Genetic Interactions Between *mei-S332* and *ord* in the Control of Sister-Chromatid Cohesion

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ABSTRACT

The Drosophila *mei-S332* and *ord* gene products are essential for proper sister-chromatid cohesion during meiosis in both males and females. We have constructed flies that contain null mutations for both genes. Double-mutant flies are viable and fertile. Therefore, the lack of an essential role for either gene in mitotic cohesion cannot be explained by compensatory activity of the two proteins during mitotic divisions. Analysis of sex chromosome segregation in the double mutant indicates that *ord* is epistatic to *mei-S332*. We demonstrate that *ord* is not required for MEI-S332 protein to localize to meiotic centromeres. Although overexpression of either protein in a wild-type background does not interfere with normal meiotic chromosome segregation, extra ORD$^+$ protein in *mei-S332* mutant males enhances nondisjunction at meiosis II. Our results suggest that a balance between the activity of *mei-S332* and *ord* is required for proper regulation of meiotic cohesion and demonstrate that additional proteins must be functioning to ensure mitotic sister-chromatid cohesion.

DURING mitosis, proper segregation of the replicated sister chromatids to daughter cells requires that each sister chromatid in a pair attach to microtubules emanating from opposite spindle poles. A stable configuration of bipolar attachment may be achieved only when tension from polar microtubule attachment is counteracted by associations between the sister chromatids. Consequently, sister-chromatid cohesion is an essential element of proper chromosome segregation. Release of cohesion appears to be the limiting event that permits anaphase chromosome movement (for review see Miyazaki and Orr-Weaver 1994). Sister chromatids are likely to be attached to one another by chromosomal proteins that are released, inactivated, or degraded at the metaphase/anaphase transition.

Sister-chromatid cohesion also is required for proper chromosome segregation during meiosis. However, its regulation is more complex than mitotic cohesion (for review see Bickel and Orr-Weaver 1996). During meiosis, two rounds of chromosome segregation follow a single doubling of the DNA. In the first division, the homologs pair and segregate, and sister chromatids migrate to the same pole. Therefore, unlike mitosis, cohesion between sisters must be maintained at the first metaphase/anaphase transition during meiosis. Not until the second meiotic division is cohesion completely released, allowing the sisters to segregate from each other.

Another difference in the regulation of cohesion in mitosis and meiosis is that sister-chromatid cohesion is lost in a two-step process during meiosis: arm and centromeric cohesion are released at different times. In meiosis I the sister chromatids are attached along their entire length, as they are in mitosis. At the metaphase I/anaphase I transition, sister-chromatid arm associations are released. However, centromeric cohesion remains intact. Sister chromatids remain stably attached at their centromeres until the metaphase II/anaphase II transition when this cohesion is abolished.

Meiotic cohesion not only ensures that sisters stay connected until anaphase II, but attachments between sister chromatids may also play a critical role in homolog behavior during meiosis I. In most cases, it is essential that the homologs pair and recombine during meiosis I so that they orient and segregate correctly (Hawley 1988). Exchange between homologs may be promoted if sisters are held together as a unit (Kleckner 1996). In addition, cohesion along the sister-chromatid arms during meiosis is postulated to stabilize the chiasmata that in turn attach the homologs together (Darlington 1932; Maguire 1993).

It is reasonable that the underlying physical basis for sister-chromatid cohesion is conserved between mitosis and meiosis. However, the requirement for cohesion to persist through the first meiotic division and to be released in a step-wise manner probably necessitates

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meiosis-specific release mechanisms. One possibility is that mitotic cohesion functions are utilized during meiosis but have been modified to: (1) facilitate homolog associations; (2) direct recombination events to occur between homologs; and (3) maintain sister-chromatid cohesion until anaphase II (Kleckner 1996).

The Drosophila *mei-S332* and *ord* genes encode proteins that are essential for sister-chromatid cohesion in meiosis (Davis 1971; Mason 1976; Goldstein 1980; Kerrebrock *et al.* 1992, 1995; Miyazaki and Orr-Weaver 1992; Bickel *et al.* 1996). When these genes are mutated, the sister chromatids prematurely separate and segregate randomly. Interestingly, the time at which premature sister separation occurs differs between the two mutants, even with null alleles. In *ord* mutants, sister chromatids separate early in meiosis I and appear to segregate randomly through both meiotic divisions. *ord* flies exhibit predominantly meiosis I nondisjunction, but also meiosis II nondisjunction. In contrast, sister-chromatid associations are normal in *mei-S332* mutants until late anaphase I when they release inappropriately. As a consequence, *mei-S332* flies display meiosis II nondisjunction. Note that we use the term "nondisjunction" in the genetic sense rather than mechanistic. Instead of failing to disjoin, chromosomes in *mei-S332* and *ord* mutants prematurely disjoin. The resulting random segregation produces gametes lacking a particular chromosome or containing two copies of it, the genetic diagnostic for nondisjunction.

These observations suggested that *ord* is necessary for cohesion early in meiosis and acts along the length of the sister chromatids, while *mei-S332* acts specifically at the centromere and therefore is essential only after arm cohesion is released at the metaphase I/anaphase I transition. Consistent with this proposal, MEI-S332 protein was found to localize specifically to the centromere regions of meiotic chromosomes (Kerrebrock *et al.* 1995). The protein binds to the centromeres in prometaphase I, persists at the centromeres during anaphase I, and is released or degraded at the metaphase II/anaphase II transition. What is required for MEI-S332 association with the chromosomes and what signals its release or degradation are currently unknown.

Although ORD and MEI-S332 are required for proper sister-chromatid cohesion during the meiotic divisions, they are not essential for mitotic divisions in somatic tissues. Flies that are null mutants for either gene are fully viable, and aberrant mitotic figures are not present in neuroblasts (Miyazaki and Orr-Weaver 1992; Kerrebrock *et al.* 1995; Bickel *et al.* 1996; J. Wu and T. Orr-Weaver, unpublished results). Despite the lack of mitotic defects, MEI-S332 indeed functions during mitosis. MEI-S332 protein is found at the centromere region of mitotic chromosomes and dissociates when the sister chromatids separate (Moore *et al.* 1998; H. Leblanc, T. T.-L. Tang, J. Wu and T. L. Orr-Weaver, unpublished results). Furthermore, if mitotic cells are arrested in metaphase by drug-induced disruption of the spindle, and arm cohesion is released by hypotonic treatment, the sister chromatids prematurely separate in *mei-S332* mutants (H. Leblanc, T. T.-L. Tang, J. Wu and T. L. Orr-Weaver, unpublished results). Although it participates in mitotic sister-chromatid cohesion, MEI-S332 may not be essential because there are redundant mitotic functions. One possibility is that ORD provides the cohesion activity that compensates for lack of MEI-S332 activity in *mei-S332* mutants during mitosis.

In this article, we address whether *mei-S332* and *ord* have redundant functions in mitosis by analyzing double mutants. We also further investigate the relationship between ORD and MEI-S332 activity during meiosis. In order to begin to dissect the role of MEI-S332 and ORD in centromeric cohesion, the localization of MEI-S332 protein is examined in spermatocytes of *ord* mutants. In addition, we evaluate the consequences of changing the relative dosage of the two gene products by monitoring meiotic chromosome segregation in flies carrying extra copies of either gene.

MATERIALS AND METHODS

Stocks: All Drosophila stocks and crosses were raised at 25° on standard cornmeal-brewer's yeast-molasses-agar food. *mei-S332^{1}* was originally described by Davis (1971). All other *mei-S332* alleles were isolated and described by Kerrebrock *et al.* (1992). Mason (1976) characterized *ord^{1}*. *ord* alleles 2 through 6 were isolated by Miyazaki and Orr-Weaver (1992) and *ord* alleles 7 through 12 were described in Bickel *et al.* (1997). The deficiency chromosome *Df(2R)WI370* deletes the *ord* gene (Bickel *et al.* 1996) and *Df(2R)X58-6* uncovers *mei-S332* (Kerrebrock *et al.* 1995). The iso-*X*/*Y*, compound-*X*, and compound-*XY* stocks were described in Kerrebrock *et al.* (1992). When possible, segregation tests were performed on stocks containing the iso-*X*/*Y* chromosomes to minimize variability in the recovery of sex chromosomes in segregation tests. Flies carrying transposon constructs were tested in a *Df(1)y w^{67c23}* background containing the iso-*y^{+}Y* chromosome.

Flies carrying four additional copies of the *ord* gene were homozygous for insertions of the pCoSpeR construct *P{ord^{+} D39}* (Bickel *et al.* 1996) on the second and third chromosomes. *P{ord^{+} D39}* contains ~18 kb of genomic DNA and fully rescues the *ord* mutant phenotype. For genetic tests analyzing the effect of extra copies of the *ord* gene in a *mei-S332* mutant background, smaller CaSpeR4 *ord^{+}* transposon constructs were utilized (Table 4). *P{ord^{+} 6.3BB}* and *P{ord^{+} 7.3BP}* contain insertions of 6.3 kb and 7.3 kb, respectively (Bickel *et al.* 1996). Both *P{ord^{+} 6.3BB}* and *P{ord^{+} 7.3BP}* have been shown to complement *ord* mutations.

Flies carrying four extra copies of the *mei-S332* gene contained homozygous insertions of *P{mei-S332^{+} 5.6KK}* (Kerrebrock *et al.* 1995) on the second and third chromosomes. This 5.6-kb *Kpn*I fragment in CaSpeR4 fully complements *mei-S332* mutations. A single insertion of *P{mei-S332^{+} 5.6KK}* on the third chromosome was tested for its effect in an *ord* mutant background.

Construction of *mei-S332^{1} ord^{10}* double-mutant chromosomes: *ord* lies 3 cM distal to *mei-S332* on the right end of the second chromosome. In order to avoid homozygosity for other mutations on the *mei-S332* chromosome when examining the phenotype of the double mutant, we constructed two different recombinant chromosomes using different starting *mei-S332^{1}* chromosomes: *pr cn mei-S332^{1} px sp* and *cn mei-S332^{1} px*. Trans-

heterozygotes carrying *pr cn mei-S332^{1} px sp* over *cn mei-S332^{1} px* were fully viable and fertile. *cn ord^{10} bw sp If/SM1* females were crossed to *pr cn mei-S332^{1} px sp/SM1* and *cn mei-S332^{1} px/SM1* males. Recombinant chromosomes were then recovered from *ord^{10}/mei-S332^{1}* mothers by mating them to *b cn px sp/SM1* fathers and scoring for males that were cn px If. *Sco/SM1* females were crossed to individual recombinant males to generate multiple lines for each recombinant chromosome. Segregation tests were performed on a small scale to test each recombinant chromosome over an *ord^{1}* chromosome as well as the *Df(2R)X58-6* chromosome. These tests confirmed that mutant alleles of *ord* and *mei-S332* resided on each recombinant chromosome. Recombinant chromosomes were crossed into an iso-*X*/*Y* background for further experiments.

To examine segregation in the double mutant, we tested different *mei-S332^{1} ord^{10}* double-mutant chromosomes as transheterozygotes and scored them separately. Because the frequency of each class of gametes was indistinguishable between the sets, the data were pooled. The values listed in Table 1 represent the pooled data.

For comparison, the *cn mei-S332^{1} px ord^{10} If* double mutant chromosome was tested over the *cn ord^{10} bw sp If* and *pr cn mei-S332^{1} px sp* chromosomes. Only one combination was used for the *ord^{10}/mei-S332^{1} ord^{10}* experiment. Three *mei-S332^{1}/mei-S332^{1} ord^{10}* combinations were tested.

Nondisjunction tests: The frequency of sex chromosome nondisjunction in males and females was measured as described in Kerrebrock *et al.* (1992). By mating mutant *y/y^{+}Y* males to attached-*X, y^{2} su(w^{a}) w^{a}* females or mutant females to attached-*XY, v f B* males, gametes bearing all normal and most exceptional sex chromosome constitutions were recoverable and distinguishable. In female tests, all regular *X* gametes but only half of the total number of exceptional gametes were recoverable. To compensate for this, the total frequency of nondisjunction was calculated by doubling the number of exceptional progeny and dividing by the adjusted total. The adjusted total equals the number of progeny in the normal class plus twice the number of progeny in the exceptional classes.

Dosage effects in mutant backgrounds: Crosses were set up to generate sibling flies of a specific genotype with or without a given transposon on the third chromosome. The experiments to test the effect of ORD overexpression in *mei-S332* mutant males were performed in three separate experiments. The same marked *mei-S332* and *Df(2R)X58-6* chromosomes were used for all three, but the flies were constructed differently. In all three cases, a more extreme phenotype was observed for all *mei-S332* alleles tested. The data in Table 4 are a subset of the total data collected and include segregation tests from all three experiments.

In the first set of tests, *y/y^{+}Y; cn mei-S332^{n} px sp/SM1* males were crossed to *y w/y w; +/+; P{ord^{+} 6.3BB}/P{ord^{+} 6.3BB}* virgins. *y w/y^{+}Y; cn mei-S332^{n} px sp/+; P{ord^{+} 6.3BB}/+* males were then mated to *y w/y w; Df(2R)X58-6/SM1* females. Sibling *y w/y^{+}Y; cn mei-S332^{n} px sp/Df(2R)X58-6; P{ord^{+} 6.3BB}/+* and *y w/y^{+}Y; cn mei-S332^{n} px sp/Df(2R)X58-6; +/+* males were assayed for sex chromosome nondisjunction in parallel. Unambiguous identification of *mei-S332^{n}/Df* flies was possible because *Df(2R)X58-6* also uncovers *px*.

For the second set of tests, *y w/y w; Df(2R)X58-6/SM1; P{ord^{+} 6.3BB}/+* virgins were mated to *y/y^{+}Y; mei-S332^{n}/SM1* males and sibling *y w/y^{+}Y; mei-S332^{n}/Df(2R)X58-6* males with and without the transposon were tested.

The third set of tests assayed the effect of a different *ord^{+}* transposon on the third chromosome. *P{ord^{+} 7.3BP}* was crossed into *mei-S332$^{1,2,7\ and\ 8}$* backgrounds. Then *y w/y^{+}Y; mei-S332^{n}/+; P{ord^{+} 7.3BP}/+* males were mated to *y w/y w; Df(2R)X58-6/SM1* females and sibling males were tested (Table 4). Females generated from this cross also were tested (data not shown).

Other transposon constructs inserted on the third chromosome that do not contain the *ord* gene have been tested previously in *mei-S332* rescue experiments and do not suppress or enhance the *mei-S332* phenotype (Kerrebrock *et al.* 1995; A. Kerrebrock, unpublished results). Because the same *y w* background was utilized, we also can rule out the possibility that modifiers on the third chromosome are responsible for any effects.

The reciprocal experiments to test whether increased dosage of *mei-S332^{+}* affected the *ord* mutant phenotype also were performed. For these experiments, we utilized a weak allele, *ord^{8}*. We chose this allele because it exhibits negative complementation, an unusual genetic property that indicates that protein-protein interactions are required for normal ORD function (Bickel *et al.* 1996, 1997; Bickel and Orr-Weaver 1998). The *ord^{8}* mutation has a high level of residual activity, but this is poisoned by other missense mutations. We were interested in what effects the increased *mei-S332^{+}* dosage would have under conditions in which negative complementation was occurring. Therefore we tested the effect of extra MEI-S332 in *ord^{8}/Df* and *ord^{8}/ord^{2}* flies. In addition, we attempted to examine the consequences of increased *mei-S332^{+}* dosage in *ord* null flies. However, the fertility of *ord^{10}/Df* flies in a *y w* background was too low to obtain statistically meaningful results.

For these MEI-S332 dosage experiments, *y w/Y; +/+; P{mei-S332^{+} 5.6KK}/P{mei-S332^{+} 5.6KK}* males were crossed to *y w/y w; Tft/SM6* virgins and *y w/y w; Tft/+; P{mei-S332^{+} 5.6KK}/+* virgins collected. These were mated to *y/y^{+}Y; cn ord^{8} bw sp If/SM1* and *y/y^{+}Y; cn ord^{10} bw sp If/SM1* males to generate *y w/y^{+}Y; ord^{n}/Tft; P{mei-S332^{+} 5.6KK}/+* males that were crossed to *y w/y w; Df(2R)WI370/CyO* or *y w/y w; ord^{2}bw/SM1* virgins. Male and female *ord^{8}/Df*, *ord^{8}/ord^{2}*, and *ord^{10}/Df* transheterozygotes with and without the *mei-S332^{+}* transposon were tested.

For all dosage experiments, a 2×2 (normal and exceptional gametes) χ^2 contingency analysis (Lindren *et al.* 1978) was used to determine if differences in nondisjunction frequencies were statistically significant when comparing siblings with and without a given transposon.

Localization of GFP-MEI-S332 protein in *ord* spermatocytes: The transposon construct carrying the *mei-S332^{+}-GFP* fusion gene is described in Kerrebrock *et al.* (1995). The wild-type spermatocyte shown in Figure 1 was isolated from males carrying a transposon on the *X* chromosome (insertion GrM13) as well as one on the second chromosome (insertion GrM1; Kerrebrock *et al.* 1995). Mutant *ord* males contained only the insertion GrM13 on the *X* chromosome. A single copy of the transposon is sufficient to rescue *mei-S332^{7}/Df(2R)X58-6* males and females.

Testes were dissected and fixed as described previously (Kerrebrock *et al.* 1995). Epifluorescence microscopy was performed on a Nikon (Melville, NY) Optiphot-2 microscope equipped with Nikon 60× and 100× oil immersion objectives and a Photometrics (Tucson, AZ) Image Point cooled CCD video camera was used to photograph the images. The images were processed using Adobe Photoshop 3.0 run on a Power Macintosh 8100/80. For Figure 1C, images from adjacent focal planes were cut and pasted together in order to show all the chromosomes.

RESULTS

Double mutants for *mei-S332* and *ord*: The genetic analysis of *mei-S332* and *ord* mutants demonstrated that the genes are essential only for meiosis. It seems likely, however, that the same or similar proteins would act to maintain sister-chromatid cohesion in mitosis and in

meiosis. MEI-S332 does localize to the centromeres of mitotic chromosomes, and, although it appears to function in mitosis, it is not essential (Moore *et al.* 1998; H. Leblanc, T. T.-L. Tang, J. Wu and T. L. Orr-Weaver, unpublished results). Because ORD is the only other protein known to be essential for sister-chromatid cohesion in Drosophila, we tested whether ORD compensates for MEI-S332 in mitosis by analyzing double mutants. Recombinant chromosomes were generated that contained likely null alleles for both genes. ord^{10} is a stop codon at the N terminus of the protein (Bickel *et al.* 1997), and the $mei\text{-}S332^{1}$ allele is a large insertion midway through the coding sequence (Kerrebrock *et al.* 1995).

If *mei-S332* and *ord* play a significant role in mitosis but have redundant activities, then we would expect the double mutants to have reduced viability. We crossed heterozygous flies and measured the recovery of double-mutant flies relative to heterozygous siblings. In these tests the double-mutant progeny were recovered at the expected frequency (517 or 65.4% $mei\text{-}S332^{1}\ ord^{10}/SM1$ siblings to 273 or 34.5% $mei\text{-}S332^{1}ord^{10}/mei\text{-}S332^{1}ord^{10}$ siblings). Thus the *mei-S332 ord* double mutants were fully viable. Drosophila development to produce viable adults can still occur despite considerable cell death, but mitotic errors can nevertheless be recognized by developmental defects in diploid imaginal tissues leading to rough eyes, missing bristles, or etched cuticles. The *mei-S332 ord* double mutants did not exhibit any of these phenotypes. Therefore the lack of an essential mitotic role for each gene cannot be explained by compensatory activity of the other gene.

The double-mutant chromosomes also permitted us to compare the roles of the *mei-S332* and *ord* genes in controlling sister-chromatid cohesion in meiosis. If these genes maintain cohesion by distinct mechanisms, then a synthetic meiotic phenotype might occur in the double mutants. A likely synthetic consequence of both genes being mutated would be that chromosome segregation would be so aberrant as to result in sterility. We scored the numbers of progeny produced by $mei\text{-}S332^{1}ord^{10}$ double-mutant females and males and found that the fertility was not depressed below the low levels observed in *ord* null mutants alone.

Because the double mutants were not synthetically sterile, we were able to investigate the meiotic functions of these genes further by scoring the meiotic nondisjunction events in the double-mutant males and females. It has been observed that in *ord* mutants sister chromatids can be found prematurely separated as early as prometaphase I in spermatocytes. Furthermore, the meiotic segregation patterns of marked sex chromosomes can be explained by separation of the sister chromatids before meiosis I followed by random segregation. Our interpretation has been that *ord* is required for sister-chromatid cohesion early in meiosis I (Miyazaki and Orr-Weaver 1992). In contrast, we proposed that *mei-S332* does not become essential until after arm cohesion has been released at anaphase I and the sister chromatids are attached only at their centromeres (Kerrebrock *et al.* 1992). If these two proposals are correct, then *ord* should be epistatic to *mei-S332*, and segregation patterns typical of *ord* mutants should occur in the double mutants.

In double-mutant males a total of 50.8% nondisjunction of the marked sex chromosomes was observed, and the ratios between the classes of exceptional sperm closely mirrored those observed in $ord^{10}/mei\text{-}S332^{1}ord^{10}$ males (Table 1). These ratios also are similar to the theoretical values predicted from random segregation of separated sister chromatids during both meiotic divisions. In contrast, $mei\text{-}S332^{1}/mei\text{-}S332^{1}ord^{10}$ control males exhibited the *mei-S332* mutant phenotype, giving rise to *XX* and nullo-*X* exceptional sperm during meiosis II.

In female sex chromosome segregation tests, we also found that the double mutant displayed levels of sex

TABLE 1

Sex chromosome nondisjunction in *ord mei-S332* males

Genotype	Regular sperm (%)		Exceptional sperm (%)				Total progeny	Total nondisjunction (%)
	X	*Y(Y)*	*O*	*XY(Y)*	*XX*	*XXY(Y)*		
$\frac{mei\text{-}S332^{1}\ ord^{10}}{mei\text{-}S332^{1}\ ord^{10}}$	25.4	23.8	28.9	16.3	5.2	0.4	3196	50.8
$\frac{ord^{10}}{mei\text{-}S332^{1}\ ord^{10}}$	29.1	21.6	31.8	14.1	3.3	0.2	2426	49.3
$\frac{mei\text{-}S332^{1}}{mei\text{-}S332^{1}\ ord^{10}}$	37.1	25.8	25.1	0.4	11.7	0.0	2272	37.1
Theoretical[a]			32.9	17.1	3.6	2.4		56.0

[a] Theoretical values for the frequencies of each exceptional gamete class resulting from random segregation of the chromatids through both meiotic divisions, taken from Miyazaki and Orr-Weaver (1992).

chromosome nondisjunction comparable to the *ord* mutant. Total nondisjunction in $ord^{10}/mei\text{-}S332^{1}ord^{10}$ females was 54.5% (1404 progeny scored). A similar frequency of 55.8% nondisjunction occurred in the 1391 progeny scored from the double-mutant females. Females carrying $mei\text{-}S332^{1}$ over the double-mutant chromosome had 40.5% total nondisjunction (2238 progeny scored). Thus the *ord* meiotic chromosome segregation phenotype is epistatic to that of *mei-S332* in both males and females.

MEI-S332 localization in *ord* mutant spermatocytes: Given that premature sister-chromatid separation occurs earlier in *ord* mutants than *mei-S332* mutants, we wanted to test whether *ord* was required for MEI-S332 localization. One possibility is that ORD protein activity is directly needed for MEI-S332 localization. In support of this hypothesis, certain alleles of *ord* indicate that *ord* function is necessary for proper cohesion at the centromere during meiosis II (Bickel *et al.* 1997). Another possibility is that ORD function is a prerequisite for MEI-S332 localization but does not directly promote it. For example, the early separation of sister chromatids that occurs in meiosis I in *ord* mutants could preclude MEI-S332 localization. Alternatively, in *ord* mutants the chromosome morphology is altered, so MEI-S332 may be unable to localize. Because the levels of MEI-S332 protein are unaffected in *ord* mutants, we were able to determine directly whether *ord* mutants affected the ability of MEI-S332 to localize to centromeres (T. Tang, S. Bickel and T. Orr-Weaver, unpublished results).

We used the MEI-S332-GFP fusion protein to analyze the localization of MEI-S332 in *ord* mutant spermatocytes. A transposon containing this fusion fully complements *mei-S332* mutants, demonstrating that the fusion protein is functional (Kerrebrock *et al.* 1995). The transposon was crossed into ord^{10}, ord^{3}, and ord^{5} mutants, and spermatocytes were examined from males transheterozygous for the *ord* allele over a deficiency. Like ord^{10}, the ord^{3} and ord^{5} mutations are stop codons that genetically appear to be null alleles (Bickel *et al.* 1996).

In wild-type spermatocytes, MEI-S332-GFP localizes to discrete foci on the chromosomes in prometaphase I (Figure 1A). In all three *ord* mutants we observed MEI-S332-GFP localized onto chromosomes in prometaphase I (Figure 1B). This was seen in 31 primary spermatocytes. To confirm that the foci of localization in *ord* mutants corresponded to the centromere regions, we examined anaphase I figures. In *ord* mutants MEI-S332-GFP localized to the centromeres in anaphase I, distinguishable because they are the chromosomal regions that lead in movement to the poles (Figure 1C). These experiments demonstrate that *ord* function is not a prerequisite for the localization of MEI-S332 protein onto meiotic centromeres.

Because MEI-S332-GFP protein normally is not detectable after the dissociation of sister centromeres in wild-type anaphase II, it was of interest to determine whether MEI-S332 could localize to separated sister centromeres during meiosis I in *ord* spermatocytes. This was technically difficult because the fixation conditions needed to permit the spermatocytes to be squashed flat enough to see separated sister chromatids did not preserve GFP fluorescence. We did, however, find rare cells in which separated sister centromeres were seen protruding from the chromosome mass and still MEI-S332-GFP was localized (Figure 2). Therefore, it appears that MEI-S332 is capable of binding to individual sister-chromatid centromeres. We cannot distinguish whether the sister-chromatid centromeres prematurely separated despite the localized MEI-S332 protein, or whether MEI-S332 assembled onto the single sister chromatids.

Overexpression of MEI-S332 and ORD proteins: We investigated whether overexpressing MEI-S332 or ORD extended sister-chromatid cohesion beyond its normal release point, resulting in aberrant meiotic chromosome segregation. Such an outcome might occur if MEI-S332 or ORD were structural components that maintain cohesion. We established stocks with additional copies of the $mei\text{-}S332^{+}$ gene or the ord^{+} gene and assayed meiotic chromosome segregation in males and females. The stocks contained six total copies of each gene. By Western blot experiments we demonstrated that the proteins indeed were overexpressed in the ovaries and

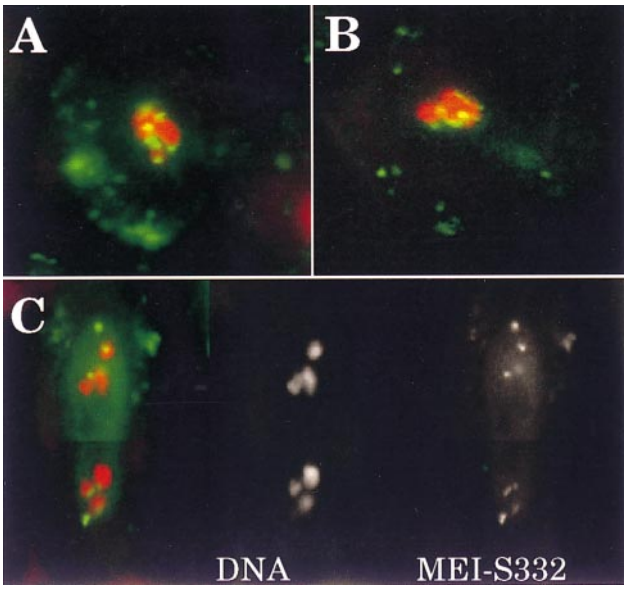

Figure 1.—Localization of MEI-S332-GFP fusion protein in wild-type and *ord* spermatocytes. (A) In wild-type males, discrete foci of MEI-S332-GFP fusion protein (green) are visible on condensed chromosomes (red) during metaphase I. (B) MEI-S332-GFP localization is visible in ord^{10}/Df primary spermatocytes. (C) In ord^{3}/Df spermatocytes, MEI-S332-GFP is seen on the leading edge of chromosomes as they move toward the poles in anaphase I, demonstrating that MEI-S332 is present at meiotic centromeres in the absence of *ord* activity. The upper and lower poles of the spindle were in adjacent focal planes. The three panels are composites that show the appropriate plane for each. Genetically, ord^{10} and ord^{3} behave as nulls.

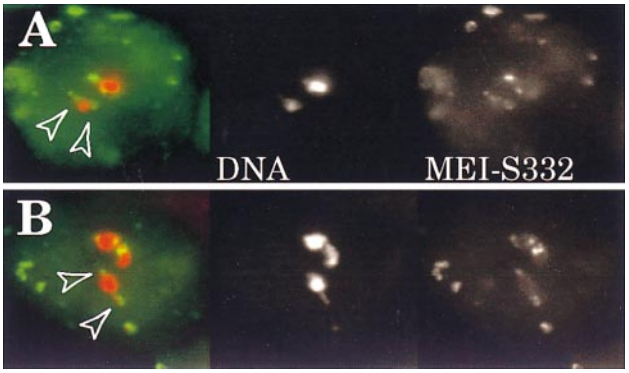

Figure 2.—MEI-S332-GFP is present on separated centromeres in *ord* mutants. (A) In ord^5/Df primary spermatocytes, individual centromeres (arrows) protruding from the chromosome mass (red) still display weak MEI-S332-GFP staining (green). (B) MEI-S332 localization to separated centromeres (arrows) was also observed in ord^1/Df males. ord^5 behaves as a null allele, while ord^1 has some residual function.

testis of these stocks (Moore *et al.* 1998; T. Tang, S. Bickel and T. Orr-Weaver, unpublished results).

In males with six copies of the ord^+ gene, 0.2% nondisjunction of the sex chromosomes was observed. In the presence of six copies of $mei\text{-}S332^+$, 0.3% nondisjunction occurred (Table 2). These numbers are similar to the level of nondisjunction observed in the original *y w* stock into which these transposons were introduced. Similarly, six copies of ord^+ or six copies of $mei\text{-}S332^+$ did not significantly increase meiotic chromosome nondisjunction during female meiosis (Table 3). Thus despite the increased protein levels, sister-chromatid cohesion did not appear to persist longer than with normal dosage. Because neither meiosis I nor meiosis II segregation errors were observed, both sister-chromatid arm and centromere cohesion seem to undergo a timely release in the presence of excess MEI-S332 or ORD.

Dosage effects in mutant backgrounds: An appealing model that is consistent with the *ord* null mutation being epistatic to *mei-S332* is that MEI-S332 protects ORD at the centromere. If MEI-S332 protected ORD, then increased levels of ORD protein might compensate for mutations in *mei-S332* by permitting sister-chromatid cohesion to persist into meiosis II. Suppression of *mei-S332* mutations by increased dosage of ord^+ would be consistent with this model. The effect of an extra copy of ord^+ during male meiosis was tested in six of the *mei-S332* mutants (Table 4). Meiotic chromosome segregation was assayed in transheterozygotes that contained the *mei-S332* allele over a deficiency, comparing siblings with and without an ord^+ transposon on the third chromosome.

Unexpectedly, increasing the dosage of ord^+ enhanced the *mei-S332* phenotype in all six alleles tested and resulted in increased nondisjunction in male meiosis (Table 4). We showed that the enhancement of the *mei-S332* phenotype was not the consequence of a particular ord^+ transposon insertion site by demonstrating similar enhancement with a different ord^+ transformant. Furthermore, this effect was not due to modifiers on the third chromosomes present in the *y w* stock in which transformants were generated (see materials and methods). Although the enhancement of the *mei-S332* phenotype by increased ord^+ indicates that the ratios of these gene products are important, it is not readily consistent with a model in which MEI-S332 solely acts to protect ORD activity at the centromere.

We also examined the effects of increased ord^+ dosage on four *mei-S332* alleles in female meiosis (see materials and methods). In contrast to the effects we observed in males, the ord^+ transposon suppressed nondisjunction in $mei\text{-}S332^7/Df$ females from 50.0 to 43.4%. However, no effect was observed in the other *mei-S332* mutant backgrounds. These results suggest that sufficient levels of ORD can partially compensate in certain *mei-S332* females, but this effect is neither as consistent nor as sensitive as the increase in nondisjunction observed in *mei-S332* males.

Reciprocal experiments to test whether increased dosage of $mei\text{-}S332^+$ affected the *ord* mutant phenotype also

TABLE 2

Sex chromosome nondisjunction in males with extra copies of ord^+ or $mei\text{-}S332^+$

Genotype	Regular sperm (%)		Exceptional sperm (%)				Total progeny	Total nondisjunction (%)
	X	*Y(Y)*	*O*	*XY(Y)*	*XX*	*XXY(Y)*		
$\frac{y\ w}{y^+Y};\frac{+}{+};\frac{+}{+}$	49.9	49.8	0.2	0.1	0.0	0.0	1039	0.3
$\frac{y\ w}{y^+Y};\frac{P\{ord^+\}}{P\{ord^+\}};\frac{P\{ord^+\}^a}{P\{ord^+\}}$	48.2	51.7	0.1	0.1	0.0	0.0	1376	0.2
$\frac{y\ w}{y^+Y};\frac{P\{mei\text{-}S332^+\}}{P\{mei\text{-}S332^+\}};\frac{P\{mei\text{-}S332^+\}^b}{P\{mei\text{-}S332^+\}}$	49.7	50.0	0.1	0.1	0.1	0.0	1383	0.3

[a] These flies contain four copies of the $P\{ord^+\ D39\}$ transposon.
[b] These flies contain four copies of the $P\{mei\text{-}S332^+\ 5.6KK\}$ transposon.

TABLE 3

Sex chromosome nondisjunction in females with extra copies of *ord*$^+$ or *mei-S332*$^+$

Genotype	Regular ova (%)	Exceptional ova (%)		Total progeny	Adjusted total[a]	Total nondisjunction (%)
	X	*O*	*XX*			
$\frac{y\ w}{y^+Y}; \frac{+}{+}; \frac{+}{+}$	99.8	0.1	0.1	2111	2113	0.2
$\frac{y\ w}{y^+Y}; \frac{P\{ord^+\}}{P\{ord^+\}}; \frac{P\{ord^+\}^b}{P\{ord^+\}}$	99.2	0.5	0.3	1878	1886	0.8
$\frac{y\ w}{y^+Y}; \frac{P\{mei\text{-}S332^+\}}{P\{mei\text{-}S332^+\}}; \frac{P\{mei\text{-}S332^+\}^c}{P\{mei\text{-}S332^+\}}$	99.4	0.0	0.6	1787	1792	0.6

[a] The progeny total is adjusted to correct for recovery of only half of the exceptional progeny.
[b] These flies contain four copies of the *P{ord$^+$ D39}* transposon.
[c] These flies contain four copies of the *P{mei-S332$^+$ 5.6KK}* transposon.

were performed (see materials and methods). One additional copy of *mei-S332*$^+$ did not affect the level of nondisjunction in *ord* males (data not shown). In females, an enhancement of the mutant phenotype was observed only in *ord*^{8}/*ord*^{2} flies. One extra copy of the *mei-S332*$^+$ transposon increased nondisjunction from 30.6 to 37.5%.

These experiments demonstrate that changing the dosage of one meiotic cohesion protein when the activity of the other protein is compromised can in some instances affect the level of nondisjunction. This suggests that the balance between ORD and MEI-S332 activity needs to be tightly regulated, with *mei-S332* males being the most sensitive to this balance.

DISCUSSION

Although the significance of sister-chromatid cohesion in ensuring proper chromosome segregation has been long recognized, identification of the responsible proteins is still in early stages. Proteins needed for cohesion in mitosis have been identified from genetic screens (Guacci *et al.* 1997; Michaelis *et al.* 1997) and in complexes isolated from Xenopus extracts (Losada *et al.* 1998). Separate genetic screens have identified genes needed for meiotic sister-chromatid cohesion (Clayberg 1959; Davis 1971; Mason 1976; Moreau *et al.* 1985; Maguire *et al.* 1991; Kerrebrock *et al.* 1992; Miyazaki and Orr-Weaver 1992; Maguire *et al.* 1993; Molnar *et al.* 1995). While it is likely that at least some common cohesion proteins will be used in mitosis and meiosis, most genes identified in one class of genetic screens have not been tested for their role in the other type of division. The Drosophila genes *ord* and *mei-S332* are exceptions in that their role in meiosis has been analyzed extensively, but by genetic and cytological criteria the genes are not essential for mitosis (Moore *et al.* 1998; H. Leblanc, T. T.-L. Tang, J. Wu and T. L. Orr-Weaver, unpublished results). We used double mutants to test whether ORD compensates for MEI-S332 in mitosis. The double mutants are fully viable and do not exhibit any phenotypes consistent with mitotic defects. Thus ORD cannot be the sole protein that is redundant for MEI-S332 in mitosis.

The relationship between ORD and MEI-S332 in controlling sister-chromatid cohesion in meiosis is interesting because recent studies demonstrate that two distinct protein complexes are needed for cohesion in mitosis. The cohesion proteins are present on the DNA during interphase and establish sister-chromatid cohesion at DNA replication (Losada *et al.* 1998). The cohesins are then replaced by condensins as the chromosomes condense in prophase (Hirano *et al.* 1997). Although we have to date been unable to localize the ORD protein onto chromosomes, premature sister-chromatid separation is detectable at prometaphase I in *ord* mutant spermatocytes. This suggests that ORD acts earlier than MEI-S332 and might play a role in establishing cohesion. Because the double mutants were viable and fertile, we were able to analyze the meiotic consequences of absence of both *mei-S332* and *ord*. The *ord* mutant is epistatic to *mei-S332*, an observation that is consistent with the interpretation that ORD is needed for sister-chromatid cohesion earlier in meiosis than MEI-S332.

Given the possibility that ORD establishes cohesion along the chromosomes while MEI-S332 maintains it at the centromere until anaphase II, it is striking that ORD is not necessary for assembly of MEI-S332 onto centromeres. In addition to revealing that ORD is not a prerequisite for MEI-S332 localization, the fact that MEI-S332 can localize onto meiotic centromeres, yet apparently fail to hold the sister-chromatids together, has implications for MEI-S332 action. An interesting possibility is that ORD is needed for MEI-S332 activity but not localization. It is also possible, however, that the localized MEI-S332 observed was assembled onto the centromeres

TABLE 4

Sex chromosome nondisjunction in *mei-S332* males with an extra copy of *ord*$^+$

Genotype	Regular sperm (%)		Exceptional sperm (%)				Total progeny	Total nondisjunction (%)	χ^2
	X	*Y(Y)*	*O*	*XY(Y)*	*XX*	*XXY(Y)*			
$\frac{mei\text{-}S332^1}{Df^a}; \frac{+}{+}$	32.0	36.5	20.5	0.1	10.9	0.0	872	31.5	$P < 0.001$[b]
$\frac{mei\text{-}S332^1}{Df}; \frac{P\{ord^+\ 7.3BP\}}{+}$	29.4	29.4	23.4	0.4	17.4	0.1	749	41.3	
$\frac{mei\text{-}S332^2}{Df}; \frac{+}{+}$	42.0	35.8	15.5	0.2	6.6	0.0	1112	22.2	$P < 0.001$
$\frac{mei\text{-}S332^2}{Df}; \frac{P\{ord^+\ 7.3BP\}}{+}$	34.6	36.8	18.7	0.0	9.8	0.1	1114	28.5	
$\frac{mei\text{-}S332^3}{Df}; \frac{+}{+}$	46.5	40.7	8.4	0.2	4.1	0.0	1316	12.8	$0.05 < P < 0.10$
$\frac{mei\text{-}S332^3}{Df}; \frac{P\{ord^+\ 6.3BB\}}{+}$	43.2	41.3	9.2	0.2	5.9	0.0	1244	15.4	
$\frac{mei\text{-}S332^6}{Df}; \frac{+}{+}$	46.6	37.9	11.3	0.0	4.2	0.0	1090	15.5	$0.01 < P < 0.05$
$\frac{mei\text{-}S332^6}{Df}; \frac{P\{ord^+\ 6.3BB\}}{+}$	49.1	31.7	13.1	0.4	5.7	0.0	1220	19.2	
$\frac{mei\text{-}S332^7}{Df}; \frac{+}{+}$	34.1	36.8	20.0	0.0	9.1	0.0	1032	29.1	$0.01 < P < 0.05$
$\frac{mei\text{-}S332^7}{Df}; \frac{P\{ord^+\ 7.3BP\}}{+}$	34.3	31.5	20.7	0.4	13.1	0.1	1049	34.2	
$\frac{mei\text{-}S332^7}{Df}; \frac{+}{+}$	42.0	34.7	13.6	0.1	9.5	0.0	1028	23.2	$P < 0.001$
$\frac{mei\text{-}S332^7}{Df}; \frac{P\{ord^+\ 6.3BB\}}{+}$	38.3	30.4	21.6	0.1	9.6	0.0	955	31.3	
$\frac{mei\text{-}S332^8}{Df}; \frac{+}{+}$	37.1	37.6	15.2	0.2	9.9	0.0	1005	25.3	$0.001 < P < 0.01$
$\frac{mei\text{-}S332^8}{Df}; \frac{P\{ord^+\ 7.3BP\}}{+}$	34.6	33.6	20.4	0.7	10.7	0.1	914	31.8	
$\frac{mei\text{-}S332^8}{Df}; \frac{+}{+}$	39.8	36.6	15.6	0.4	7.5	0.0	959	23.6	$0.01 < P < 0.05$
$\frac{mei\text{-}S332^8}{Df}; \frac{P\{ord^+\ 6.3BB\}}{+}$	40.6	31.9	17.4	0.5	9.7	0.0	963	27.6	

[a] *Df(2R)X58-6.*

[b] χ^2 contingency analysis was performed for each set of sibling tests to determine whether differences in nondisjunction were statistically significant when comparing flies with and without an *ord*$^+$ transposon.

of sister chromatids that were already separated because of the *ord* mutation. Thus MEI-S332 may have been localized and active, but unable to reattach sister chromatids that were apart.

Although the epistasis results are most simply explained by ORD acting prior to MEI-S332, they also are consistent with the model that ORD is downstream from MEI-S332. One possibility is that the role of MEI-S332 is to maintain ORD activity at the centromere at the metaphase I/anaphase I transition. At this transition cohesion is released along the chromatid arms but persists at the centromeres until anaphase II. If ORD protein acts to attach the sister chromatids, perhaps it requires protection against inactivation at the centromeres at anaphase I. MEI-S332 could maintain cohesion by stabilizing ORD until anaphase II. We did an experiment to test the model that ORD is downstream of MEI-S332 by examining whether overexpression of ORD could suppress *mei-S332* mutations. Although the results from this experiment do not support the model, it remains an intriguing formal possibility that can be evaluated by molecular analysis of the ORD protein.

The dosage studies provide additional insights into the mechanisms by which *ord* and *mei-S332* act. There was no effect when either gene was overexpressed in a background in which the other was wild type and functional. This suggests that the ratio between the wild-type proteins is not critical. An unexpected observation was that, when the *ord* gene was overexpressed in *mei-S332* mutants, the male meiotic phenotype consistently was worsened. Higher chromosome nondisjunction occurred in meiosis II, but the levels of nondisjunction in meiosis I were not elevated. This result is compelling because it was observed with all *mei-S332* alleles tested and with different ord^+ transposons. While overexpressing ord^+ in wild type has no effect, perhaps if MEI-S332 protein is absent or compromised, extra ORD protein is able to play a more pronounced role in promoting cohesion at the centromere. For example, ORD could be similar to the cohesins in assembling early onto the chromosomes and subsequently be replaced by MEI-S332 at the centromeres. When MEI-S332 is not fully functional, the persistent cohesion from ORD at the centromeres may not be properly released at the metaphase II/anaphase II transition, leading to the increased meiosis II nondisjunction observed.

The fact that sister chromatids prematurely separate in meiosis in the single *mei-S332* or *ord* mutants shows that the two proteins do not compensate for each other in meiosis. The double mutants reveal that MEI-S332 and ORD are not redundant for each other in mitotic sister-chromatid cohesion. The results presented lead to several new possibilities for how these proteins maintain cohesion, models that can now be evaluated experimentally. Moreover, it is reasonable to conclude that other proteins contribute to cohesion at least in mitosis, and possibly in meiosis as well. The *mei-S332* and *ord* mutants provide the means to isolate mitotic cohesion functions by screening for synthetic effects on mitosis.

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