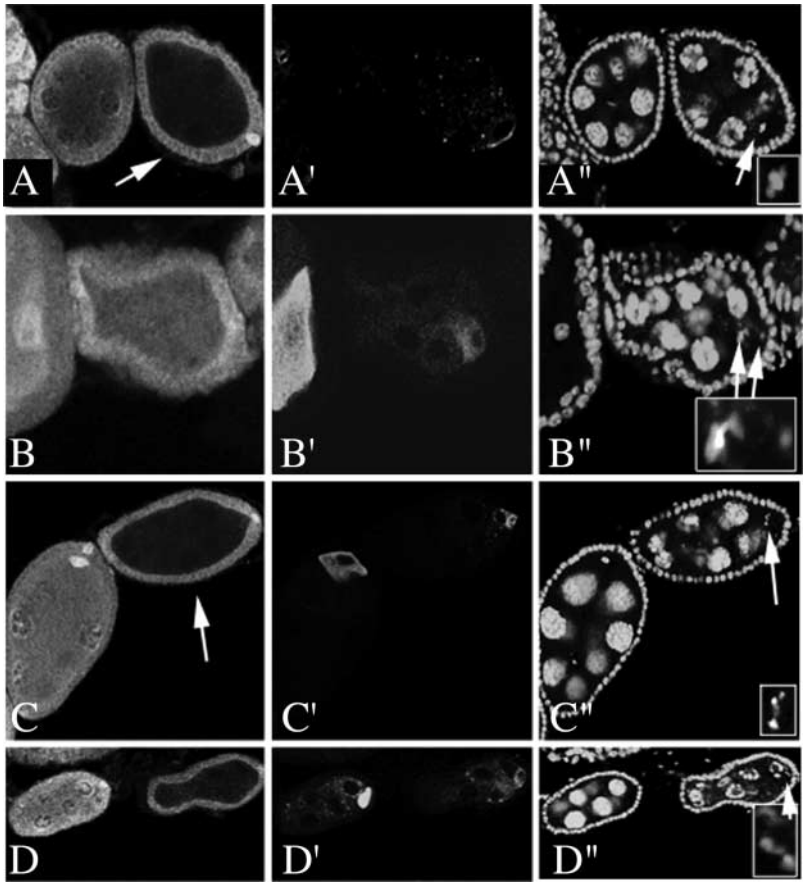

FIGURE 4.—Phenotypes of class II mutants. Anterior is left. (A, B, C, and D) Anti-β-galactosidase. (A′, B′, C′, and D′) Anti-ORB. (A″, B″, C″, and D′′) DAPI (a larger image of the karyosome is shown in the inset). A, A′, and A″ show a *Doa*$^{12\text{-}44}$ mutant clone (A, arrow). ORB is faint and incompletely trafficked to the oocyte (A′), the nurse cell chromosomes still exhibit the distinct polytene morphology characteristic of earlier stages, and the karyosome is incompletely condensed (A″, arrow, inset). B, B′, and B″ show an arrested *bob*$^{38\text{-}2}$ clone. ORB is localized to the oocyte, but is localized to the anterior (B′). The karyosome is fragmented and decondensed (B″, arrows, inset). C, C′, and C″ show an arrested *benedict*$^{50\text{-}40}$ clone (C, arrow). ORB is patchy within the oocyte (C′) and the karyosome is decondensed (C″, arrow, inset). (D, D′, and D″) A *fried*$^{212\text{-}11}$clone (D, arrow) shows low levels of ORB and retains a significant fraction of total ORB in the nurse cells (D′). The karyosome is not condensed (D″, arrow, inset) and the nurse cells are underreplicated.

phenotype, although we observed some chambers that appeared normal as late as stage 8.

The two *sunny side up* alleles, *114-11* and *133-46*, displayed a very similar phenotype (Figure 3, A, A′, and A″): egg chambers usually arrested by stage 6, although we observed one wild-type chamber as late as stage 12. ORB was very faint or even undetectable in most chambers.

Class II: Phenotypic class II egg chambers exhibited ORB localization defects and early oogenesis arrest similar to those observed in mutants of class I. In addition, egg chambers from class II exhibited unusual defects in the oocyte and nurse cell nuclear morphology and in ORB localization within the oocyte. This phenotype is quite distinct from the phenotype reported for the weak *cyclin E* allele, *cycE*^{01672}. In that mutant, two cells form karyosomes and the nurse cells endoreplicate their chromosomes (LILLY and SPRADLING 1996), suggesting that our mutations affect the nurse cell cycle in a different manner from *cycE*^{01672}. Class II includes mutants in four genes: *Doa*, *bob*, *benedict*, and *fried* (Table 2).

As described above, we isolated two weak alleles of *Doa*, *Doa*$^{12\text{-}44}$ and *Doa*$^{198\text{-}14}$. The phenotype of *Doa* null germline clones is cell-autonomous lethality after the encapsulation of the germ cells by follicle cells. The observation that the germ cells are viable during the initial stages of oogenesis may be due to perdurance of DOA protein that was inherited from a heterozygous stem cell when the clone was initially generated. The defects we observed in *Doa*$^{12\text{-}44}$ and Doa$^{198\text{-}14}$ egg chambers suggested a role for *Doa* in regulating nurse cell replication and karyosome stability. The nurse cell chromosomes in *Doa* chambers were often strikingly underreplicated and nurse cell nuclei of older egg chambers had the morphology of younger, stage 3 wild-type chambers. When nurse cells did replicate their chromosomes, they often appeared to remain polytene beyond stage 5, in contrast to wild-type chambers, which lose their condensed appearance at this stage (Figure 4A″). In addition, the karyosome was sometimes incompletely condensed, suggesting that the oocyte may not be properly maintaining meiotic arrest. Also, ORB localization was often quite weak (Figure 4A′). Rarely, we observed 16-nurse-cell chambers.

The most prominent *bob* phenotype was observed during midoogenesis. In contrast with wild-type egg chambers at this stage, ORB was still localized anterior to the oocyte nucleus, suggesting that the microtubule cytoskeleton did not rearrange appropriately at stage 1 (Figure 4B′). We isolated three *bob* alleles: *38-2*, *43-21*, and *8-37*. The *8-37* allele produced only a few clones, all of which arrested very early. In addition to defective relocalization of ORB to the posterior of the oocyte, *bob* chambers exhibited fragmented karyosomes. This

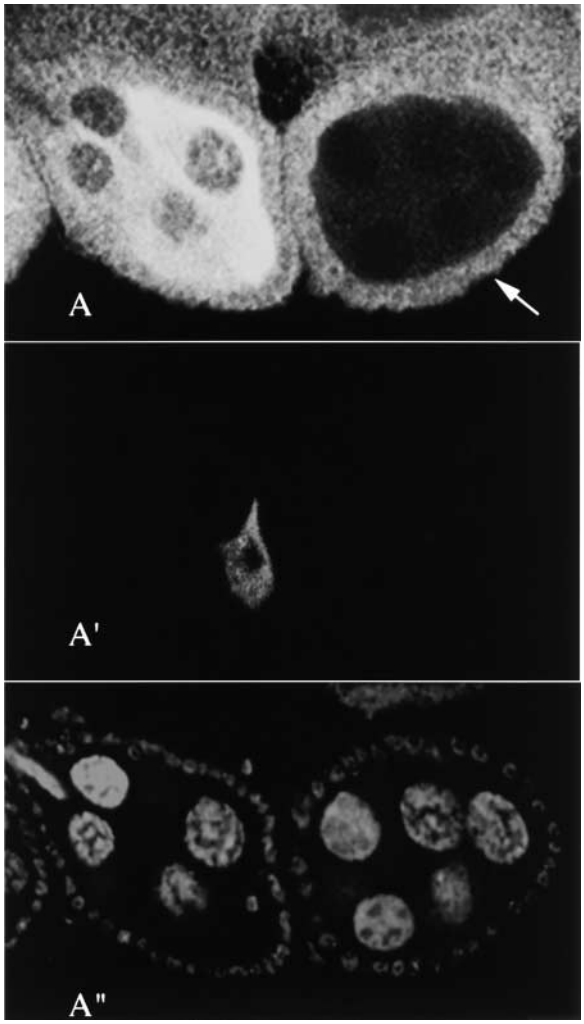

FIGURE 5.—Phenotype of the class III mutant, *soft boiled*$^{6\text{-}57}$. Anterior is left. A, A′, and A″ show a *soft boiled*$^{6\text{-}57}$ clone. (A) Anti-β-galactosidase. (A′) Anti-ORB. (A″) DAPI. *soft boiled* clones (A, arrow) show no ORB localization (A′) and possess 16 endoreplicated nurse cell nuclei and no oocyte (A″).

karyosome defect may indicate a problem in meiotic maintenance or perhaps in double-stranded break repair (see DISCUSSION). The nurse cell nuclei in *bob* chambers appeared to replicate normally. Most *bob* chambers exhibited poor accumulation of ORB in the oocyte and arrested between stage 3 and 6, although we observed one normal chamber as late as stage 8.

We isolated two alleles of *benedict*, *50-40* and *112-38*. *benedict* chambers display a number of salient defects. Both alleles produced clones with underreplicated nurse cells whose nuclei retained polytene morphology beyond stage 5. *benedict* karyosomes were fragmented or stringy. We also observed allele-specific phenotypes. The *50-40* mutation predominantly affected the karyosome (Figure 4C″) while the *112-38* mutation had a strong effect on the nurse cell nuclei (not shown, but similar to *fried* defect shown in 4D″). ORB staining was also unusual in *benedict*$^{50\text{-}40}$ chambers: it appeared patchy and distributed unevenly throughout the oocyte cytoplasm instead of collecting evenly at the posterior of the oocyte (Figure 4C′). This localization phenotype was observed much less frequently in *benedict*$^{112\text{-}38}$. This patchy localization phenotype within the oocyte is distinct from the anterior localization defect we observed in *bob*, but the two phenotypes are grouped together as defects in ORB localization within the oocyte in Table 2. ORB accumulation in the oocyte was sometimes weak in *benedict* (Figure 4C′), and chambers usually arrested by stage 6.

fried egg chambers specify an oocyte, but the karyosome is often stringy or not properly condensed. We isolated three alleles: *212-11*, *150-66*, and *63-500*. In addition to the karyosome defect, the *212-11* allele also showed a defect in nurse cell endoreplication and perdurance of polyteny (Figure 4D″), as does the *63-500* allele at lower frequency. ORB localization in *fried* chambers is usually weak (Figure 4D′). In contrast to the other mutants we isolated, we did not observe obviously growth-arrested *fried* chambers although we did not detect any chambers older than stage 8.

Class III: The third phenotypic class is the 16-nurse-cell phenotype. This phenotype is characteristic of several female sterile mutations that have been identified in other screens, including *egl*, *BicD*, *orb*, and *stonewall* (*stwl*; TEARLE and NÜSSLEIN-VOLHARD 1987; SCHUPBACH and WIESCHAUS 1989; CLARK and MCKEARIN 1996). While most of our mutants have defects in oocyte determination and fail to localize the ORB marker within the oocyte, we isolated only one gene, *soft boiled*, that exhibits a 16-nurse-cell chamber with high penetrance (Table 2). We isolated two alleles in our screen that are lethal *in trans* to each other and *in trans* to the same set of deficiencies that uncover the region between 91C7 and 91D1. Almost all *soft boiled*$^{6\text{-}57}$ chambers failed to specify an oocyte: all 16 cells endoreplicate their DNA and none of them accumulated ORB (Figure 5, A′ and A″). We observed only one or two chambers in which ORB was trafficked to one cell, but in both cases the chambers were too young to determine if that cell condensed its DNA into a karyosome. The other allele that was lethal *in trans* to *6-57*, *30-1*, generated many normal clones. We retained *30-1* in our collection of 208 mutants only because one or two chambers exhibited a weak ORB localization phenotype. Thus, at present, it is unclear if the *6-57* and *30-1* mutants are indeed allelic or simply share a second lethal mutation that maps to the 91C7-D1 interval. In addition to *soft boiled*, *bob* and *Doa* chambers also occasionally displayed a 16-nurse-cell phenotype (not shown, but similar to Figure 5, A, A′, and A″), but at much lower frequency (see DISCUSSION).

DISCUSSION

We report here the results of a clonal screen designed to isolate mutants defective in oocyte specification and early oogenesis on the right arm of chromosome 3. Out

of 9992 single female crosses screened, we identified 11 multiallelic complementation groups that we mapped to a small region or a specific genetic locus. These 11 groups include the previously described oocyte-specification factor, *orb* (Lantz *et al.* 1992), the known genes, *bob* and *Doa*, for which we have provided further analysis of oogenesis phenotypes, and eight new genes, *soft boiled, omelet, fried, poached, benedict, sunny side up, hard boiled*, and *over easy*. With the exception of the *orb* alleles we isolated, all alleles found in the screen are lethal as homozygotes and *in trans* to all other alleles in their complementation groups, indicating that these genes would not have been identified in a traditional female sterile screen for oogenesis mutants.

The screen: One important question is whether our screen saturated for mutations that cause oocyte defects on 3R. The fact that three of our most interesting mutants are represented only by a single allele (Table 1) and that we isolated no *bam* alleles at all would argue against saturation, in spite of the screen's relatively high lethal hit rate (C. Yohn and R. Lehmann, unpublished observations). It is possible that the primary screen was saturating, but that we lost many alleles in our subsequent tests. In particular, the cell lethality screen would have selected against mutations in genes that are required for eye cell viability as well as for a specific function in oogenesis. We believe that *Doa* is one example of such a gene since strong alleles of *Doa* are cell lethal in every tissue tested (Yun *et al.* 1994) while the weak alleles we isolated cause very specific phenotypes in cell cycle regulation and ORB localization. Although by eliminating the lines carrying lethal mutations we may have discarded alleles of some of our most interesting genes, the sacrifice was necessary to make the screen technically feasible. Cell lethal mutations would not generate visible clones and we would not have been able to study these mutations further.

Another question is why the clone frequency we observed for the mutants in our screen was so low. In general, the clone frequency we observed in our mutant lines was quite low compared to the isogenized strain we used for the screen, and clone frequency varied significantly from line to line. One possibility is that clone frequency was reduced because mutagenesis induced multiple lesions on 3R. Alternatively, most of our mutants may somehow interfere with germline viability, as suggested by those *omelet* and *over easy* alleles, that completely failed to produce clones.

Analysis of the mutant phenotypes: Our mutants fall into different classes with respect to oocyte specification, karyosome condensation, nurse cell endoreplication, and localization of ORB within the oocyte. Only two of the genes we describe here, *bob* and *soft boiled*, produce phenotypes that resemble those described previously for other genes. The other genes reveal novel phenotypes or combinations of phenotypes that had previously not been observed.

Mutations in *bob* disrupt posterior localization of ORB within the oocyte and also show a 16-nurse-cell phenotype. These phenotypes show striking similarity to mutations in *par-1*, which disrupt the posterior localization of the microtubule organizing center and induce the oocyte to exit meiotic arrest and enter the nurse cell endoreplication program (Huynh *et al.* 2001). In *par-1*, the oocyte has been shown to revert to a nurse cell fate between stage 2 and stage 6, long after the oocyte fate has been specified (Huynh *et al.* 2001). Similarly, the 16-nurse-cell phenotype we observe in *bob* may be the result of a reversion of the oocyte to a nurse cell fate rather than an initial failure in oocyte specification. The 16-nurse-cell phenotype occurred rarely in *bob* alleles and was not observed in very early chambers, suggesting that these mutants are defective primarily in processes that occur after the oocyte is specified. The earlier ORB localization phenotype followed by the oocyte-specification phenotype observed in *bob* and *par-1* suggests that microtubule organization in the oocyte may play a role in maintaining the meiotic cell cycle in the oocyte.

Mutations in one of our two *soft boiled* alleles consistently produced a 16-nurse-cell phenotype and we rarely detected any sign of oocyte specification in mutant egg chambers. Genes with a similar phenotype, such as *BicD*, *egl*, *Lissencephaly-1*, and *Dhc* have been implicated in microtubule-mediated transport (Theurkauf *et al.* 1993; McGrail and Hays 1997; Swan *et al.* 1999). It has been proposed that *BicD* and *egl* are required for the transport of RNA and protein from the nurse cell to the future oocyte (Suter *et al.* 1989; Mach and Lehmann 1997). It is thus possible that *soft boiled* has a similar role. The observation that *6-57* and *30-1* fail to complement for lethality may suggest a more general role for *soft boiled* in microtubule-directed transport, similar to *BicD*, for which both maternal-effect sterile alleles and lethal alleles have been identified (Schupbach and Wieschaus 1989; Ran *et al.* 1994). However, mutations in genes not known to regulate microtubule-dependent transport, such as *stwl*, also cause a 16-nurse-cell phenotype (Clark and McKearin 1996), leaving open the possibility that *soft boiled* acts via another mechanism.

Our remaining mutants display phenotypes or combinations of phenotypes that have previously not been described. With the possible exception of *fried*, all of the mutants we report here arrest or exhibit dramatically slowed growth by midoogenesis. Also, even in class I mutants where the oocyte is specified (on the basis of at least some criteria for oocyte determination, such as karyosome condensation and enrichment of ORB in a single cell), we observe failure to traffic ORB from the nurse cells to the oocyte and to accumulate normal ORB levels in the oocyte. We do not believe that nonspecific defects cause ORB localization failure and egg chamber arrest because the vast majority of the lines we examined in the screen did not exhibit these phenotypes even though almost all of them carried lethal mutations on

chromosome 3. The growth arrest and ORB localization phenotypes we observed in *poached, hard boiled*, *omelet*, *over easy*, and *sunny side up* are therefore likely to result from specific defects in oogenesis such as defects in nurse-cell-to-oocyte transport that do not affect other aspects of oogenesis such as karyosome formation.

The phenotypes we observed in *Doa*, *benedict*, and *fried* suggest a link between nurse cell endoreplication and meiotic arrest of the oocyte. *Doa*, *benedict*, and *fried* disrupt both karyosome condensation and nurse cell endoreplication, suggesting roles for these genes in regulating the cell cycle in nurse cells and oocytes. In contrast, mutations in *bob* and in the *spindle* and *par-1* pathways cause defects in karyosome condensation but not in nurse cell endoreplication. The wider range of defects observed in *Doa*, *fried*, and *benedict* may indicate that these genes act more globally in regulating chromosome behavior in the germline, while *bob* and the *spindle* genes specifically function in meiosis. Alternatively, *Doa*, *fried*, and *benedict* may simply be required before the nurse cell and oocyte fates have been specified within the cyst, causing both cell types to exhibit defects in these mutants.

The phenotypes observed in the two alleles of *benedict* are distinct but overlapping. *benedict*$^{50\text{-}40}$ chambers often exhibit a unique ORB localization phenotype. Rather than clearly accumulating ORB at the anterior or posterior of the oocyte, as *fried* and *Doa* chambers do, many *benedict*$^{50\text{-}40}$ chambers localize ORB in a patchy pattern within the oocyte. It is unclear what could be causing this localization pattern, since this phenotype has not been reported in mutations that disrupt microtubule stability or microtubule repolarization such as *par-1*. The *50-40* allele very rarely disrupts nurse cell endoreplication. *benedict*$^{112\text{-}38}$ disrupts nurse cell endoreplication, like *fried* and *Doa* mutants, but rarely exhibits the ORB localization defects observed in *50-40* chambers. Both *benedict* alleles exhibit the karyosome defects that are also observed in *fried* and *Doa*. The differences in the phenotypes of the *benedict* alleles may point to separable functions for this gene. One function, disrupted in the *50-40* allele, localizes ORB within the oocyte, probably in a process that is distinct from microtubule reorganization. The other function, disrupted in the *112-38* allele, acts in nurse cell endoreplication, like *fried* and *Doa*. Both *benedict* functions appear to be required for proper karyosome condensation.

A major advantage of our clonal screen over a recessive female sterile screen is the capacity to uncover a function in oogenesis for homozygous lethal genes. Consequently, screening 20% of the Drosophila genome enabled us to isolate eight new lethal genes required for oogenesis and to uncover a requirement in oogenesis for two previously described lethal genes. Furthermore, two of the phenotypes we describe, the failure of nurse cells to endoreplicate properly and the patchy ORB localization we observed in *benedict*$^{50\text{-}40}$, have not been reported previously. It is likely that entire phenotypic classes can be uncovered only in a clonal screen because certain phenotypes result from defects in processes required for viability. Similar screens covering the remaining 80% of the genome are therefore likely to prove very useful in identifying the genes required for nurse cell/oocyte fate specification and oogenesis.

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