Cytoplasmic Localization and Evolutionary Conservation of MEI-218, a Protein Required for Meiotic Crossing-over in *Drosophila*

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During *Drosophila* oogenesis, the oocyte is formed within a 16-cell cyst immediately after four incomplete cell divisions. One of the primary events in oocyte development is meiotic recombination. Here, we report the intracellular localization of the MEI-218 protein that is specifically required for meiotic crossing-over. To understand the role of *mei-218* in meiosis and to study the regulation of genes required for meiotic recombination, we characterized the expression pattern of its RNA and protein. Furthermore, we cloned and sequenced *mei-218* from two other *Drosophila* species. The *mei-218* RNA and protein have a similar expression pattern, appearing first in early meiotic prophase and then rapidly disappearing as prophase is completed. This pattern corresponds to a specific appearance of the *mei-218* gene product in the region of the ovary where meiotic prophase occurs. Although *mei-218* is required for 95% of all crossovers, the protein is found exclusively in the cytoplasm. Based on these results, we suggest that *mei-218* does not have a direct role in recombination but rather regulates other factors required for the production of crossovers. We propose that *mei-218* is a molecular link between oocyte differentiation and meiosis.

INTRODUCTION

Meiosis is essential to sexual reproduction in all multicellular organisms because it is the process whereby the chromosome complement is precisely divided in half. The fusion of the two gametes at fertilization creates a complete diploid genome. Meiotic crossing-over is the most important mechanism for ensuring the proper segregation of homologous chromosomes at meiosis I (Hawley, 1988). Crossovers, and the resulting chiasmata, link and orient homologous chromosomes so that they segregate properly. Crossing-over also increases the genetic variation between progeny and parents. A failure to produce a crossover between a pair of homologous chromosomes can result in nondisjunction, and the consequent aneuploidy in most organisms causes zygotic lethality.

In *Drosophila* females, the process of meiotic recombination occurs within the context of a developing oocyte. The steps involved in meiotic recombination happen early, shortly after the oocyte finishes the premeiotic S-phase (Carpenter, 1979). After this stage, the oocyte begins a develop-

mental program of growth and definition of cell polarity. Thus, it is expected that there will be a molecular link between the proteins intimately involved in meiotic recombination and others required in a regulatory role for oocyte differentiation. These regulatory processes ensure that meiotic recombination is initiated and completed within a specific time frame. Delays in this process can have disastrous consequences on development of the oocyte (Ghabrial and Schupbach, 1999).

Genetic studies have shown that the number and distribution of crossovers are tightly regulated. The “precondition defective” class of genes in *Drosophila* was originally defined as those that reduce crossing-over and alter the distribution of the residual crossovers (Sandler *et al.*, 1968). *mei-218* mutants are an example of this class; >90% of all crossing-over during meiosis is eliminated and the residual crossovers are abnormally distributed (Carpenter and Sandler, 1974; McKim *et al.*, 1996). In contrast, the frequency of gene conversion is not reduced, demonstrating that the initiation of recombination (double-strand break [DSB] formation) still occurs (Carpenter, 1982, 1984). Thus, *mei-218* and others in its class are required specifically to generate the crossovers from a DSB event. Previous experiments failed to detect any mitotic, zygotic, or oogenesis phenotypes or sensitivity to methyl methanesulfonate and x-ray mutagenesis in *mei-218* mutants. By these criteria, *mei-218* mutant defects are limited

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Figure 1. Developmental analysis of *mei-218* expression. (A) The *mei-217/mei-218* region showing the structure of the dicistronic message (Liu *et al.*, 2000), the location of primers used in RT-PCR, and the regions used to generate antibodies. The MEI-217-coding region is gray and the MEI-218 is black. The A5 antibody was generated against a 391-amino acid peptide from the 3'-end of *mei-218*, whereas the PX antibody was generated against a 315-amino acid peptide from the middle of *mei-218*. The FLAG epitope was fused to the beginning of the *mei-218*-coding region. In the *hsp83::mei-218^{FLAG}* constructs, the upstream region of the normal *mei-218* transcript, including the *mei-217*-coding region, is replaced by the *hsp83* promoter. For the *hsp83::mei-218^{FLAG-SV40}* experiments, the 3'-UTR was replaced by the SV40 3'-UTR. (B) RT-PCR reveals that *mei-218* is transcribed in embryos, larvae, and testis, all tissues in which no mutant phenotype has previously been observed.

to female meiosis (Baker *et al.*, 1976, 1978; Lutken and Baker, 1979). These results, combined with the specific requirement for crossing-over during meiosis, suggest that *mei-218* encodes a meiosis-specific gene product.

We have previously described the cloning of *mei-218* (McKim *et al.*, 1996) and found that it is part of a dicistronic message with another gene, *mei-217* (Liu *et al.*, 2000). Despite the requirement of these genes for most crossover events, their sequence has not provided insights into protein function because homologues do not exist in the databases. Therefore, to learn more about the function of *mei-218*, we have determined the expression patterns of the RNA and protein product, and we have sequenced the homologues from two other *Drosophila* species. Analysis of the transcription pattern in *Drosophila melanogaster* shows a specific program of meiotic gene expression. Based on the drastic effects of *mei-218* mutants on crossing-over, one prediction was that MEI-218 would be a nuclear protein. However, we found by immunocytochemical analyses that MEI-218 can only be detected in the cytoplasm. The protein localization patterns suggest that MEI-218 has a vital regulatory role in meiotic crossing-over.

MATERIALS AND METHODS

Isolation of RNA, Reverse Transcriptase (RT)-PCR Analysis, and in Situ Hybridization

Total RNA was collected from dissected ovaries or testis by grinding the tissue in 50% RNA lysis buffer (0.3 M sodium acetate, 4 mM EDTA, 50 mM Tris-HCl, pH 9.0, 1% SDS)/50% acid phenol followed by two extractions in acid phenol. mRNA was isolated from dissected ovaries using the Poly(A)pure Isolation kits (Ambion, Austin, TX). RT-PCR was carried out using the single tube methodology and reagents from Invitrogen (Carlsbad, CA) or Roche Molecular Biochemicals (Summerville, NJ). The location of primers is shown in Figure 1. Digoxigenin-labeled RNA probes for in situ hybridization were made from the linearized *mei-218* cDNA clone pHA-15

using the Roche Molecular Biochemicals RNA-labeling kit and hybridized as described by Tautz and Pfeifle (1989).

Generation of Polyclonal Anti-MEI-218 Antibodies

The A5 (391 amino acids) and PX (315 amino acids) fragments were subcloned from *mei-218* cDNA (Figure 1) into the Novagen (Madison, WI) pET-30c or pET-30b vectors and expressed in *Escherichia coli*. The proteins were purified under denaturing conditions over Ni²⁺-binding columns using His-binding Ni-nitrilotriacetic acid resins (Qiagen, Valencia, CA, or Novagen). After electroelution, proteins were concentrated and used to raise antibodies in rats (Pocono Rabbit Farm & Laboratory, Canadensis, PA). Antibodies were affinity purified in 1-ml aliquots using Affi-Gel 10 agarose beads (Bio-Rad, Hercules, CA). Beads (1.5 ml) were washed with 3 ml cold, 10-mM sodium acetate pH 4.5, then they were washed two times with 5 ml of 50 mM-HEPES, pH 7.5, before overnight incubation at 4°C with 5 ml antigen protein (dialyzed into 50-mM HEPES, pH 7.5). Resin was then blocked with 100 μ l 1-M ethanolamine, pH 8.0, for 1 h at room temperature. The beads were washed into the column with 50 mM HEPES, pH 7.5, and rinsed with PBST (PBS + 0.1% Tween-20) until $A_{280} = 0$. 1 ml sera was diluted to 5 ml with PBST and incubated in a column, rocking for 2–4 h at room temperature. The column was washed several times with PBST until $A_{280} = 0$. Antibody was eluted with 0.2 M glycine, pH 2.7, in 800- μ L fractions, into 200 μ L of 1 M Tris, pH 8.0.

Transgenic Constructs

The *hsp83::mei-218* construct was originally described by McKim *et al.* (1996). For the FLAG-tagged version (*hsp83::mei-218^{FLAG}*), PCR was used to introduce an *EcoRI* site in place of the *mei-218* ATG. The plasmid pFLAG83 is a derivative of pBluescript containing the *hsp83* promoter upstream of a sequence encoding an initiator ATG and the FLAG tag (MDYKDDDDK). The *mei-218*-coding region was fused in-frame to the FLAG tag using the *EcoRI* site introduced by PCR. The entire construct was cloned with *KpnI* and *NotI* into pCasper4 for transformation into *Drosophila* (Rubin and Spradling, 1982). A derivative with the simian virus (SV)40 3'-untranslated region (UTR) (*hsp83::mei-218^{FLAG-SV40}*) was constructed using PCR

to introduce an *Xba*I site after the *mei-218* stop codon and removing the 3'-UTR in the process. The SV40 3'-UTR was cloned out of pCasPeR-AUG- β -Gal using *Xba*I - *Sal*I and cloned downstream of *mei-218*. As before, the entire construct was cloned with *Kpn*I and *Nof*I into pCasPeR 4.

The X-chromosome nondisjunction frequency in each transgenic was determined by crossing *mei-218*⁶; *hsp83::mei-218*^{FLAG} females to C(1:Y), *v f B* males.

Confocal Microscopy

A fixation method based on buffer A (Belmont *et al.*, 1989) was used most often and is briefly summarized below. Ovaries were dissected from 1-d-old virgins in 1× Robbs solution and fixed for 10 min in 500 μ L of buffer A (150 mM piperazine-*N,N'*-bis[2-ethanesulfonic acid], pH 7.4, 0.8 M KCl, 200 mM NaCl, 20 mM EDTA, 5 mM ethyleneglycol-bis(β -aminoethyl ether)-*N,N'*-tetraacetic acid, final pH 7.0) plus 4% formaldehyde. Ovaries were washed for 15 min each: two times in BAT (buffer A + 0.1% Triton X-100) and two times in BAT-NGS (BAT + 10% 60 mg/ml normal goat serum). Ovaries were incubated in primary antibody (1:50 or 1:100 anti-MEI-218, 1:20 or 1:50 M5 anti-FLAG monoclonal [Kodak IBI, New Haven, CT], 1:30 anti-ORB 6H4 and 4H8 [Lantz *et al.*, 1994] or 1:50 nuclear lamin) in BAT-NGS at 4°C overnight. Ovaries were then washed with four changes over 2 h in BAT plus 0.2% bovine serum albumin, followed by a 30-min wash in BAT-NGS while the secondary (all 1:250 dilutions; horse anti-mouse fluorescein isothiocyanate [Vector Laboratories, Burlingame, CA] for anti-FLAG and anti-ORB, goat anti-rat CY3 [Amersham Pharmacia Biotech, Piscataway, NJ] or goat anti-rat fluorescein isothiocyanate [Vector] for anti-MEI-218) was preabsorbed against methanol-fixed embryos. Secondary antibodies were added with RNase if necessary (final concentration 5 μ g/ml) and left to incubate at room temperature for 4 h, followed by a 30-min wash in BAT. Ovaries were left in 1× buffer A overnight at 4°C. If tertiary labeling was used, the incubation with the secondary antibody (10 μ g of biotinylated anti-rat [Vector]) was followed by four 30-min BAT-bovine serum albumin washes and a 30-min BAT-NGS wash. The tertiary antibody, 1:500 streptavidin-Cy3, was left to incubate overnight at 4°C. The ovaries were then washed four times over 1 h with BAT before DNA stain was added. Ovary chromosomes were stained with Hoechst, 4,6-diamidino-2-phenylindole, Yo-Pro (Molecular Probes, Eugene, OR) or propidium iodide, washed for 15 min in BAT, quickly rinsed in 1× buffer A, and mounted in Vectashield (Vector). Images were collected on an LSM 510 (Zeiss, Thornwood, NY) or TCS SP (Leica, Wetzlar, Germany) confocal microscope.

Cloning and Sequencing *mei-218* from Other Species

Genomic phage libraries were screened with either a probe for *mei-218* (*Drosophila yakuba*) or the neighboring gene *RpS5* (*Drosophila virilis*). Previous attempts to use an *mei-218* clone to probe the *D. virilis* library were unsuccessful because, at low stringency, clones carrying repetitive sequences were isolated that did not contain the *mei-218* gene. A probe for *RpS5* was used because this gene is highly conserved and adjacent to the *mei-218*-coding region (McKim *et al.*, 1996). Subclones containing *mei-218* sequences were identified by Southern blot of phage DNA and then sequenced using the University of Medicine and Dentistry of New Jersey core sequencing facility. The sequence was analyzed using the Wisconsin Package, version 10.0 (Genetics Computer Group, Madison, WI). The accession number for the *mei-217/mei-218 D. virilis* sequence is AF426408 and for *D. yakuba* is AF426409.

RESULTS

Overview of Oocyte Development and Meiosis

The *Drosophila* ovariole is composed of two prominent regions: the germarium and the vitellarium. The germarium

comprises stem cells that give rise to cystoblasts that undergo four incomplete divisions to produce a 16-cell cyst in which all of the cells are connected through ring canals (Figure 3; de Cuevas *et al.*, 1997). In the 16-cell cyst, there are two cells that have four ring canals, whereas the other 12 cells have three, two, or one ring canal(s). Both of the four-ring canal cells form complete synaptonemal complexes (SC) and recombination nodules (RN). Both SC and RN are cytological markers that the cell is in the pachytene stage of meiosis, during which it is believed that meiotic recombination occurs. However, only one of these cells is maintained in meiosis as the oocyte, whereas the other 15 become polyploid nurse cells. The pro-oocyte, the four-ring canal cell that maintains meiosis, moves to the posterior end of the 16-cell cyst as it continues out of the germarium. The vitellarium consists of the successive stages of oocyte development leading up to the mature stage 14 oocyte, at which meiosis arrests in metaphase.

mei-218 Transcription Is Not Limited to the *Drosophila* Ovary

Meiotic prophase occurs in the germarium; thus, it is the region where we would expect meiotic recombination genes to be expressed (Carpenter, 1979). We used *in situ* hybridization to examine the expression pattern of *mei-218* transcription in the ovary using an antisense RNA probe. In the germarium, RNA expression is not localized to any one cell, although it is highly enriched in regions 2 and 3 where the oocyte is in meiotic prophase. After this stage there is a rapid reduction of transcript, beginning in stage 2 of the vitellarium (Figure 2). This pattern seems to reflect a program of meiosis-specific gene expression.

Despite its meiosis-specific mutant phenotype, *mei-218* is transcribed in all tissues that we have studied. RT-PCR analysis has shown that *mei-218* RNA is expressed in tissues in which no mutant defects in phenotype have been observed, including embryos, larvae, and testis (Figure 1). Although the male products appear larger, sequencing of one of these products failed to detect a difference. Consistent with these results, we have observed *mei-218* RNA by *in situ* hybridization in testis (Figure 2). These transcripts are also translated; in the testis we have detected MEI-218 protein using antibodies to the native protein (E.A. Manheim and K.S. McKim, unpublished results).

Localization of the *mei-218* Protein within Germarial 16-Cell Cysts

To analyze the *mei-218* protein expression patterns, we used three different antibodies: two antibodies raised against *E. coli*-expressed MEI-218 fragments and the M5 antibody that recognizes the protein from a FLAG epitope-tagged transgene (Figure 1). The epitope-tagged constructs of *mei-218* were generated using an *hsp83* promoter to express a full-length *mei-218* 4.2-kb cDNA fused at the amino terminus to the FLAG epitope (*hsp83::mei-218*^{FLAG}). In all experiments, expression from the *hsp83* constructs was observed without heat shock because transcription from this promoter occurs at a high level without heat shock in the ovary (Ding *et al.*, 1993). We were able to detect the epitope-tagged protein expressed from the *hsp83* promoter but not the endogenous

Figure 2. Transcription of *mei-218* in wild-type, *egl*, and *orb* mutants by in situ hybridization using antisense *mei-218* RNA probes. In all cases, a sense probe failed to detect a signal. In each image the anterior end of the ovariole or testis is to the left. (A) In wild-type ovaries, *mei-218* RNA appears at a high concentration in the germarium and then is reduced in abundance early in the vitellarium. Whereas the induction of transcription is consistent, the reduction in the vitellarium is variable between ovarioles. Localization to the vitellarium oocyte (arrow) is sometimes visible. (B) *mei-218* transcript in the testis. Unlike oogenesis, the transcript does not show a preference for any particular stage in spermatogenesis. (C) In *egl* mutant ovaries, the transcripts appear at approximately the same time as in wild type but persist longer in the vitellarium. (D) In *orb*^{F343} mutant ovaries, the *mei-218* transcript was detected at a much lower level. This image is at a lower magnification to show low levels of RNA in at least four ovarioles.

protein, by Western blotting using either anti-FLAG or anti-MEI-218 antibodies (E.A. Manheim and K.S. McKim, unpublished results). The cause of this difference can be attributed to the different promoters used. The native protein is limited to the germarium and early vitellarium, whereas the *hsp83*-driven FLAG-tagged protein was expressed at high levels in the germarium and most vitellarium stages. The inability to detect native MEI-218 on a western blot correlates with the notion that small amounts of protein are necessary for wild-type function, and it is subjected to a tightly restricted period of expression.

We performed additional controls by immunofluorescence to ensure that the affinity-purified anti-MEI-218 antibodies were specific to MEI-218. First, the preimmune sera did not recognize MEI-218. Second, the signal from the purified antibody was either reduced or eliminated by incubation with an excess of purified PX antigen (10- or 100-fold molar excess, respectively) but not by a 100-fold molar excess of an unrelated protein also expressed in bacteria and containing the same 6× HIS tag. Furthermore, the same signal was seen with antibodies raised against two different MEI-218 antigens (Figure 3). Finally, qualitatively similar results were obtained using the epitope-tagged version of MEI-218.

We also examined 12 *mei-218* mutants for effects on protein levels or localization. Using the anti-MEI-218 antibodies, we looked at alleles 1, 4, 5, 6, 6-7, 8, *hfn*d, *g2*, *g4*, *g9*, *j1*, and *j2* and found no significant differences in expression or staining pattern from the wild type. These alleles have not been molecularly characterized, but because none of these mutations result in a detectable loss of MEI-218, they are likely not protein-null mutations.

Native MEI-218 Demonstrates a Distinct Pattern of Localization within the Cytoplasm The most striking feature of MEI-218 localization was that it was entirely cytoplasmic (Figure 3). In most experiments MEI-218 was observed at a low but detectable level in region 1 of the germarium. Higher levels of MEI-218 were observed in region 2a and accumulated to their highest levels in regions 2b and 3. This increase in MEI-218 corresponds well with the stage at which the oocyte enters meiosis, region 2a. Rather than clear localization to a single cell, MEI-218 was found in the cytoplasm of several cells within the 16-cell cyst, with more protein in some cells than others. Double-labeling experiments with an antibody to nuclear lamin (Harel *et al.*, 1989) revealed MEI-218 clearly adjacent to, but not overlapping, the lamin (Figure 3). MEI-218 appeared punctate and was not uniform within the cytoplasm, perhaps reflecting accumulations in subcellular compartments. In most of our experiments with the MEI-218 antibodies, weak staining was observed in region 1, suggesting that *mei-218* is expressed at a low level in premeiotic cells. This staining also was reduced or abolished by incubation with excess antigen.

In later stages of wild-type germaria, MEI-218 was present in several cells of the cyst (Figures 3–5). The cytoplasmic MEI-218 staining in stages 3 and 4 of the vitellarium typically showed one of two patterns. In some ovarioles the protein mostly disappeared, except for a small amount of unlocalized punctate staining. In others, however, the reduction of MEI-218 levels in most cells of the cyst was accompanied by strong localization to the oocyte (Figure 3). It is clear that the criterion of oocyte

Figure 3. MEI-218 protein is in the cytoplasm of the germlarium and early vitellarium stage oocytes. In all images the tip of the germlarium is pointed toward the top left. (A) Schematic of the germlarium, showing the stages of meiosis relative to the stages of cyst development. The green shading represents ORB, and the SC in the four-ring canal cells is shown in red. (B) Low magnification view of a wild-type female ovariole showing MEI-218 (red) detected with the PX antibody relative to the chromosomes (blue) and ORB (green) localization. The germlarium region 2b and 3 cysts and the first vitellarium cyst, stage 2, are labeled. This is one of the examples where MEI-218 shows distinct localization to the oocyte in stage 3 and 4 cysts. In other ovarioles, however, the protein gradually disappears in early vitellarium stages without the transient oocyte localization. (C) Similar MEI-218 staining was observed using the A5 antibody. Only the MEI-218 staining is shown. (D) MEI-218 (red) does not overlap with the nuclear lamin (green), showing that it is outside the nucleus. This is a higher magnification view of regions 2 and 3 of a *hsp83::mei-218^{FLAG}* germlarium. Because the PX antibody was used, both the endogenous and transgene protein are observed. Localization of the MEI-218^{FLAG} to the oocyte is visible in region 3 (arrow).

localization does not strictly correlate with *mei-218* function because of the contrast between the absence of discreet oocyte localization when MEI-218 is required in the germlarium (Carpenter, 1979) and its localization when it is not required in the vitellarium.

To investigate if the MEI-218 staining pattern shows a preference for any of the cells within the 16-cell cyst, we performed double-labeling experiments to compare the localization of MEI-218 to ORB. ORB is found in the cytoplasm of each cell of the 16-cell cyst; furthermore, it shows enrichment in the oocyte in late region 2a or early region 2b (Figure 4). In colocalization experiments using anti-MEI-218 and anti-ORB in wild-type flies, a complex staining pattern emerged, and it was difficult to discern any oocyte-specific overlap. The earliest time at which an increase in MEI-218 levels was observed correlated with the earliest unlocalized ORB staining. In addition, only in these earlier stages, i.e., region 2a, was there evidence for a restricted pattern of MEI-218 expression, which may have been enrichment to the oocyte. However, this was difficult to determine in the absence of ORB localization at this stage. MEI-218 clearly showed extensive colocalization with ORB, but there were many cells with abundant

ORB and very little MEI-218. By region 3 there was no oocyte specificity; in fact, in some cases, there was a dearth of staining around the oocyte. In experiments in which cortical actin was stained by phalloidin, the distribution of MEI-218 was clearly within the boundaries and throughout the cytoplasm of the cell (Figure 4).

MEI-218^{FLAG} Shows a Distinct Cytoplasmic Staining Pattern Genetic experiments assaying nondisjunction frequencies were performed to test whether the *hsp83::mei-218^{FLAG}* constructs produced functional protein. All transgenic lines tested rescued the *mei-218* nondisjunction phenotype (Table 1). Similar transgenic constructs that lacked the FLAG epitope tag also reduced nondisjunction to wild-type levels. Furthermore, we were able to visualize MEI-218 in the ovaries of the *hsp83::mei-218^{FLAG}* transgenic flies using the anti-FLAG M5 antibody, as well as anti-MEI-218 antibodies, further demonstrating that the transgenics produced functional protein.

The results using the M5 antibody to detect MEI-218^{FLAG} were similar to the endogenous protein: the pro-

Figure 4. MEI-218 localization relative to the development of the germarium; in each image the anterior end of the germarium is toward the top left, and the bottom panels show only the MEI-218 staining. (A) ORB (green), MEI-218 (red), and DNA (blue) localization in wild-type ovaries (orange color reflects protein overlap). In wild type, both ORB and MEI-218 are visible in region 2a. Later, MEI-218 does not have the same oocyte specificity as ORB. (B) MEI-218 (red) relative to cortical actin (green) and DNA (blue), demonstrating the localization of MEI-218 relative to the cell boundaries.

tein was entirely cytoplasmic, its first appearance was variable but was often visible in region 2a, and it gradually accumulated to higher levels in region 3. There was a striking difference between native and transgenic protein-staining patterns. Unlike the endogenous protein, the transgenic MEI-218 was enriched in a single cell (Figures 3 and 5). This cell probably was the oocyte based on its posterior position in later cysts and, in a separate experiment, colocalization with the ORB protein. Oocyte localization was also observed when the PX antibody was used to detect protein in *hsp83::mei-218^{FLAG}* ovaries (Figure 3–5). To test whether the FLAG tag was responsible for the stronger oocyte localization than seen with the antibodies to the endogenous protein, we used the PX antibody to detect *hsp83*-driven MEI-218 that did not have an epitope tag. This transgenic protein showed the same cytoplasmic staining pattern and strong localization to the oocyte as MEI-218^{FLAG} (Figure 5). It is likely that the difference in localization of the native protein in contrast to the transgenic proteins is regulatory and attributable to the lack of native 5'-UTR and intron sequences in *hsp83* constructs.

Table 1. Rescue of *mei-218⁶* by *hsp83::mei-218^{FLAG}* constructs

Transgenic line	Chromosome	% X ND	% 4 ND	Total
None	–	30.5	10.24	1018
<i>mei-218^{FLAG}</i>				
3	3	0	0	276
4	3	0	0	250
7	2	0	0	198
10	3	0	0	372
<i>mei-218^{FLAG-SV40}</i>				
1A	3	3.45	1.31	1652
2A	3	16.36	4.18	1033
2B	2	2.48	0.48	1087
3B	2	6.05	1.39	769
4C	3	27.84	7.52	511
5A	2	9.34	1.76	1102
6A-1	2	0.35	0.82	1713
6A-2	3	0.17	0.13	2339
7D	3	0.57	0.5	1399

ND, Nondisjunction.

Figure 5. Localization of MEI-218 expressed from the *hsp83::mei-218^{FLAG}* transgene is qualitatively similar to wild type but with specific localization to the oocyte. The anterior ends of the germaria are pointed toward the top left of the frame, and the bottom row shows only the MEI-218 staining. (A) MEI-218^{FLAG} was detected using the M5 antibody (red) and DNA stain (blue). The arrow is pointing to localized staining in the cell most likely to be the presumptive oocyte in region 2a. (B) *hsp83::mei-218⁺* germarium stained with the PX antibody to MEI-218 (red). In this section oocyte-specific staining is visible early in region 2 (arrow). The endogenous MEI-218 is difficult to observe because the genetic background expresses MEI-218 at a low level (see RESULTS and Figure 6). (C) A volume projection of an *egl^{PV27}; hsp83::mei-218^{FLAG}* germarium showing uniform MEI-218 staining.

mei-218 Protein Expression Levels Are Sensitive to Genetic Background

MEI-218 in *egl* and *BicD* Mutants The cytoplasmic nature of MEI-218 led us to investigate whether genes that have a role in germ line differentiation regulate its expression and localization. The *egl* and *BicD* genes are required to localize many factors to the oocyte and restrict oocyte development to a single cell (Theurkauf, 1994). In both *egl^{PV27}* and *BicD^{R26}* females, *mei-218* was transcribed at a higher level than wild type and persisted at this high level into the vitellarium stages (Figure 2). Thus, as compared with wild type, in these mutant backgrounds *mei-218* was turned on normally, but the RNA expression continued much later in oocyte development.

In the course of our experiments with these developmental mutants, we discovered that the MEI-218 expression level was extremely low in some genetic backgrounds. In general, the wild-type Oregon R showed the same levels of MEI-218 expression as several other laboratory stocks. In contrast,

using the same preparation conditions, similarly low levels of MEI-218 were observed in *BicD/CyO*, *BicD/BicD*, *egl/CyO*, and *egl/egl* females. Because of the low signal to noise ratio in these stocks, it was difficult to determine whether *egl* and *BicD* mutants affected the expression and/or localization of MEI-218.

There was a higher signal to noise ratio in *egl; hsp83::mei218^{FLAG}* ovaries that allowed us to evaluate the effect of *egl* on MEI-218. Under these conditions, MEI-218 failed to localize to the oocyte (Figure 5). These results suggest that MEI-218 may respond to gradients in the 16-cell cyst, although this is not obvious in the low-expressing genetic backgrounds. In some cases, we could visualize MEI-218 using a tertiary-labeling technique (MATERIALS AND METHODS). We used this technique on several laboratory stocks and found that a small amount of MEI-218 was enriched in a single cell of the 16-cell cyst, most likely the oocyte in region 2a and b (Figure 6). Although in most genetic backgrounds enrichment of MEI-218 in the oocyte was not observed, these results suggest that in some cases,

Figure 6. Mutational analysis of MEI-218 expression. The anterior end of each germaria is toward the top left of the frame. (A) The MEI-218 staining pattern in an *orb^{F343}/TM3* germarium, an example of a stock with low protein levels detected by tertiary labeling. (B) In an *orb^{F343}* homozygote, variably low MEI-218 protein was observed but with clearly less localization to single cells. (C) Expression of MEI-218 in *hsp83::mei-218^{FLAG-SV40}* transgenic 6A-2, which contains the SV40 3'-UTR. MEI-218 (red) was detected using the M5 anti-FLAG antibody to distinguish the artificially expressed protein from the native MEI-218 (DNA is stained blue). This line rescues the *mei-218* mutant nondisjunction phenotype and shows oocyte localization in the later stages of the germarium (anterior end toward the top left of frame). (D) The same image showing only the MEI-218 channel.

such as when protein levels are low, specific localization to the oocyte within the germarium does occur.

The low protein levels prevented us from determining the role of *egl* and *BicD* in MEI-218 localization. The tertiary labeling necessary to visualize the proteins decreased the signal to noise ratio too significantly to allow a qualitative assessment of the MEI-218 expression and localization defects in the mutant backgrounds.

***mei-218* expression in *orb* mutants** The ORB protein is necessary to promote formation of the 16-cell cyst from the eight-cell stage as well as oocyte differentiation (Lantz *et al.*, 1994). We studied the weaker *orb^{F303}* and stronger *orb^{F343}* alleles to ascertain whether the ORB protein was necessary for proper transcription, translation, or localization of MEI-218. Even although a 16-cell cyst is not formed in *orb^{F343}* ovaries, by in situ hybridization a low level of *mei-218* RNA was detected (Figure 2). In *orb^{F303}* ovaries, in which the 16-cell cyst is formed with an abnormal oocyte that is located in the center of the cyst instead of at the posterior end, there were high levels of *mei-218* transcript qualitatively similar to wild type. RT-PCR analysis confirmed that *mei-218* mRNA was present in both alleles, and consistent with the in situ

hybridization results, lower amounts of RNA were present in *orb^{F343}* as compared with wild type or *orb^{F303}* (E.A. Manheim and K.S. McKim, unpublished results). These results demonstrated that the generation of the 16-cell cyst, but not necessarily normal oocyte differentiation, is required for high levels of *mei-218* transcription.

The genetic background effects on protein levels described above were also observed in the *orb* mutant stocks. In this case, however, we were able to observe low levels of staining in heterozygotes of both alleles, and significantly, this included localization to the oocyte (Figure 6). Consistent with the existence of detectable *mei-218* transcript in both *orb* mutants, we observed small amounts of protein. The localization of MEI-218 appeared abnormal and was not localized to single cells, although, because of the developmental and morphological defects in *orb* mutant germaria, we could not accurately relate MEI-218 staining to the position of the abnormal oocyte (in the case of *orb^{F303}*).

The Role of the 3'-UTR in *mei-218* Expression

Several genes involved in early oocyte development and embryogenesis require their 3'-UTR for proper translation

and localization (Lantz *et al.*, 1994; St. Johnston, 1995). To investigate the relationship between the 3'-UTR, oocyte localization, and protein function, we generated a derivative of the *hsp83::mei-218^{FLAG}* in which the endogenous 3'-UTR was replaced with that from SV40 (*hsp83::mei-218^{FLAG-SV40}*). Unlike the *hsp83::mei-218^{FLAG}* transgenes, these transgenic constructs showed variable results in genetic rescue experiments. In some lines there was complete rescue of the *mei-218* nondisjunction phenotype, whereas in other lines there was partial or no rescue at all (Table 1).

Immunofluorescence analysis of the *hsp83::mei-218^{FLAG-SV40}* transformants that had full rescue of the mutant phenotype showed protein staining similar to the original *hsp83* transgenes with the endogenous 3'-UTR (Figure 6). MEI-218 appeared in region 2 with enrichment in the pro-oocyte in region 2B; this oocyte localization continued into later stages of the vitellarium. On the other hand, in the transformants that showed a complete lack of rescue, there was very little MEI-218^{FLAG-SV40} present, and it was difficult to discern if the protein localized. These results show that the MEI-218 protein has an inherent ability to localize to the oocyte. Therefore, the 3'-UTR may only be important for efficient translation of *mei-218*. Furthermore, position effects were probably responsible for the differences in rescue of the mutant phenotype. Although the *hsp83::mei-218^{FLAG}* transgenes with the endogenous 3'-UTR were also susceptible to position effects, as shown by protein levels assayed by western blot (data not shown), even in the *hsp83::mei-218^{FLAG}* line expressing the least amount of protein, there was enough to detect by immunofluorescence and rescue *mei-218* mutants.

Sequence of *mei-218* in *D. virilis* and *D. yakuba*

No known homologues of *mei-218* have been found in any of the sequence databases. We have cloned and sequenced *mei-218* from *D. yakuba* and *D. virilis* to study the evolutionary conservation of the gene and to identify the important domains for function. *D. yakuba* diverged from *D. melanogaster* ~10–15 million years ago, and *D. virilis* so diverged 40–60 million years ago (Powell and DeSalle, 1995). *mei-218* is situated ~2 kb from the end of *RpS5* (Figure 1), a highly conserved ribosomal protein. Using probes to both *mei-218* and *RpS5*, phage clones containing *mei-218* from *D. yakuba* and *D. virilis* were isolated. The use of the *RpS5* probe was necessary to screen out false positives picked up by the *mei-218* probe detected at low stringency. We obtained the complete sequence from the *D. virilis* gene and a partial sequence from *D. yakuba*.

With the complete sequence of the *D. virilis* gene, we could determine that the structural features of this locus are conserved (Figure 1). The dicistronic nature of *mei-217* and *mei-218* was conserved in *D. virilis*. In fact, the two proteins overlap in both species, and the distance from the *mei-218* ATG to the stop codon of *mei-217* is 20 bp in both species. There are eight introns located at the same positions, and two of these (nos. 2 and 3) are larger than the rest in both species (>100 bp). There are also conserved sequences within the first intron that may be involved in posttranscriptional regulation.

D. virilis MEI-217 shows high similarity (72.25%) and identity (63.87%) to the *D. melanogaster* protein, including sequences upstream of the MEI-217 ATG we had originally predicted (Liu *et al.*, 2000). Based on the sequence of RT-PCR products and conservation in *D. virilis*, it now appears that

the *D. melanogaster* MEI-217 open reading frame begins with an ATG 250 bp upstream of the previously predicted start codon. This is possible because of a splicing event between the two ATGs. One implication of these findings is that the *mei-217^{r1}* mutation, originally thought to be in the 5'-UTR, is actually in the coding sequence and changes a Lys residue to a stop codon. A nonsense mutation was unexpected because this allele is a hypomorph. We also compared the promoter and 3'-UTR sequences in an effort to find regulatory sequences. The promoter region, defined as the sequences between the coding regions of *mei-217* and *RpS5*, did not have significant homology. Only weak similarity was found in the 3'-UTR regions, and it was far less extensive than that observed for some other germ line transcripts such as *nanos* (Gavis *et al.*, 1996).

The amino acid alignment corresponds approximately to visible domains in the *mei-218* protein. Although not conserved and lacking known motifs, the protein can be divided into three domains: a basic amino-terminal domain, a central acidic domain, and a carboxyl-terminal hydrophobic domain (Figure 7). It was not possible to accurately align the amino acids of the basic domain of MEI-218 with the corresponding *D. virilis* sequence because of the poor homology. In addition, the *D. virilis* sequence is 191 amino acids shorter than *D. melanogaster*. However, the chemical properties of this region are conserved: in both proteins this region is basic, hydrophilic, and rich with Gln and Ser. In *mei-218*, the motif (Q)₂₋₅K is repeated six times and the *D. virilis* sequence is also Q-rich but with a different structure of repeats. These similarities show that the lack of conservation does not mean that this region is not important but signifies that the region's crucial features are chemical properties rather than the sequence of amino acids.

The central region of both proteins again is not highly conserved but is slightly acidic and hydrophilic. The only highly conserved region is the more hydrophobic C-terminal region with 71.8% similarity and 63.8% identity. Similarly, this same pattern of conservation was found in *D. yakuba* with a more highly conserved C-terminal domain than the central domain.

DISCUSSION

Meiosis in *Drosophila* occurs within a complex developmental context. The occurrence of meiosis at a specific time within a unique tissue implies that the genes required for meiotic recombination and oocyte differentiation are regulated by the same factors. Our observations of MEI-218 support this view and suggest that both transcriptional and posttranscriptional modes of regulation are important for meiotic recombination genes.

Even though *mei-218* has a critical role in meiosis (it is required for >90% of meiotic crossing-over), the protein sequence has evolved rapidly. Our sequence analysis suggests that there are three separate domains within MEI-218 diverging at different rates. The sequences from *D. yakuba* and *D. virilis* show that the C terminus of MEI-218 is under selection within the genus of *Drosophila*. The other domains of *mei-218*, however, appear to be evolving at a rapid rate because they are less conserved and in some cases unrecognizable based on simple sequence comparisons. Begun and Whitley (2000) made a similar conclusion regarding the

Figure 7. Schematic showing the alignment of the *mei-218* region in the three *Drosophila* species. The percentage of identity or similarity reflects a comparison of the species to *D. melanogaster* only. The *D. yakuba* sequence is not complete, and therefore, the amino acid coordinates are not shown.

evolution of *mei-218* based on a partial sequence of *mei-218* from several isolates of *Drosophila simulans*.

Regulation of *mei-218* RNA and Protein Expression

Transcriptional Regulation Our analysis of *mei-218* shows a specific pattern of transcription in the ovary. We believe that activation of *mei-218* transcription occurs when the 16-cell cyst forms. In support of this hypothesis, *mei-218* transcription occurs at a very low level in a strong *orb* mutant that fails to make a 16-cell cyst. ORB is proposed to be a translational regulator (Lantz *et al.*, 1992), and its effects on *mei-218* expression may be indirect. For example, transcription of *mei-218* and other genes required for meiotic recombination may occur at a high level only if a 16-cell cyst forms.

Consistent with a role limited to meiotic recombination, the *mei-218* RNA appears in region 2a of the germarium and rapidly disappears early in the vitellarium, by which time most steps of meiotic recombination have been completed (Carpenter, 1979). Therefore, the timing of *mei-218* transcription is coincident with the period in oogenesis during which recombination is occurring. The reduction in *mei-218* expression is sensitive to meiotic progression; as prophase continues, *mei-218* expression is reduced. Turning off *mei-218* transcription may require the completion of a specific meiotic event, and this signal may not materialize in either *egl* or *BicD* because the meiotic prophase is terminated early. Although the SC rapidly disappears in *egl* mutants with the reversion to nurse cells in the germarium (Carpenter, 1994; Huynh and St. Johnston, 2000), *mei-218* RNA and protein expression persist at higher levels than normal.

The *mei-218* pattern of transcription may be a paradigm for genes specifically required for meiotic recombination. We have observed the same pattern with other meiotic recombination genes, albeit at different levels of expression (McKim and Hayashi-Hagihara, 1998; Liu and McKim, unpublished data). In contrast, genes like *mei-9* and *spo76* that are required for both meiotic recombination and more general functions such as DNA repair have a ubiquitous pattern of expression throughout the vitellarium (Jang, Liu, and McKim, unpublished results).

Whereas transcription of *mei-218* within the germ line appears to be tightly controlled, transcription is not limited to female meiosis. We have detected the transcript by RT-PCR in embryos, larvae, and testis. A recurring theme in *Drosophila* is that genes required only for meiosis are transcribed in a wide variety of tissues. DSB formation is meiosis specific, but the genes required for this event, such as *mei-W68* and *mei-P22*, are transcribed in testis where there is no meiotic recombination (McKim and Hayashi-Hagihara, 1998). cDNA clones for the *c(3)G* gene product, a component of the meiosis-specific SC, have also been isolated from several tissues, including the head (Rubin *et al.*, 2000). Thus, transcriptional regulation does not appear to be the mechanism to restrict meiotic recombination to the presumptive oocyte. We have failed to detect conserved sequences in the promoters of *Drosophila* meiotic recombination genes (McKim, unpublished results) or between the *mei-218* promoter of *D. melanogaster* and *D. yakuba*. It is likely that most meiotic genes are regulated by generic promoters because, unlike *Saccharomyces cerevisiae* (Vershon and Pierce, 2000), there does not seem to be meiosis-specific promoter sequences and gene regulation.

Posttranscriptional Regulation Although MEI-218 did not show specificity to the oocyte in the germarium in most genotypes, it did show a restricted pattern, including uneven distribution between cells in a cyst and punctate appearance within each cell. This MEI-218 localization may reflect subcellular structures such as the Golgi apparatus or endoplasmic reticulum. In addition, whereas the RNA is rapidly induced in region 2a, the protein is initially present at very low levels and then gradually accumulates to a peak in region 3. The pattern of protein accumulation followed by a rapid decline is similar to the observation of Carpenter (1979) that late RNs first appear in region 2a, accumulate to higher numbers in region 2b, and then decline in region 3. It is striking, however, that distinct enrichment to the oocyte is not required for native MEI-218 to function. A similar situation has been observed with BICD and EGL, which are required to restrict SC formation to the two four-ring canal cells before they are localized (Carpenter, 1994; Huynh and St. Johnston, 2000). Thus, it may be common that proteins

required for oocyte differentiation may not demonstrate oocyte localization at the time they are first required.

Because, at least in part, of transport via a polarized microtubule network, many proteins and RNAs are asymmetrically distributed to the oocytes (Theurkauf, 1994). Genes like *egl* and *BicD* are required for the microtubule gradient and the localized expression of oocyte determinants. In most cases preferential localization to the oocyte of native MEI-218 was not observed, but there was evidence that MEI-218 has the capacity for oocyte localization. The protein was often found localized to the oocyte in early vitellarium cysts and in the germarium of some genetic backgrounds. In addition, strong oocyte localization was always observed with *hsp83*-driven transgenes; this localization was disrupted in *egl* mutants. The differences in native MEI-218 localization could be related to protein levels, but given that the transgenes express high levels of protein and that the *mei-218*-coding region is part of a complex transcription unit, posttranscriptional regulation may have an important role in MEI-218 localization.

Whereas 3'-UTR sequences are required for RNA and protein localization during *Drosophila* oogenesis (e.g., Lantz and Schedl, 1994), our data showed that the 3'-UTR was not essential for MEI-218 localization. We have also shown that the endogenous 3'-UTR is not absolutely essential for expression and rescue of the mutant phenotype; its presence seems only to increase the level of protein expression. This explains why *hsp83::mei-218^{FLAG}*, but not *hsp83::mei-218^{FLAG-SV40}*, transgenes always fully rescue both the *mei-218* crossing-over and nondisjunction mutant phenotypes. Because we can see oocyte localization of the *hsp83::mei-218^{FLAG-SV40}*-produced protein in the rescuing lines that lack any endogenous UTR sequences, our data suggest that MEI-218 likely has an inherent ability to localize to the oocyte. Alternatively, localization may solely depend on efficient translation of the message.

Models for the Role of *mei-218* in Meiotic Crossover Formation

The original DSB repair model proposed that gene conversion and crossing-over were alternative resolutions of the same intermediate whose fate depends on which strands of the Holliday junction are cut. Evidence from several organisms is inconsistent with this feature of the DSB model. Studies of a variety of organisms have shown that the alternative outcomes of the Holliday junction do not occur with equal frequency (Carpenter, 1990; Kleckner, 1996). The relative frequency of gene conversion to crossover events at the *rosy* locus of *D. melanogaster* is ~5:1 (Hilliker *et al.*, 1988). These results suggest that the alternative outcomes, gene conversions and crossovers, result from different repair and resolution reactions. Thus, crossing-over may require a specific set of proteins to orchestrate a modified pathway of DSB repair that promotes crossover resolution of the Holliday junction but is not required for gene conversion.

The strong phenotype of *mei-218* mutants, in which crossing-over but not gene conversion (Carpenter, 1982) or DSB formation (Liu *et al.*, 2000; McKim *et al.*, 2000) are reduced by >90%, is consistent with a meiosis-specific repair pathway for generating crossovers. We propose that crossing-over in *Drosophila* is the result of a *mei-218*-dependent meiosis-

specific pathway of DSB repair and resolution. Cytoplasmic localization of MEI-218 is consistent with an indirect role in crossover formation, perhaps in the regulation of other gene products required for crossing-over. In this model, MEI-218 functions in the cytoplasm to regulate other proteins or possibly control the access of proteins involved in building late RNs and generating crossovers to the oocyte nucleus. A regulatory role is not without precedent: hypomorphic alleles of *Sxl* (K. Cook, personal communication; Bopp *et al.*, 1999) and *mei-P26* (Page *et al.*, 2000), both genes with primary roles in germ line differentiation, have crossover phenotypes similar to *mei-218*. Therefore, it is possible that MEI-218 functions in the pathway between germ line differentiation and realization of meiotic recombination events. The lack of oogenesis defects in *mei-218* mutants suggests that the position of *mei-218* is after the point at which the meiotic and developmental pathways diverge.

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