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Mutational Analysis of the Drosophila Sister-Chromatid Cohesion Protein ORD and Its Role in the Maintenance of Centromeric Cohesion

Sharon E. Bickel, Dudley W. Wyman and Terry L. Orr-Weaver

Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142

Manuscript received January 22, 1997
Accepted for publication April 21, 1997

ABSTRACT

The *ord* gene is required for proper segregation of all chromosomes in both male and female Drosophila meiosis. Here we describe the isolation of a null *ord* allele and examine the consequences of ablating *ord* function. Cytologically, meiotic sister-chromatid cohesion is severely disrupted in flies lacking ORD protein. Moreover, the frequency of missegregation in genetic tests is consistent with random segregation of chromosomes through both meiotic divisions, suggesting that sister cohesion may be completely abolished. However, only a slight decrease in viability is observed for *ord* null flies, indicating that ORD function is not essential for cohesion during somatic mitosis. In addition, we do not observe perturbation of germ-line mitotic divisions in flies lacking ORD activity. Our analysis of weaker *ord* alleles suggests that ORD is required for proper centromeric cohesion after arm cohesion is released at the metaphase I/anaphase I transition. Finally, although meiotic cohesion is abolished in the *ord* null fly, chromosome loss is not appreciable. Therefore, ORD activity appears to promote centromeric cohesion during meiosis II but is not essential for kinetochore function during anaphase.

WHEN eukaryotes undergo cell division, proper chromosome segregation requires maintenance of cohesion between sister chromatids until the metaphase/anaphase transition. Only if sister chromatids stay connected to each other are they able to form stable bipolar attachments to spindle microtubules. Cohesion prevents individual chromatids from segregating randomly to either pole and thereby guarantees that chromosomes are partitioned correctly. Although it is imperative that sister-chromatid cohesion be regulated precisely, the nature of the physical association between sisters is poorly understood. In addition, very little is known about the molecules that control temporal and spatial changes in cohesion, allowing chromosomes to separate from each other at the proper time. Genetic analysis of mutations that disrupt cohesion has been invaluable in the recent identification of proteins that regulate this process (KERREBROCK *et al.* 1995; MOLNAR *et al.* 1995; BICKEL *et al.* 1996).

Sister-chromatid cohesion is necessary during both mitosis and meiosis; however, it remains to be demonstrated how closely the regulation of cohesion is conserved between these two types of cell division (for review see BICKEL and ORR-WEAVER 1996). One major difference is the manner in which cohesion is released. During mitosis, cohesion along the entire length of the chromatids is abolished so that chromosomes may segregate away from each other. In contrast, during meiosis, cohesion is lost in a step-wise manner. At the first metaphase/anaphase transition, attachment between the arms of sister chromatids is destroyed, allowing homologues that have undergone reciprocal exchange to move apart from each other. However, centromeric cohesion remains intact, facilitating proper segregation during the second meiotic division. At anaphase II, this last association is released, enabling sister chromatids to separate and move to opposite poles. Determining the basis for differential control of arm and centromeric cohesion during meiosis will be an important step in understanding both the differences and the similarities between mitotic and meiotic cohesion mechanisms.

During meiosis, in addition to ensuring proper disjunction of sister chromatids, cohesion also may serve to modulate homologue association and segregation. In most systems, reciprocal exchange between homologues is necessary to achieve proper segregation during the first meiotic division (for review see HAWLEY 1988). Meiotic crossover events occur preferentially between homologues, not between sisters. It has been proposed that connections between sister chromatids make them act as a unit, thereby promoting homologue exchange (SCHWACHA and KLECKNER 1994; KLECKNER 1996). Moreover, following the resolution of recombination intermediates, a chiasma would not be inherently stable, since it merely represents the site of overlap between two DNA duplexes. At this time, sister-chromatid cohesion distal to the site of reciprocal exchange may be necessary to keep homologues associated until the first metaphase/anaphase transition (DARLINGTON 1932; MAGUIRE 1993).

The Drosophila *ord* (*orientation disruptor*) gene encodes one of the few proteins that are known to be essential for cohesion. In strong *ord* mutants, segrega-

Corresponding author: T. L. Orr-Weaver, Whitehead Institute, 9 Cambridge Center, Cambridge, MA 02142. E-mail: weaver@wi.mit.edu

tion defects first become manifest during meiosis I, when cohesion is normally maintained along the entire length of the chromatids (MASON 1976; GOLDSTEIN 1980; MIYAZAKI and ORR-WEAVER 1992). In *ord* spermatocytes, premature separation of sister chromatids has been observed prior to metaphase I. Defects in cohesion in *ord* males result in segregation errors during both meiotic divisions. Meiotic recombination is also affected by *ord* mutations. Although wild-type Drosophila males do not undergo meiotic exchange, recombination frequencies are severely reduced in *ord* females (MASON 1976; MIYAZAKI and ORR-WEAVER 1992). It has remained unclear whether ORD possesses two separate activities in females (cohesion and recombination) or whether reduction of exchange reflects aberrant homologue interactions that indirectly are caused by sister-chromatid cohesion defects. In addition, cytological evidence has suggested that the ORD protein might be necessary for proper segregation during the mitotic divisions of germ cells (LIN and CHURCH 1982; MIYAZAKI and ORR-WEAVER 1992). The pleiotropic effects observed in *ord* mutants make it likely that determining the molecular details of ORD function will provide valuable insight into basic principles that govern chromosome segregation, including the differences between meiotic and mitotic control of cohesion as well as the contribution of cohesion to homologue behavior during meiosis.

To better understand cohesion at the molecular level, we cloned the *ord* gene (BICKEL *et al.* 1996). Since ORD is a novel protein, an excellent way to gain information about its mechanism of action is by investigation of mutant alleles. Through genetic and molecular analysis of several *ord* mutations, we determined that the C-terminal part of the ORD protein is critical for both recombination and cohesion functions. Furthermore, certain alleles are able to poison the residual activity of weaker alleles (BICKEL *et al.* 1996). This behavior, termed negative complementation, has been observed for genes whose protein products are known to require protein interactions for activity (FINCHAM 1966; FOSTER 1975; PORTIN 1975; RAZ *et al.* 1991). Therefore, we have proposed that the C-terminal domain of ORD participates in protein-protein interactions that are necessary for ORD function.

In this article, we identify and describe the behavior of several new *ord* alleles that provide significant insight into the requirement for ORD function in regulating proper chromosome segregation. Our analysis indicates that ORD function is required for proper cohesion during both meiotic divisions and therefore is necessary to maintain centromeric as well as arm cohesion. Flies lacking ORD protein show no evidence of meiotic cohesion, but exchange is not completely abolished in *ord* null females. In addition, neither somatic nor germline mitotic divisions appear to be affected by absence of ORD activity. Therefore, ORD function differentiates the control of meiotic and mitotic cohesion.

MATERIALS AND METHODS

Stocks: All Drosophila stocks and crosses were raised at 25° on standard cornmeal-brewer's yeast-molasses-agar food. ord^1 was originally described by MASON (1976). *ord* alleles 2–6 were isolated by their failure to complement ord^1 (MIYAZAKI and ORR-WEAVER 1992). For all tests requiring a chromosome for which the *ord* gene was deleted, the deficiency chromosome *Df(2R)WI370* was used (BICKEL *et al.* 1996). The iso-*X/Y*, *cv v f car*, compound-*X* and compound-*XY* stocks were described in KERREBROCK *et al.* (1992). All new *ord* alleles were crossed into the iso-*X/Y* background to minimize variability in the recovery of sex chromosomes in segregation tests.

EMS mutagenesis and screen for noncomplementers: A screen was designed to identify new alleles of *mei-S332* and *ord* (second chromosome) and simultaneously to allow isolation of second-site noncomplementers on the third chromosome (Figure 1). Adult males containing a *Y* chromosome marked with y^+ and isogenized for marked second and third chromosomes were mutagenized with 0.035 M EMS (LEWIS and BACHER 1968) and mated to y^+ virgins containing a $mei\text{-}S332^1$ ord^1 double-mutant tester chromosome. Male progeny containing the *mei-S332 ord* chromosome were then individually tested for sex chromosome missegregation by crossing to homozygous y^- virgins. In this scheme, missegregation giving rise to nullo-*XY* male gametes yields y^-/O males (yellow body color). All other flies have wild-type body color. A total of 11,152 males were tested, 9719 of which were fertile. Forty-eight vials gave rise to two or more yellow males. Of these, 17 were confirmed to be hits to y^+ on the *Y* chromosome by a simple fertility test. For each of the 31 remaining putative positives, a series of crosses were set up to retest and stock both the second and third mutagenized chromosomes.

Eight mutagenized second chromosomes failed to complement *ord*. Two second chromosomes failed to complement *mei-S332* (S. BICKEL and T. ORR-WEAVER, unpublished observations). However, no second-site noncomplementers on the third chromosome were identified.

Sequence analysis of mutant *ord* alleles: Genomic DNA was isolated from females carrying the mutagenized *cn bw sp If* chromosome over *Df(2R)WI370*. PCR and sequence analysis were carried out as previously described (BICKEL *et al.* 1996). Briefly, for each allele, two clones containing mutant DNA were generated from independent PCR reactions and sequenced. In this way, an EMS mutation that was found in both clones could be differentiated from PCR errors that appeared in only one of the two clones. Each of the eight mutants was found to contain a single base pair change within the open reading frame of *ord*. Interestingly, the ord^6 mutation was reisolated in this screen. To limit background differences, all tests using this allele made use of the new isolate since it was carried on the isogenized *cn bw sp If* chromosome on which the other new *ord* alleles were induced. In addition, sequence analysis also confirmed that two independent ord^{12} alleles were isolated.

Viability tests: *cn* ord^{10} *bw sp If/SM1* males were crossed to *Df(2R)WI370, cn/CyO* virgins in bottles. Parents were discarded on day 7 and progeny were counted until day 18. As a control, males carrying the isogenized *cn* ord^+ *bw sp If* chromosome over *SM1* were tested simultaneously. Non-Balancer flies were distinguished phenotypically as Curly$^+$. Flies carrying *SM1/CyO* are not viable. A standard χ^2 test was performed to determine if the difference in viability was statistically significant.

Missegregation tests: The frequency of sex chromosome missegregation in males and females was measured as de-

scribed 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 male tests, mono-*Y* and diplo-*Y* sperm were indistinguishable. In addition, diplo-*Y* sperm are not efficiently recovered in diplo-*X* females (LINDSLEY and GRELL 1968; GOLDSTEIN 1980). Therefore, total missegregation values underrepresented actual levels. 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 missegregation 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.

Recombination tests: Recombination on the *X* chromosome was measured by crossing *y/cv v f car* females to attached-*XY*, *v f B* males. In this cross, one half of the regular *X* gametes give rise to *XO* males in which recessive *X* chromosome markers can be scored. Only crossovers in the intervals *y-cv* and *cv-f* were measured because the dominant eye mutation *If* precluded scoring eye color.

Cytology of meiosis in males: Testis squashes to analyze meiotic chromosome behavior were prepared as described in GOLDSTEIN (1980) with modifications. Acetic acid/orcein was prepared by refluxing 3 g synthetic orcein (Sigma) in 100 ml of 60% acetic acid for 30 min. Lactic acid/acetic acid/orcein was prepared by mixing 500 μl of the above acetic acid/orcein with 333 μl of 85% lactic acid and 167 μl water. Within 4 hr after eclosion, males were dissected in 0.7% NaCl and each set of testes was transferred to 20 μl acetic acid/orcein, covered and allowed to stain for 5 min. The testes were moved to a 2-μl drop of 60% acetic acid on a clean slide and cut with tungsten needles at a point approximately halfway between the apical tip and the first turn of the testis. A coverslip containing 2 μl lactic acid/acetic acid/orcein was lowered onto the tissue and allowed to sit for ~5 min before excess liquid was drawn away by placing the slide between sheets of bibulous paper.

Hypotonic treatment of testes to disentangle chromosomes before fixation was based on MIYAZAKI and ORR-WEAVER (1992). Testes were incubated in 0.5% sodium citrate dihydrate for 5 min and then processed as above. Application of finger pressure when drawing liquid away resulted in preparations that facilitated ploidy determination, since individual chromosomes were more readily visible.

Phase-contrast microscopy was done using a Zeiss Axiophot equipped with plan Neofluar 20, 40 and 100× objectives or a Nikon Optiphot-2 with a plan Neofluar 40× objective.

RESULTS

Defects arising from complete loss of ORD function are restricted to the meiotic divisions: Using a strategy that scored for failure to complement the original ord^1 mutation (Figure 1), we screened 9719 EMS mutagenized chromosomes and identified six new *ord* alleles. To determine the molecular lesion associated with each mutation, PCR amplification of mutant genomic DNA was performed as previously described (BICKEL *et al.* 1996). For each allele, sequence analysis uncovered a single base pair change within the *ord* open reading frame. Figure 2 indicates the amino acid and nucleotide changes in *ord* alleles 7–12 as well as their locations relative to previously described mutations.

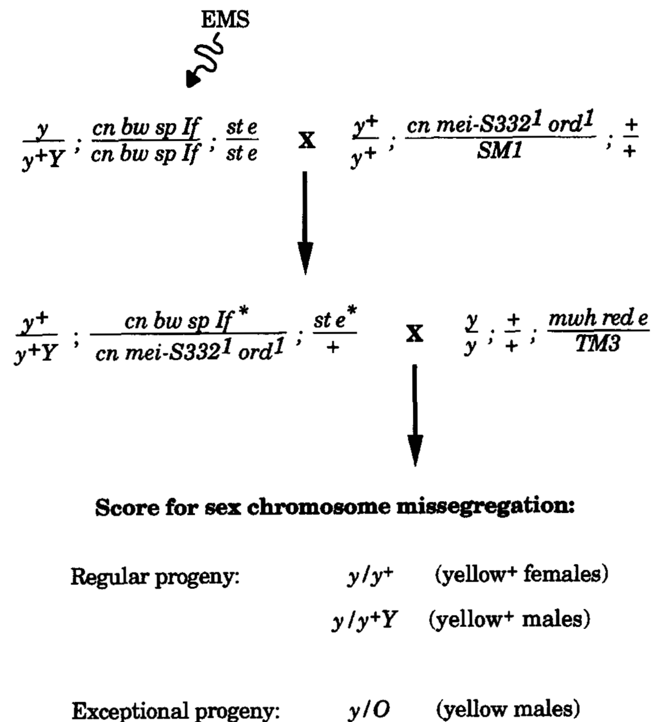

FIGURE 1.—Screen for noncomplementers of *ord* and *mei-S332*. Males containing a y^+Y chromosome and isogenized second and third chromosomes were mutagenized with EMS and crossed to ord^1 $mei\text{-}S332^1$ virgins. Single males were tested for missegregation by crossing to y^- females and scoring for *XO* male progeny. In this screen, other exceptional progeny arise but cannot be distinguished from regular progeny or are not viable.

In this screen we isolated not only additional interesting partial loss-of-function alleles that disrupt the C-terminal part of the ORD protein, but also ord^9 and ord^{10}, the first two *ord* mutations in the N-terminal region. Moreover, ord^{10} resulted in a stop codon after 23 amino acids, allowing us for the first time to unambiguously determine the consequences of ablating ORD function.

Analysis of the null phenotype of *ord* was critical to conclude definitively whether ORD was essential for normal cohesion during mitotic division in somatic tissues. Failure of previously isolated alleles to display somatic defects was inconclusive since it remained unclear whether strong alleles entirely eliminated ORD activity. At the molecular level, the most severe mutation, ord^5, resulted in a stop codon midway through the open reading frame (BICKEL *et al.* 1996). Therefore, the possibility existed that the remaining 50% of the protein possessed partial activity.

Analysis of mitotic defects: We examined whether ORD function was necessary for viability by crossing *Df(2R)WI370/CyO* females to ord^{10}/*SM1* males. Of the 1027 progeny that eclosed, 299 were non-Balancer ord^{10}/*Df* transheterzygotes (29.1%), clearly indicating that ORD activity was not essential for survival. In parallel control crosses in which *Df/CyO* females were mated

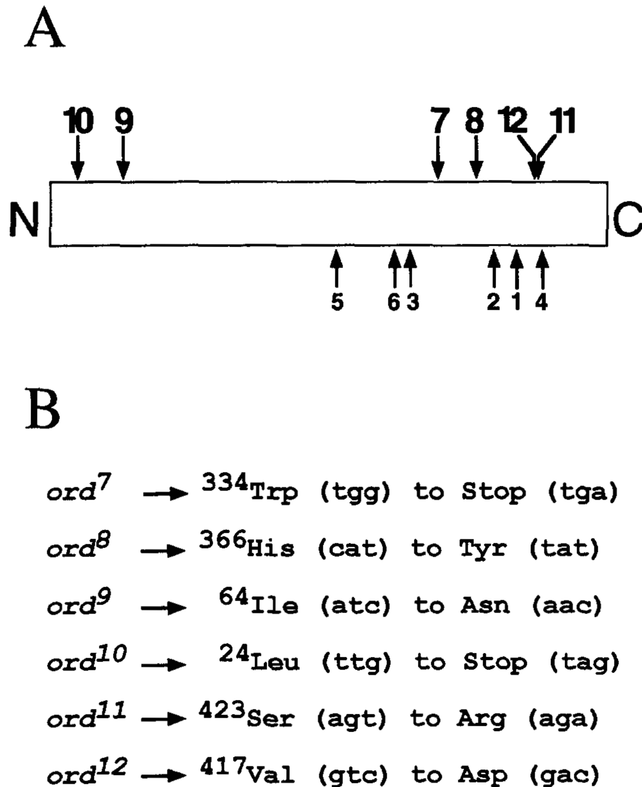

FIGURE 2.—Position and nature of new *ord* alleles. (A) A schematic of the ORD protein is depicted. The locations of *ord* mutations 7–12 (top) are shown relative to those of previously characterized alleles (bottom). (B) The nucleotide and corresponding amino acid change associated with each new allele is indicated.

to males containing the original isogenized ord^+ chromosome over *SM1*, ord^+/Df flies accounted for 33.4% of the progeny (518/1553 flies). Although a statistical analysis indicated the difference between ord^+ and ord^{10} to be significant ($P < 0.005$), the decrease in viability was minimal. In addition, we observed no cuticular, bristle or eye phenotypes indicative of aberrations in the somatic mitotic divisions of ord^{10}/Df flies. Although we could not rule out the possibility that the small decrease in viability arose from subtle defects in cohesion, these results indicated that ORD function is not absolutely required to maintain sister-chromatid cohesion during the mitotic divisions of somatic cells.

Previous cytological analysis has indicated that, in addition to causing meiotic defects, particular *ord* mutations also disrupt germ-line mitotic divisions in the male (LIN and CHURCH 1982; MIYAZAKI and ORR-WEAVER 1992). However, such experiments suffer the drawback that the mitotic divisions are not observed directly. During cytological preparation, chemical treatment is used to loosen the association between meiosis I bivalents (pairs of homologous chromosomes). This allows the number of chromosomes to be counted in primary spermatocytes prior to the first meiotic division. In this way, one may infer mitotic defects based on the chromosome complement of cells following mitosis but preceding meiosis. Technically, this approach requires that all chromosomes be separated sufficiently so they may be counted.

LIN and CHURCH (1982) have used this procedure to analyze the behavior of ord^1 homozygotes by light microscopy and noted that, although segregation of the sex and fourth chromosomes was normal, 31% of the primary spermatocytes observed were aneuploid for one of the large autosomes. Similarly, autosomal aneuploidy was observed in 15% of ord^2/Df primary spermatocytes examined (MIYAZAKI and ORR-WEAVER 1992). Both ord^1 and ord^2 are recessive mutations that result from a single amino acid change in the C-terminal region of the protein.

We undertook a similar analysis with the expectation that if ORD function is required for normal segregation during the germ-line mitotic divisions, then the ord^{10} mutation would result in comparable or perhaps even higher levels of aneuploidy than observed with either of the two missense alleles previously analyzed. In addition to examining primary spermatocytes from ord^{10}/Df males, we also analyzed ord^{10}/ord^5 males that, in segregation tests, display the null phenotype (see below). In our examination of 32 primary spermatocytes with adequately separated chromosomes (19 of which were from ord^{10}/Df males), we were unable to detect any gross abnormalities reflecting aberrant segregation during the germ-line mitotic divisions (Figure 3). Lack of ORD activity might cause subtle defects beyond our capability to identify. However, if ORD were playing an important role, absence of its function during the four consecutive mitotic divisions immediately preceding meiosis would be expected to yield observable defects in primary spermatocytes.

Examination of meiotic cytology: We further analyzed the *ord* null phenotype by preparing orcein-stained testes squashes to examine the meiotic divisions of ord^{10}/Df males. As noted previously for other *ord* mutants (GOLDSTEIN 1980; MIYAZAKI and ORR-WEAVER 1992), striking defects were evident in the morphology of bivalents during prophase I. In wild-type spermatocytes, the structure of each bivalent is compact (Figure 4A). In contrast, ord^{10}/Df bivalents appeared more loosely organized with single chromatids protruding from the chromosome mass (Figure 4B). In some instances, completely separated sister chromatids were observed apart from the bivalent (Figure 4C). However, three darkly stained clumps corresponding to the large autosomal and sex chromosome bivalents were always visible, indicating that, in the absence of ORD protein, initial homologue association was not compromised severely in males.

Unlike the wild-type situation (Figure 4D), precocious separation of sister chromatids was readily apparent in ord^{10}/Df males during meiosis II (Figure 4E and F). In most secondary spermatocytes, cohesion ap-

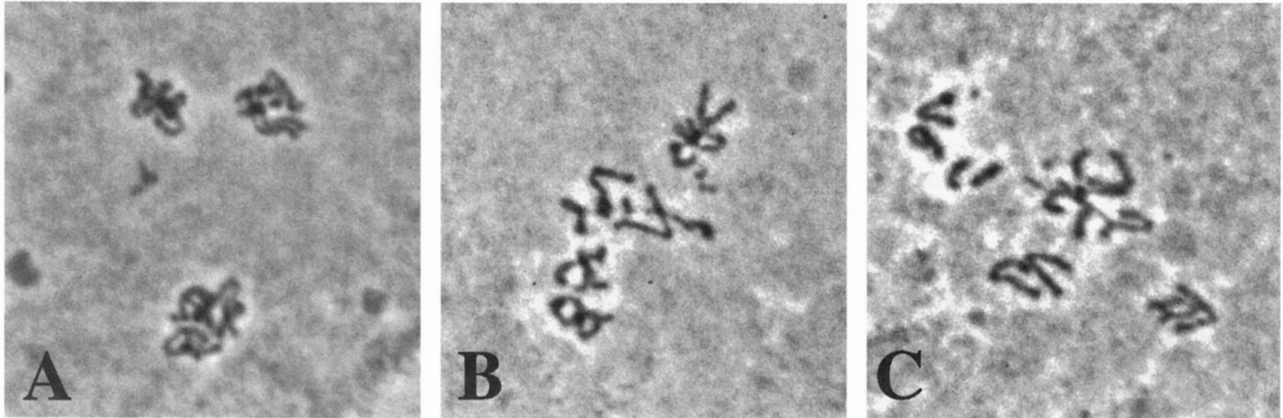

FIGURE 3.—Cytological examination of hypotonically treated *ord* primary spermatocytes to determine ploidy. (A) In addition to the correct number of large chromosomes (12), homologue pairing is evident in ord^{10}/Df primary spermatocytes that are gently squashed. (B and C) The correct ploidy also is observed in primary spermatocytes of ord^{5}/ord^{10} testes that have been prepared with more vigorous pressure to facilitate the spreading and counting of chromosomes. Evidence of segregation defects during germ-line mitotic divisions is not apparent for either genotype. The small fourth chromosomes were excluded from this analysis.

peared to be totally absent before anaphase II. In addition, aberrant anaphase II figures with unequal polar distribution of chromatids were seen often, consistent with the production of aneuploid gametes (data not shown). No metaphase II figures were observed, presumably because stable bipolar microtubule attachments on the metaphase plate could not arise in the absence of sister-chromatid cohesion.

Decrease in meiotic recombination: In addition to meiotic segregation defects, *ord* females undergo lower levels of reciprocal exchange. However, recombination cannot be the sole defect leading to missegregation in *ord* mutants, since even recombinant chromosomes nondisjoin in *ord* oocytes (MASON 1976). In other words, unlike its effect in wild-type flies, exchange does not ensure proper segregation in *ord* females. One possibility consistent with these data is that ORD protein is required for two separate functions during female meiosis: cohesion and recombination. Alternatively, if cohesion were the principal defect in *ord* females, disruption of the association between sisters might indirectly reduce the number of crossover events.

We investigated whether the complete loss of ORD activity would entirely block exchange in ord^{10}/Df oocytes. Recombination levels have been monitored for ord^{1}, ord^{3} and ord^{6}, but two of the strongest mutations, ord^{2} and ord^{5}, were not assayed for exchange, since females displayed significantly reduced fertility (MASON 1976; MIYAZAKI and ORR-WEAVER 1992). Similarly, we found ord^{10}/Df females to be quite infertile. However, we proceeded to monitor their level of exchange, reasoning that positive evidence of reciprocal recombina-

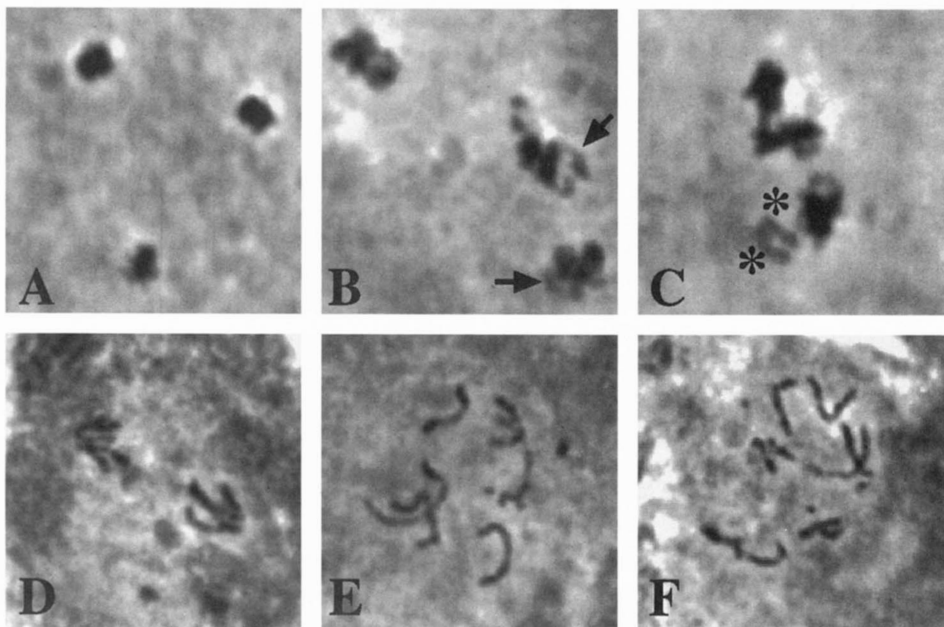

FIGURE 4.—Precocious separation of sister chromatids in ord^{10}/Df spermatocytes. (A) The large autosomal and sex chromosome bivalents appear as tightly condensed masses in primary spermatocytes of $ord^{10}/+$ heterozygotes. (B) In ord^{10}/Df males, the bivalents are more loosely organized with single chromatid arms (arrows) extending from the chromosome mass. (C) In some cases, individual chromatids (*) are seen during meiosis I. (D) During meiosis II, cohesion between sisters is not released in $ord^{10}/+$ males until anaphase II when chromatids move to opposite poles. (E and F) Individual sister chromatids are seen in ord^{10}/Df secondary spermatocytes before the anaphase II transition. Sister-chromatid cohesion appears to be completely ablated.

TABLE 1

Sex chromosome missegregation in *ord* male transheterozygotes

Genotype	Regular sperm (%)		Exceptional sperm (%)				Total progeny	Total missegregation (%)
	X	*Y(Y)*	*O*	*XY(Y)*	*XX*	*XXY(Y)*		
$ord^{+}/Df(2R)WI370$	47.4	51.7	0.5	0.3	0.0	0.0	1523	0.8
$ord^{8}/Df(2R)WI370$	51.7	44.2	3.3	0.4	0.3	0.0	2744	4.0
ord^{8}/ord^{1}	36.5	35.8	18.6	7.0	2.0	0.0	3766	27.6
ord^{8}/ord^{2}	38.5	41.7	11.0	3.0	5.5	0.3	3801	19.8
ord^{8}/ord^{6}	46.0	42.8	6.9	2.7	1.7	0.0	2420	11.3
$ord^{11}/Df(2R)WI370$	35.6	36.3	20.8	3.6	3.6	0.0	2061	28.0
ord^{11}/ord^{1}	30.5	31.0	25.4	10.0	3.4	0.2	3690	39.0
ord^{11}/ord^{2}	26.6	30.0	23.8	10.4	8.3	1.0	3364	43.5
ord^{11}/ord^{6}	37.4	35.4	16.5	6.2	4.5	0.1	1809	27.3
$ord^{6}/Df(2R)WI370$	42.2	36.9	15.7	3.7	1.5	0.0	2702	20.9
ord^{6}/ord^{1}	33.3	32.7	20.3	10.4	3.2	0.0	2808	33.9
ord^{6}/ord^{2}	35.2	31.1	19.1	7.7	6.4	0.5	3350	33.7

tion would be informative even if absolute numbers were low. From 300 ord^{10}/Df females, only 114 regular male progeny were recovered, seven of which showed evidence of a crossover event. Overall map distance measured within the *y-cv* and *cv-f* intervals was calculated to be 6.1 cM or 16% of that observed in $ord^{10}/+$ females (37.1 cM). Given the small sample size, a sixfold decrease in exchange in *ord* null females is consistent with the 7–10-fold reduction observed for other *ord* mutants. Our results demonstrate that, although the frequency of reciprocal exchange is diminished in the *ord* null, it is not eliminated. Homologues are still able to undergo recombination in the absence of ORD. If a separate recombination function exists for ORD protein, it is not absolutely required for crossovers to occur.

Leaky *ord* alleles reveal novel aspects of ORD function: In contrast to ord^{10}, which truncates the open reading frame, we isolated two new missense alleles, ord^{8} and ord^{11}, which display residual activity when placed over a deficiency (Tables 1 and 2). Both mutations were localized to the C-terminal region of the protein where we previously uncovered several other mutations (Figure 2). The ord^{8} mutation resulted in the weakest phenotype we have observed for any allele except ord^{4}, while the phenotype of ord^{11} was moderate. Interestingly, the amino acid change in ord^{11} immediately precedes Ala^{424}, which is mutated in ord^{4}.

In our analysis of the effects of moderate alleles, we noticed that leaky *ord* alleles consistently resulted in higher levels of missegregation in females than in males. ord^{8}/Df and ord^{11}/Df females exhibited segregation defects that were 2.7 and 1.3 times higher than those seen in respective males (Tables 1 and 2). Similarly, the effect of ord^{6} is considerably more severe in females (Tables 1 and 2). Even the near wild-type activity of ord^{4}/Df flies is slightly more disrupted in females (2.2%) than in males (0.6%) (Bickel *et al.* 1996). These effects suggest that females are more sensitive to decreased activity of ORD protein than males.

The phenotype of strong *ord* alleles clearly indicates that ORD function is required early in meiosis to establish and/or maintain proper chromatid cohesion. However, the severe loss of cohesion during meiosis I in strong alleles has made it impossible to ask whether ORD function is also required later, specifically after the metaphase I/anaphase I transition when arm cohesion is destroyed and sisters are held together solely at their centromeric regions. The isolation of new weak and moderate alleles of *ord* permitted us to investigate whether ORD plays an important role in controlling centromeric cohesion during meiosis II.

We used the information attained in testing for sex chromosome missegregation in males to address this question. The ratio of diplo-*XY* to diplo-*XX* gametes is informative in determining when segregation defects are first manifest. Because the *X* and *Y* homologues segregate away from each other during meiosis I, aberrant diplo-*XY* gametes arise only if segregation is disrupted during this division. In contrast, if meiosis I proceeds normally but cohesion defects become manifest during the second meiotic division, diplo-*XX* gametes will be recovered much more frequently than *XY* gametes. In a strong *ord* mutant such as the ord^{5} homozygote, sister-chromatid cohesion is lost during meiosis I. Therefore, single chromatids segregate aberrantly during both divisions, resulting in *XY* as well as *XX* gametes. In these flies, *XY* gametes outnumber *XX* gametes by greater than threefold (Miyazaki and Orr-Weaver 1992).

Not only did some of the new *ord* alleles exhibit moderate segregation defects, but the ratios of *XY* to *XX* gametes were quite different from those observed pre-

TABLE 2

Sex chromosome missegregation in *ord* female transheterozygotes

Genotype	Regular ova (%) X	Exceptional ova (%) O	Exceptional ova (%) XX	Total progeny	Adjusted total[a]	Total missegregation (%)
$ord^{+}/Df(2R)WI370$	99.6	0.0	0.4	2020	2024	0.4
$ord^{8}/Df(2R)WI370$	89.1	4.6	6.3	2963	3134	10.9
ord^{8}/ord^{1}	56.0	19.6	24.5	2752	3529	44.1
ord^{8}/ord^{2}	79.2	7.8	13.1	3224	3599	20.9
ord^{8}/ord^{6}	86.1	5.6	8.3	1880	2021	13.9
$ord^{11}/Df(2R)WI370$	64.1	18.9	17.0	2294	2796	35.9
ord^{11}/ord^{1}	46.3	28.6	25.1	1764	2412	53.7
ord^{11}/ord^{2}	53.2	24.5	22.4	1651	2156	46.9
ord^{11}/ord^{6}	58.5	24.1	17.4	856	1080	41.5
$ord^{6}/Df(2R)WI370$	49.9	25.8	24.2	1225	1634	50.0
ord^{6}/ord^{1}	45.9	30.6	23.6	1858	2547	54.2
ord^{6}/ord^{2}	49.4	19.5	31.1	1244	1665	50.6

[a] The progeny total is adjusted to correct for recovery of only half of the exceptional progeny.

viously. Certain transheterozygous combinations resulted in almost equal numbers of *XY* and *XX* gametes (Table 1). For example, ord^{8}/ord^{2} males gave rise to *XX* gametes at a slightly higher rate than *XY* gametes. In a similar fashion, ord^{11}/Df, ord^{11}/ord^{2} and ord^{11}/ord^{6} males generated approximately equal levels of *XY* and *XX* gametes. ord^{6}/ord^{2} males behaved the same.

These results indicate that mutant *ord* alleles in certain combinations appear to be disrupting cohesion in meiosis I to a much lower degree than normally seen with strong alleles. Segregation defects do not become manifest until later in meiosis, specifically during meiosis II when sisters are normally held together only at their centromeric regions.

One argument opposing this interpretation is that the total level of missegregation is lower in these mutants and that the ratio of *XY* to *XX* gametes merely reflects this weaker phenotype. However, we argue against this idea based on a comparison of specific mutant combinations that give rise to total levels of missegregation that are almost identical. For instance, the difference in total missegregation between ord^{8}/ord^{1} and ord^{11}/Df is negligible, but the ratio of *XY* to *XX* gametes dramatically changes from 3.5:1 to 1:1. The same trend is observed when comparing ord^{6}/ord^{1} and ord^{6}/ord^{2} males or ord^{11}/ord^{1} and ord^{11}/ord^{2} males. In addition, because diplo-*Y* and normal sperm cannot be differentiated in our assay, the level of meiosis II missegregation is underrepresented. Therefore, the manifestation of segregation disorders after anaphase I is probably more pronounced than experimentally indicated.

Several *ord* alleles exhibit negative complementation: Previously, we described unusual behavior in which the residual activity of one *ord* allele was poisoned when in trans to another allele (Bickel *et al.* 1996). This phenomenon, termed negative complementation, has been observed for genes whose products are known to require protein-protein interactions for proper function. Our analysis demonstrated that a very weak allele, ord^{4}, functions almost as well as wild-type *ord* when homozygous or in trans to a deficiency. However, three missense alleles, each with an altered residue in the C-terminal part of the protein (ord^{1}, ord^{2} and ord^{6}) are able to poison the activity of ord^{4}. In contrast, mutations that prematurely truncate the open reading frame of ORD are unable to elicit these effects. This indicates that the negative complementation behavior is mediated through the C-terminal part of ORD and suggests that protein-protein interactions involving this part of the protein are necessary for proper activity.

Here we extend our analysis of negative complementation and demonstrate that these effects are not restricted to interactions with ord^{4}. In several cases we found that a missense mutation (such as ord^{1}) had a more severe effect than a deficiency when placed over an allele that exhibited residual activity (Tables 1 and 2). For example, the missegregation in ord^{8}/ord^{1} or ord^{8}/ord^{2} flies was much greater than that observed in ord^{8}/Df flies. Like the negative complementation of ord^{4}, we observed this phenomenon in both males and females. In fact, although ord^{2} did not affect the activity of ord^{4} in males (Bickel *et al.* 1996), its ability to poison ord^{8} and ord^{11} in males was pronounced (Table 1). Similarly, in females, ord^{6} compromised the activity of ord^{8} and ord^{11} but not ord^{4} (Table 2) (Bickel *et al.* 1996). In addition, for particular allelic combinations, negative complementation was predominant in one sex. For instance, ord^{6} interfered with the activity of ord^{8} in males significantly more than in females.

These data make clear that nearly every missense mu-

TABLE 3

The effect of an N-terminal missense mutation on ORD activity in males

Genotype	Regular sperm (%)		Exceptional sperm (%)				Total progeny	Total missegregation (%)
	X	*Y*	*O*	*XY(Y)*	*XX*	*XXY(Y)*		
$ord^9/Df(2R)WI370$	26.0	25.1	42.3	5.3	1.3	0.0	1078	48.9
ord^9/ord^4	54.6	44.9	0.4	0.1	0.0	0.0	4357	0.5
$ord^4/Df(2R)WI370$[a]	51.7	47.7	0.4	0.1	0.1	0.0	6132	0.6

[a] Data taken from BICKEL *et al.* (1996).

tation in the C-terminal part of the ORD protein is capable of participating in negative complementation behavior. Strong alleles typically show poisoning activity, whereas weaker alleles are poisoned. However, one moderate allele, ord^6, is capable of functioning in both capacities. These results confirm that negative complementation is not merely an allele-specific effect of ord^4, but rather a general phenomenon exhibited by several *ord* alleles. This strengthens the argument that protein-protein interactions are necessary for wild-type ORD function.

Functional analysis of the N-terminal region of ORD: The majority of *ord* mutations are clustered within the C-terminal half of the ORD protein. Missense mutations in this region show strong defects in both meiotic cohesion and exchange, indicating that this portion of the protein is absolutely required for both aspects of ORD function. In addition, we have shown that the C-terminal part of ORD is required to elicit negative complementation effects. In contrast to all other *ord* missense mutations, ord^9 lies near the N-terminus of ORD, making it possible for the first time to determine which types of activity are dependent on this domain of the protein.

Sequence analysis of ord^9 indicated that an Asn was substituted for Ile^{64}. Like the region of the C-terminus where ord^{12}, ord^{11} and ord^4 mutations are clustered, the ord^9 change lies within a stretch of amino acids (54–71) that is considerably hydrophobic (11/18 residues) and might promote protein-protein interactions. In segregation tests ord^9 behaved as a strong allele, resulting in 48.9% total missegregation of the sex chromosomes in males and 58.5% missegregation of the *X* chromosome in females (Tables 3 and 4).

Figure 5 shows orcein-stained testes squashes from ord^9/Df males. Like other strong *ord* alleles, abnormalities are evident in the structure of bivalents during early meiosis I, and precocious separation of sister chromatids is seen in secondary spermatocytes. Therefore, as with other *ord* mutations, the segregation defects in ord^9/Df males appear to be arising from inadequate cohesion.

If recombination and cohesion could be mutated separately, it would provide strong evidence that ORD protein does indeed carry out two distinct functions in females. Our isolation of new *ord* alleles was performed by testing for noncomplementation in males, so it was possible that a mutation that caused strong cohesion defects in males might not perturb exchange efficiency appreciably in females. Therefore, we tested whether the ord^9 missense mutation in the N-terminal region of the protein lowered recombination between homologues. Like ord^{10}/Df females, we found ord^9/Df females to be considerably infertile. Two hundred fifty ord^9/Df females produced 61 regular male progeny, three of which showed evidence of a crossover event. Because the sample size was so small, an accurate assessment of the absolute decrease in exchange was not possible. However, this test still provided clear evidence that reciprocal exchange is definitely reduced but not completely ablated in these females. More importantly for our model of ORD action, both recombination and cohesion functions are affected by the N-terminal ord^9 mutation.

The last activity of ord^9 that we analyzed was its ability to participate in negative complementation. Our previous finding that the truncation mutations ord^3 and ord^5 did not interfere with ord^4 function indicated that the C-terminal region of the protein mediated protein interactions that were instrumental in eliciting negative complementation effects. The fact that the C terminus was required for negative complementation and that all the alleles tested so far were missense changes in the C terminus did not preclude the possibility that the N terminus could also participate in this behavior. Therefore, we tested the ability of ord^9 to poison the activity of ord^4.

Phenotypically, ord^9/ord^4 flies were indistinguishable from ord^4/Df flies, (Tables 3 and 4), indicating that ord^9 was unable to antagonize ord^4 activity. One simple explanation for the similarity of effects between ord^9 and a deficiency is that the ord^9 missense mutation renders the protein unstable; lack of ORD^9 protein would be identical to deleting the gene. However, by Western analysis of testes extracts from ord^9/Df males, ORD protein was easily detectable (S. BICKEL and T. ORR-WEAVER, unpublished observations). Therefore, although the N-terminal mutation seriously compromises

TABLE 4

The effect of an N-terminal missense mutation on ORD activity in females

Genotype	Regular ova (%)	Exceptional ova (%)		Total progeny	Adjusted total[a]	Total missegregation (%)
	X	*O*	*XX*			
ord^9/*Df(2R)WI370*	41.5	30.5	28.0	798	1128	58.5
ord^9/*ord*^{4}	97.8	0.8	1.4	3636	3676	2.2
ord^4/*Df(2R)WI370*[b]	97.8	1.1	1.1	7308	7389	2.2

[a] The progeny total is adjusted to correct for recovery of only half of the exceptional progeny.
[b] Data taken from BICKEL *et al.* (1996).

the ability of ORD^9 to function, it does not allow the competitive behavior necessary to induce negative complementation.

Absence of ORD function results in random segregation of sister chromatids with no significant chromosome loss: Theoretical values for the recovery of exceptional gametes from flies lacking sister-chromatid cohesion can be determined by assuming random segregation of single chromatids through both meiotic divisions (MIYAZAKI and ORR-WEAVER 1992). Such calculations rely on the important assumption that each chromatid will reach one pole or the other; no chromosome loss will occur. In comparing the values obtained for *ord*^{10}/*Df* females to those predicted theoretically (Table 5), we found the numbers to correlate well. These data support the conclusion that, in the absence of ORD protein, cohesion is missing, thereby allowing individual chromatids to segregate randomly. In addition, this comparison also indicates that chromosome loss is not occurring at an appreciable rate in *ord* null females. Moreover, *ord*^{10}/*ord*^{5} females behave similarly, demonstrating that the behavior of *ord*^{5} is the same as that of a deficiency that removes the entire *ord* gene. Therefore, based on these criteria, we believe that ORD activity also is completely eliminated in *ord*^{10}/*ord*^{5} flies.

Table 6 displays the missegregation values obtained

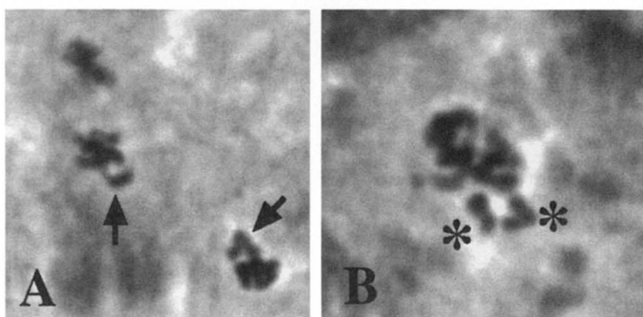

FIGURE 5.—Defects in sister-chromatid cohesion result from a missense mutation in the N terminus of ORD. (A) During meiosis I, the chromosomes in *ord*^{9}/*Df* spermatocytes are less densely packed and single chromatid arms (arrows) are visible protruding from the bivalent mass. (B) In *ord*^{9}/*Df* secondary spermatocytes, individual chromatids (*) are visible before anaphase II.

in the analysis of *ord*^{10}/*Df* and *ord*^{10}/*ord*^{5} males. Initially, it appeared that in males the null phenotype induced quite large amounts of chromosome loss. Over 50% of the gametes recovered from *ord*^{10}/*Df* males contained neither an *X* nor a *Y* chromosome. However, additional tests suggested that the loss seen in *ord*^{10}/*Df* males was caused not by lack of ORD activity per se, but specifically by the *Df(2R)WI370* chromosome, which we have used exclusively in this study. This hypothesis is supported by several observations. In theoretical calculations, the ratio of nullo-*XY* gametes to the sum of all other exceptional gametes equals 1.42. There is an excess of nullo-*XY* gametes over the sum of other exceptional gametes because random segregation takes place during two consecutive divisions. For instance, the production of an *XXYY* gamete at the end of meiosis would simultaneously result in three gametes void of sex chromosomes. However, when *ord* moderate alleles are placed over *Df(2R)WI370*, nullo gametes are obtained at higher than expected levels (Table 1). Therefore, even when the amount of ORD protein is not limiting, the presence of the *Df(2R)WI370* chromosome appears to be inducing loss in males. In addition, *Df(2R)bw-S46*, another deficiency that does not extend as far proximally as *Df(2R)WI370*, has not resulted in chromosome loss when placed over several *ord* alleles (MIYAZAKI and ORR-WEAVER 1992). Tests with both deficiencies measured missegregation of the same iso-*X* and iso-*Y* chromosomes (KERREBROCK *et al.* 1992), ruling out any chromosome specific differences that might lead to variations in recovery.

We do not observe high levels of chromosome loss in *ord*^{10}/*ord*^{5} males. This finding is significant because, in segregation tests, *ord*^{10}/*Df* and *ord*^{10}/*ord*^{5} females behave similarly, suggesting that, at least in females, the *ord*^{5} mutation eliminates ORD activity. Therefore, the levels of missegregation exhibited by *ord*^{10}/*ord*^{5} males probably represents a truer example of the *ord* null phenotype in males than that observed for *ord*^{10}/*Df*. Our observations raise the intriguing possibility that an additional locus uncovered by *Df(2R)WI370* plays an essential role in regulating chromosome segregation during meiosis in the male.

TABLE 5

Sex chromosome missegregation in females lacking *ord* function

Genotype	Regular ova (%)	Exceptional ova (%)		Total progeny	Adjusted total[a]	Total missegregation (%)
	X	*O*	*XX*			
ord^{10}/*Df(2R)WI370*	41.2	35.8	23.0	750	1062	58.8
ord^{10}/*ord*^{5}	41.0	36.4	22.7	697	989	59.1
Theoretical[b]		33.3	22.2			55.5

[a] The progeny total is adjusted to correct for recovery of only half of the exceptional progeny.
[b] Theoretical values taken from Miyazaki and Orr-Weaver (1992).

DISCUSSION

Role of ORD in centromere behavior: The behavior of moderate alleles provides unexpected new information about the nature of ORD's function. It has been clear from the analysis of strong *ord* alleles that defects early in meiosis were arising either from an inadequacy in establishing cohesion or the inability to stabilize attachments between sisters once they initially were formed. Distinguishing between these two roles has not been possible. However, our present study indicates that specific transheterozygous combinations exhibit segregation defects that are manifest later than previously observed for strong *ord* alleles. These experiments suggest that ORD function is required for maintenance of centromeric cohesion between sisters after the metaphase I/anaphase I transition. Therefore, ORD function appears to be involved in two types of cohesion that are regulated very differently during the course of meiosis, arm and centromeric cohesion. The time at which ORD activity is required cannot be determined from our analysis. One possibility is that ORD functions after anaphase I to promote cohesion. Conversely, ORD may be required much earlier than the time at which segregation defects occur.

In our analysis, we are assuming that increased levels of diplo-*X* gametes arise because of premature loss of cohesion during meiosis II. However, one also might obtain diplo-*X* gametes in this assay if homologous chromosomes segregated to the same pole in meiosis I and reductional segregation was delayed until the second meiotic division. Although this is a formal possibility, we do not think it is a likely explanation for the segregation defects we observe in certain *ord* transheterozygotes. For this to occur, leaky *ord* alleles would need to exhibit the opposite behavior of strong alleles. Moreover, premature separation of sister chromatids has been observed cytologically for two of the mutations that give rise to increased levels of diplo-*X* gametes in certain allelic combinations (Miyazaki and Orr-Weaver 1992).

Another novel Drosophila protein, MEI-S332, also is required to maintain centromeric cohesion during meiosis in both males and females. Defects in *mei-S332* mutants become manifest early in anaphase I when arm cohesion has been released and sisters are held together at their centromeric regions only (Davis 1971; Goldstein 1980; Kerrebrock *et al.* 1992). This is consistent with recent localization of a MEI-S332-GFP fusion protein to the centromeres of meiotic chromosomes, indicating that the site of MEI-S332 action is indeed at the centromere (Kerrebrock *et al.* 1995). Although, like MEI-S332, ORD appears to be required for proper centromeric cohesion, mutations in either gene elicit defects. This indicates that neither protein is sufficient to provide centromeric cohesion alone and suggests they are not carrying out the same function.

Analysis of the *ord* null phenotype provides additional evidence that the mode of action of ORD and MEI-S332 is different. Lack of *ord* function does not cause significant levels of chromosome loss. This observation indicates that, although ORD is required for centromeric cohesion, it does not affect the ability of kinetochores to attach to microtubules and migrate to spindle

TABLE 6

Sex chromosome missegregation in males lacking *ord* function

Genotype	Regular sperm (%)		Exceptional sperm (%)				Total progeny	Total missegregation (%)
	X	*Y(Y)*	*O*	*XY(Y)*	*XX*	*XXY(Y)*		
ord^{10}/*Df(2R)WI370*	23.5	17.7	51.7	5.0	2.1	0.0	1305	58.8
ord^{10}/*ord*^{5}	27.9	23.4	26.6	18.2	3.8	0.1	2063	48.7
Theoretical[a]			32.9	17.1	3.6	2.4		56.0

[a] Theoretical values taken from Miyazaki and Orr-Weaver (1992).

poles. For ORD activity a clear separation of cohesion and kinetochore function is evident. This differs from what has been observed for MEI-S332. In female segregation tests, strong *mei-S332* alleles not only disrupt segregation but also have been observed to cause significant levels of chromosome loss (DAVIS 1971; KERREBROCK *et al.* 1992). Chromosome loss has also been observed in *mei-S332* males, but analysis of loss in male tests is more difficult since equational exceptions are underestimated. These data suggest that, unlike ORD, the function of MEI-S332 is not restricted to promoting cohesion but also is necessary for some other activity involving the kinetochore. In contrast, ORD is essential to secure the attachment between sisters at their centromeric region as well as along the arms.

Functional domains of the ORD protein: Isolation and analysis of ord^9 provide significant information about regions that are critical for ORD function. These data supply the first evidence that ORD activity is dependent on residues located within the N-terminal region of the protein.

On the basis of the negative complementation behavior of ord^4 and ord^1, we have proposed a model in which two separable activities are required for ORD to be fully functional. ORD requires homo- or heterotypic protein-protein interactions in order to carry out its cohesion function. We suggested that the ord^4 mutation diminishes protein binding. In contrast, ord^1 allows normal protein-protein interactions but disrupts the "active site" required for cohesion activity once binding has taken place (BICKEL *et al.* 1996). Because ORD^1 binds more effectively, it can poison the cohesive ability of ORD^4.

By both genetic and cytological criteria, ord^9 behaves as a strong mutation. Debilitation of the protein could arise through failure in either protein binding or cohesion function or both. However, the finding that ORD^9 protein is stable but does not compete with ORD^4 implies binding activity must be impaired. If only the active site was compromised by the ord^9 amino acid change, then ORD^9 should be able to bind competitively in the presence of ORD^4, resulting in negative complementation. Therefore, these data suggest that, while the C-terminal part of the protein is necessary, it is not sufficient for binding. Binding activity requires participation of part of the N terminus also. It is interesting that ord^4 and ord^9, two mutations that both appear to compromise binding activity, each are located within a stretch of hydrophobic amino acids. In addition, ord^{12}, a C-terminal missense mutation that severely disrupts ORD function but exhibits minimal negative complementation, also resides within the C-terminal hydrophobic interval (BICKEL and ORR-WEAVER 1997). Perhaps protein binding requires hydrophobic interactions that involve these N- and C-terminal regions of ORD.

The negative complementation effects that we have observed for several *ord* alleles supports our hypothesis that protein-protein interactions are required for the proper activity of ORD^+ protein. Furthermore, comparison of the poisoning behavior of ord^1 and ord^2 reveals differences during meiosis I and meiosis II. ord^1, when placed over moderate alleles, consistently resulted in diplo-*XY* gametes that outnumbered diplo-*XX* gametes by a factor of three or more. In contrast, the same alleles when in trans to ord^2 exhibited weaker disruption of segregation during meiosis I as evidenced by the recovery of relatively equal numbers of *XY* and *XX* gametes. Both ord^1 and ord^2 elicit strong meiosis I effects when homozygous. However, the ORD^2 protein in combination with another partly functional ORD protein is more efficient at maintaining cohesion during meiosis I than ORD^1. This difference in negative complementation could be reflecting distinct activities of ORD in the regulation of cohesion during these two divisions.

Control of homologue segregation in the absence of ORD: The *ord* null mutation allowed us to analyze whether defects in sister-chromatid cohesion affect the association between homologues. Our results indicate that, in Drosophila males, the two processes are independent. Homologue association occurs whether or not sister chromatids are held together properly. In cytological preparations of ord^{10}/Df and ord^{10}/ord^5 testes, three large masses of chromatin corresponding to the large bivalents are observed, indicating that the initial association of homologues early in meiosis I appears to be normal. Male and female homologue segregation in Drosophila has been shown to be under different genetic control. In males, recombination does not take place. Thus, unlike most meiosis I divisions, proper segregation of homologues in Drosophila males does not require reciprocal exchange to secure a stable association between homologous chromosomes. Instead, cis-acting sequences have been shown to be necessary for proper pairing and segregation of homologues during the first meiotic division in Drosophila males (COOPER 1964; MCKEE and KARPEN 1990; MCKEE 1996). Our data suggest that this system for controlling homologue association functions well in the absence of sister-chromatid cohesion and may account in part for the observation that ord^{10}/ord^5 males display lower levels of missegregation than predicted for random chromatid behavior.

Unlike males, in Drosophila females the association between homologues does not appear to function independently of sister cohesion. Females lacking *ord* function exhibit missegregation frequencies that correspond to the levels predicted by theoretical calculations that assume independent segregation of completely separated chromatids. Such random segregation of individual chromatids can occur only if both homologue and sister attachments are ablated.

Because in females proper homologue connections depend on recombination, failures in homologue association might arise from defects in the exchange pathway or by the inability to maintain stable attachments

between homologues after recombination intermediates are resolved. Loss of ORD function clearly depresses the frequency of crossovers between homologues, indicating early processes are aberrant. However, exchange is not completely eliminated in females lacking ORD protein. The observation that segregation appears random even in the presence of residual exchange suggests that, in the absence of ORD protein, the connections between exchange homologues are not maintained.

Although it is possible that ORD protein is required for two different functions in females, we have been unable to separate recombination and cohesion activity genetically in *ord* mutants. All alleles tested, including a missense allele in the N-terminal region, exhibit defects in both functions. It is important to note that if mutations that affected only cohesion but not recombination failed to elicit a mutant phenotype in females, we would still have recovered them, since we have performed our screens in males. While a sufficient number of mutations may not have been isolated, we favor the hypothesis that the cohesion activity of ORD is required for normal levels of homologue exchange.

Another interesting observation consistent with this idea is that males and females exhibit different sensitivities to partial loss of ORD function. In all cases, weak and moderate alleles result in stronger defects in chromosome segregation in females than in males, indicating that females have a more stringent requirement for ORD activity than males. The greater sensitivity of females could simply reflect an additive effect on segregation that arises because both recombination and cohesion are compromised. However, another explanation again relies on the premise that homologue association is independent of ORD function in males but not in females. In females, the loss of cohesion coupled with the lack of exchange could seriously decrease the ability of homologues to maintain their connections after exchange. In other words, in females, a mechanism must exist to ensure that connections between exchange homologues are stabilized after recombination takes place and this could depend on sister-chromatid cohesion.

ORD may be a special player that ensures proper temporal and spatial regulation of cohesion during meiosis: In our analysis of ord^{10} flies, we find no convincing evidence that ORD is required for proper segregation during mitotic divisions. Viability of ord^{10}/Df transheterozygotes indicates that ORD protein is not necessary for survival. If ORD function were essential to maintain cohesion during the mitotic divisions of somatic tissues during development, the resulting random chromosome segregation would most likely lead to gross aneuploidy and therefore death. We cannot definitively conclude that ORD has no role in the mitotic divisions. It is possible that the small effect on viability we observe arises because lack of ORD protein does cause a slight perturbation of segregation. However, if ORD is functioning during these divisions, it cannot be the sole player ensuring cohesion.

In addition, our experiments are not consistent with previous studies that concluded ORD function is required during the mitotic divisions of the germ cells. Cytological examination of ord^1 and ord^2 have documented significant levels of aneuploidy (Lin and Church 1982; Miyazaki and Orr-Weaver 1992). We do not see convincing evidence of this in ord^{10}/Df or ord^{10}/ord^5 males. It could be that the nature of ord^1 and ord^2 missense mutations result in a mitotic phenotype, whereas total loss of *ord* function does not. Perhaps ord^1 and ord^2 have neomorphic activity that disrupt mitotic segregation in the germ line. However, it should be noted that not all investigations of ord^1 primary spermatocytes have concluded that ORD function is required for germ-line mitosis (Goldstein 1980). Some variability in scoring may lead to discrepancies because individual chromatids can be counted accurately only if the chromosomes are adequately disentangled. However, if ORD were functioning in a significant capacity to regulate segregation through four consecutive mitotic divisions, it is likely that a male completely lacking ORD protein would exhibit unambiguous defects cytologically. Therefore, we conclude from our analysis that if ORD plays a role in the mitotic divisions of germ cells, it must be a minor one.

It is evident that cohesion functions must operate in both mitosis and meiosis. What remains less clear is whether any proteins are required for cohesion in both types of divisions. If ORD were responsible for the basic cohesive action along the length of chromatids in both mitosis and meiosis, loss of ORD function would be expected to have a serious effect on mitotic divisions, which we have not detected. Because the regulation of the release of meiotic cohesion requires more complex control, it is possible that completely different mechanisms have evolved. Another possibility, however, is that the mechanical basis for cohesion is the same and that merely its regulation is different. One could extend this idea to hypothesize that ORD represents a meiosis-specific function that facilitates the specialized regulation of cohesion required for proper homologue association as well as the step-wise release of the attachments between sisters.

Understanding how ORD functions will provide significant insight into how arm and centromeric cohesion are established and then differentially regulated. We have provided evidence that ORD is absolutely required for both of these functions. What remains to be demonstrated is how ORD is involved in the sequential release of cohesion. Negative complementation behavior suggests that protein interactions are required for ORD function and that these may be regulated differently during the two meiotic divisions. Identification of ORD's binding partner(s) coupled with biochemical

analysis of their activity will provide valuable clues into this essential prerequisite for proper cell division.

We thank CAROLYN COMBS for assistance with the screen for *ord* and *mei-S332* noncomplementers; MICHAEL BICKEL for helpful discussions about theoretical calculations and ANNE KERREBROCK, JACQUELINE LOPEZ, DANIEL MOORE and ANTHONY SCHWACHA for critical reading of the manuscript. This work was supported by Damon Runyon-Walter Winchell Cancer Research Fund Postdoctoral Fellowship DRG 1137 to S.E.B. and the Council for Tobacco Research, Inc.

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Communicating editor: R. S. HAWLEY