Chromosomal Position Effects Reveal Different *cis*-Acting Requirements for rDNA Transcription and Sex Chromosome Pairing in *Drosophila melanogaster*

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ABSTRACT

In *Drosophila melanogaster*, the rDNA loci function in ribosome biogenesis and nucleolar formation and also as sex chromosome pairing sites in male meiosis. These activities are not dependent on the heterochromatic location of the rDNA, because euchromatic transgenes are competent to form nucleoli and restore pairing to rDNA-deficient X chromosomes. These transgene studies, however, do not address requirements for the function of the endogenous rDNA loci within the heterochromatin. Here we describe two chromosome rearrangements that disrupt rDNA functions. Both rearrangements are translocations that cause an extreme *bobbed* visible phenotype and XY nondisjunction and meiotic drive in males. However, neither rearrangement interacts with a specific Y chromosome, Y*mal*$^{+}$, that induces male sterility in combination with rDNA deletions. Molecular studies show that the translocations are not associated with gross rearrangements of the rDNA repeat arrays. Rather, suppression of the *bobbed* phenotypes by Y heterochromatin suggests that decreased rDNA function is caused by a chromosomal position effect. While both translocations affect rDNA transcription, only one disrupts meiotic XY pairing, indicating that there are different *cis*-acting requirements for rDNA transcription and rDNA-mediated meiotic pairing.

ACTIVE transcription of ribosomal DNA (rDNA) is required in all organisms for both nucleolar formation and ribosome biogenesis. Thus, it is somewhat paradoxical that in many multicellular eukaryotes, the rDNA loci reside in the heterochromatin, which is generally transcriptionally quiescent. This localization suggests that some property of heterochromatin is important for rDNA function and/or maintenance. It is possible that a heterochromatic environment is required to suppress recombination between the rDNA genes, which are tandemly repeated within each locus. Localization of the transcriptional silencing protein Sir2 to the nucleolus in yeast and increased rDNA recombination in *sir2* mutants (Gotta *et al.* 1997) support this hypothesis. A heterochromatic environment may also be functionally important. A number of cell cycle regulatory molecules have recently been shown to reside in the nucleolus (Straight *et al.* 1999), suggesting that the rDNA may direct the formation of privileged regulatory sites within the nucleus (for review, see Garcia and Pillus 1999). Location of the nuclear organizing region within heterochromatin may be critical for establishing such nuclear architecture.

The functional significance of a heterochromatic location of the rDNA has best been addressed in *Drosophila melanogaster*, where the availability of rDNA deletions and transgenes has allowed the assessment of various aspects of rDNA function. In Drosophila, the rDNA resides in two roughly equally sized clusters: in the heterochromatin of the X chromosome and on the short arm of the entirely heterochromatic Y chromosome (Cooper 1959; Ritossa *et al.* 1966). In terms of ribosome biogenesis, these two arrays are redundant, and either array is sufficient to encode enough rRNA for ribosome synthesis (Ritossa 1976). Each sex chromosome in wild-type flies usually contains 200–250 tandem repeats of the polycistronic genes encoding 18s and 28s rRNA (Ritossa and Spiegelman 1965). However, many of these genes are interrupted by insertions that render them transcriptionally inactive under most conditions (Glover and Hogness 1977; Pellegrini *et al.* 1977; Wellauer and Dawid 1977; White and Hogness 1977; Labella *et al.* 1983). On the X, ~60% of the genes have type I insertions, while type II insertions are found in 15% of the rDNA genes on both the X and the Y (Dawid *et al.* 1978; Wellauer and Dawid 1978). How the organization of intact and insertion-bearing genes influences the overall function of the rDNA is unknown.

Considering the vital role of rDNA, the maintenance of insertion-bearing cistrons is a conundrum. Their retention may suggest that they have a function that is not dependent on transcription. In this regard, the rDNA arrays in Drosophila have been shown to serve an additional function in male meiosis, where they act as pairing sites between the X and Y (McKee and Karpen 1990). While the X and Y chromosomes share homology at several other loci (*e.g.*, the repeated *stellate* locus; Livak 1984, 1990) and satellite repeats (Peacock *et al.* 1977;

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Brutlag 1980) only the rDNA is able to mediate pairing. In addition, not all homologous sequences within the rDNA arrays participate equally. Individual rDNA cistrons differ not only by type I and II insertion polymorphisms, but also in the number of short repeat sequences present in the intergenic spacer (IGS) between each transcription unit. These IGS repeats have been classified into three families on the basis of length and sequence identity: the 95-, 240-, and 330-bp repeats (Coen and Dover 1982). The 240-bp IGS repeats bear homology to the rRNA promoter (Coen and Dover 1982; Simeone *et al.* 1982; Miller *et al.* 1983) and act as transcriptional enhancers both *in vitro* and *in vivo* (Hayward and Glover 1988; Grimaldi *et al.* 1990). The functions of the 95- and 330-bp repeats are undefined. Assays of pairing ability conferred by rDNA transgenes and deletion derivatives show that the coding regions themselves do not facilitate pairing. Rather, the ability to pair is correlated to the number of 240-bp IGS repeats (McKee *et al.* 1992; Merrill *et al.* 1992; Ren *et al.* 1997).

The 240-bp IGS sequences must possess some property in addition to sequence homology that allows them to function as pairing sites. This may be related to their ability to act as enhancers of rDNA transcription, but may not depend on transcription *per se*. For example, the 240-bp repeat sequences may form an open chromatin domain that is required for both pairing and transcription.

The influence of the heterochromatic environment on all three rDNA functions (XY pairing, nucleolar formation, and ribosome biogenesis) has been partially addressed by transgene studies in Drosophila. Remarkably, a single euchromatic rDNA cistron is capable of directing each of these activities, including formation of a mininucleolus, ameliorating a bobbed phenotype (Karpen *et al.* 1988; McKee and Karpen 1990), and restoring pairing to an rDNA-deficient X chromosome (McKee and Karpen 1990). These observations seem to belie a requirement for a heterochromatic environment for rDNA function. However, it is important to note that these transgene studies have been performed in a genetic background with at least a partially functional endogenous, heterochromatic rDNA locus. It remains to be tested if single euchromatic rDNA cistrons are competent to carry out these functions independently or if they rely on *trans*-acting contributions from the endogenous locus. Furthermore, these studies do not address any additional requirements that might exist for repetitive rDNA sequences in their normal, heterochromatic location. The regulation and activities of single transgenes may significantly differ from rDNA genes embedded in large, complex arrays.

Here we investigate the relationship between the different activities of the rDNA loci in Drosophila through the study of two rearrangements with breakpoints that cause a position effect on the X chromosome rDNA locus. We characterize the effects of the rearrangements on sex chromosome pairing and recovery, rDNA copy number, organization, and transcription. Our results suggest that there are *cis*-acting requirements for both rDNA transcription and rDNA-mediated pairing at the endogenous locus and that these may differ for each activity. We find that full function of the endogenous rDNA locus depends not merely on the presence of the rDNA cistrons but also on chromosomal context.

MATERIALS AND METHODS

Drosophila culture: Drosophila stocks and crosses were maintained at 25° on standard cornmeal-yeast-agar food. The compound-*X* (*C(1)RM, In (1)EN, y v*), the compound-*XY* (*YS-X.YL, y*), and the compound-*4* (*C(4) ci ey*) stocks are described in Lindsley and Zimm (1992). Flies bearing X-linked (*[rib7]1A1-4, X[rib7]*HJ+B, X[rib7]*DM211*) and autosomal (*[rib7]1A1-4(68B)* and *[rib7]1A1-4(94B)*) rDNA transgenes were kindly provided by B. McKee (Karpen *et al.* 1988; McKee and Karpen 1990; McKee *et al.* 1992). The *Df(Y)S12* stock was kindly provided by P. Dimitri.

Mutagenesis and screen for mutations causing sex chromosome nondisjunction: We performed a screen to recover mutations that cause sex chromosome nondisjunction in males. Adult males bearing an X chromosome marked with *yellow* (*y*) and the balancer second and third chromosomes *SM2, Cy* and *TM3, Ubx* were mutagenized with 10 mm ethyl methanesulfonate (EMS; Lewis and Bacher 1968). A *Muller-5* test (Baker and Carpenter 1972) indicated that this dose of EMS produced ~7.5% sex-linked lethal mutations. This dose is lower than that typically used (25 mm) to avoid multiple mutations per chromosome. Results of a previous screen had suggested that X-linked genes with small effects on sex chromosome segregation may be very common (Baker and Carpenter 1972), and we wanted to avoid isolating collections of modifiers that we might not be able to map.

Mutagenized males were mated to compound-*X* females that carried a Y chromosome marked with *y+* (*i.e.*, $X\hat{}X/y^{+}Y$). These females also carried isogenized *cn* second and ry^{506} third chromosomes. Sons bearing the *SM2, Cy* and *TM3, Ubx* chromosomes were individually mated to compound-*X* females that lacked a *Y* (*i.e.*, $X\hat{}X/O$). From this latter cross, all regular *X/O* sons lacked the Y chromosome fertility factors and thus were sterile. The exceptional *X/Y* sons that received both X and Y chromosomes as a result of paternal sex chromosome nondisjunction had wild-type body color and were fertile. These fertile sons were allowed to mate with their $X\hat{}X/Y$ sisters to establish stocks of the mutagenized X chromosomes. The *cn ry* F_2 progeny were selected to remove the *SM2, Cy* and *TM3, Ubx* chromosomes from the stocks. All 116 first-round positives were retested in triplicate, and two mutant X chromosomes were identified that consistently caused >1% nondisjunction.

Chromosome cytology: Prophase chromosomes from larval neuroblast squashes were prepared as described by Gatti *et al.* (1976), stained with Hoechst 33258, and examined by fluorescence microscopy. Breakpoints were localized to specific heterochromatic bands using the maps of Gatti *et al.* (1976). Salivary gland chromosomes were fixed in 45% acetic acid, squashed in acetic acid/orcein, and examined by phase microscopy.

For examination of meiotic chromosomes, testes from adults or third instar larvae were dissected in Schneider's Drosophila media (GIBCO BRL, Gaithersburg, MD), trans-

ferred into acetic acid/orcein for 5 min, teased apart, then squashed under a silanized coverslip in 45% acetic acid (Lifschytz and Hareven 1977).

All chromosome preparations were examined at ×1000 magnification using a Nikon Optiphot light microscope. Images were captured using a Sensys cooled CCD camera (Photometrics, Tuscon, AZ) and IP lab software (Scanalytics, Fairfax, VA).

Recombination mapping of mutants: Both X chromosome mutations were mapped with respect to *m wy sd* and *os*^{o}, which are at positions 36.1, 40.7, 51.1, and 59.2 on the genetic map, respectively. The phenotypes scored for mapping were sex chromosome nondisjunction in *X*/*Y* males and bobbed in *X*/*O* progeny. These two phenotypes cosegregated in all individuals tested. The number of recombinant chromosomes tested for *mscd1* was 406, and for *mscd2* was 197. The following map distances in centimorgans were obtained: *m*–3.7–*wy*–10.1–*sd*–8.9–*os*–29.1–*mscd1* and *m*–3.6–wy–9.1–sd–7.6–os–10.7–*mscd2*. The expansion of the proximal recombination map in *mscd1* is unlikely to be a reflection of low penetrance of the phenotype. In unrelated crosses involving *mscd1* males, the phenotypes were completely penetrant and nonoverlapping with wild type. Recombination is normally suppressed in proximity to the centromere (Dobzhansky 1930; Beadle 1932; Yamamoto and Miklos 1977) and this map expansion likely reflects a deletion of the X centromeric sequences normally involved in this suppression.

Measurement of rDNA copy number and expression: Genomic DNA was isolated from 100 adult *X*/*O* male flies of each indicated genotype by the method of Bender *et al.* (1983). DNA was measured by OD_{260} on a DU Series 64 spectrophotometer (Beckman Instruments Inc., Fullerton, CA). PCR amplification was performed using 5 ng genomic DNA as template, 40 pmoles of each primer, 200 μm dNTPs, 2 mm $MgCl_2$, 1× biolase buffer, and 0.2 units biolase (Intermountain Scientific, Kaysville, UT) in a 50-μl reaction volume. Internal sequences of the 18S and 28S rDNA genes were amplified using 18S primers CTGGTTGATCCTGCCAGT and GTCTTACGACGG TCCAAG and the 28S primers GCCTCTAACTGGAACGTA and ATCTCTCGACGGCTTCTT. Primers were based on sequences reported by Tautz *et al.* (1988). To control for amount of template DNA, sequences including part of exon 1 and 2 of the testis-specific β2-tubulin isoform were amplified using primers CCACGCGCAATTCTCGTGGAC and CACC AGCTGATGCACACTCAG, on the basis of sequence determined by Rudolph *et al.* (1987). The number of PCR cycles was empirically determined such that amplification was in the linear range. Final PCR conditions for 18S rDNA amplification were 24 cycles of 94° for 30 sec, 56° for 1 min, then 72° for 1 min. PCR conditions for 28S rDNA were 24 cycles of 94° for 30 sec, 53° for 1 min, then 72° for 1 min. PCR conditions for β2-tubulin were 26 cycles of 94° for 30 sec, 58° for 1 min, then 72° for 1 min. Reactions were performed using a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT).

For measuring transcript levels, total RNA was isolated using the GLASSMAX RNA isolation kit (GIBCO BRL) from 100 pairs of testes dissected from *X*/*O* or *X*/*Y* males. RNA was quantitated by measuring the OD_{260}, and 1 μg was used from each sample to generate cDNA. First-strand synthesis was performed using 2 pmoles of each of the reverse primers for 18S, 28S, and β2-tubulin described above and 200 units SuperScript II RNase H-reverse transcriptase as per the manufacturer's instructions (GIBCO BRL). A reaction lacking reverse transcriptase was performed in parallel for each sample. First-strand cDNA was amplified by PCR as above using 1/20 of the first-strand reaction as template. All reactions were performed in duplicate.

PCR products were separated by electrophoresis through 1.0% agarose gels containing 0.4 μg/ml ethidium bromide. Gels were photographed using a Kodak Digital Science electrophoresis documentation and analysis system 120 (Eastman Kodak Co., Rochester, NY) and band intensities measured using IPLab Spectrum software (Scanalytics). A plot of intensity *vs.* amount was generated, and the best-fit line was determined by linear regression. The slope and Y intercept were used to calculate an average value for each plot.

Comparison of IGS length variants: IGS profiles from the X chromosome rDNA from *mscd1*/*O*, *mscd2*/*O*, and wild-type *X*/*O* males were compared by examining ethidium bromide-stained PCR products on agarose gels. The wild-type X chromosome used was the progenitor chromosome on which the mutations had been induced. Complete IGS were amplified using a forward primer at the 3′ end of the 28S rDNA gene (IGSF) and a reverse primer located in the external transcribed spacer region (ETSR) described by Polanco *et al.* (1998). Thirty amplification cycles were performed using the following conditions: 94° for 1 min, 48° for 30 sec, 72° for 30 sec. Identical profiles for each sample were obtained from three independent PCR amplifications (data not shown).

DNA sequencing: The intergenic spacers from wild-type and mutant stocks were amplified by PCR using IGSF- and ETSR primers as above. DNA excised from agarose gels was purified by spinning through silanized glass wool, phenol:chloroform extraction, and ethanol precipitation. DNA was sequenced by the Molecular Core Facility of the Wayne State University Center for Molecular Medicine and Genetics, using the same primers used for PCR amplification and dye terminator PCR cycle sequencing on an ABI prism 377 DNA sequencer (Perkin-Elmer, Norwalk, CT) as per manufacturer's instructions. Sequences of IGS PCR products have been submitted to GenBank, accession nos. AF191293, AF191294, and AF191295.

RESULTS

Screen for mutations affecting meiotic chromosome segregation in males: We performed a screen to identify mutations on the X chromosome that increase the frequency of sex chromosome nondisjunction at meiosis I in Drosophila males. Of 5357 males bearing an EMS-treated X chromosome tested, 116 (2.2%) produced one or more progeny that received both paternal sex chromosomes. Lines were established from each of these 116 initial positives, and mutant males from 21 of these reproducibly showed increased rates of XY nondisjunction in subsequent tests (>0.5%). The majority of these mutations were unstable, however, and retested with wild-type levels of nondisjunction after being kept in stock for a period of 6 months to 1 year. A similar instability associated with X-linked male meiotic mutants in Drosophila has been previously reported (Baker and Carpenter 1972). Only two of our mutations were stable and consistently produced more than 1% sex chromosome exceptions among progeny. We named these *mscd1* and *mscd2* for *m*ale *s*ex *c*hromosome *d*isjunction. Neither mutation increased X chromosome nondisjunction in females when homozygous or heterozygous with the multiply inverted X chromosome balancer *Muller-5*.

***mscd1* and *mscd2* are translocations between the X and fourth chromosome:** Salivary gland chromosomes from

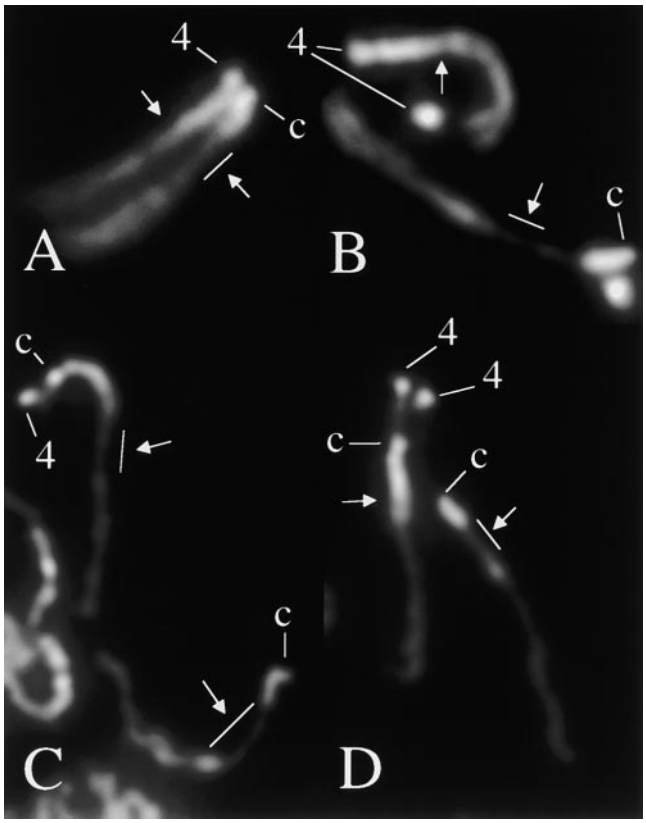

Figure 1.—Hoechst 33258-stained larval neuroblast chromosome squashes. A and B, *mscd1*/ + females; C and D, *mscd2*/+ females. The X centromere (c) and chromosome 4 are indicated. The arrow points to h29, the position of the X chromosome rDNA locus, which is noticeably more extended on the wild-type X than either translocation.

female larvae heterozygous for *mscd1* or *mscd2* and the wild-type progenitor X chromosome were examined, and no abnormalities were detected in the euchromatin. However, examination of prophase larval neuroblast chromosomes stained with Hoechst 33258 indicated that both *mscd1*- and *mscd2*-bearing chromosomes were translocations with one breakpoint on the fourth chromosome and the other in X heterochromatin (Figure 1). Only the 4^{P} X^{D} halves could be analyzed, as the reciprocal translocation halves were not recovered. We mapped the X heterochromatic breakpoints onto the mitotic maps of Gatti *et al.* (1976). The X translocation breakpoint for *mscd1* is h32 while for *mscd2* it is h34. Cytologically, *mscd1* and *mscd2* have been defined as *T(1;4)32h; 102* and *T(1;4)34h; 102*, respectively. For simplicity, we will refer to these chromosomes henceforth as *mscd1* and *mscd2*.

The majority of the X heterochromatin is retained on *mscd1* with the exception of the regions corresponding to the X centromere and the right arm, h33 and h34. In neuroblast chromosome spreads of 20 individuals heterozygous for *mscd1*, all had two free fourth chromosomes, suggesting that the haplo-insufficient *Minute* locus *M(4)101* may be deleted or inactive on the translocated chromosome 4.

The second translocation, *mscd2*, appears to retain nearly all of the X heterochromatin, including the centromere region, and the entire fourth chromosome attached to the short arm of the X. It also retains a functional allele of *M(4)101*, as 16 of 24 larval neuroblast spreads examined had only one free fourth chromosome, but adults bearing the translocation do not appear Minute.

In agreement with our cytology, *mscd* males exhibited a transmission pattern expected of pseudolinkage. As shown in Table 1, a single cross between $Xy/y^{+}Y$ males and *y w sn; C(4) ci ey* females can be used to simultaneously monitor sex and fourth chromosome disjunction. From such matings, paternal sex chromosome nondisjunction results in y^{+} daughters (from XY-bearing sperm) or y sons (from nullo-XY sperm). Sperm lacking a fourth chromosome as a result of nondisjunction or loss produce ci ey progeny, whereas progeny produced from diplo-4 sperm cannot be distinguished. In these crosses, *mscd1* and *mscd2* males produced 4.2 and 10.9% XY exceptions, respectively. The *mscd2* males also produced ~5% ci ey progeny. Because we could detect only half of fourth-chromosome exceptional gametes (the nullo-4 class) in only one sex (the nontranslocation-bearing males) this corresponds to ~20% fourth-chromosome nondisjunction, assuming no chromosome loss. The presence of two free fourth chromosomes in *mscd1* males precluded detection of fourth-chromosome nondisjunction, as nullo-exceptional progeny would have resulted only if all three fourth chromosomes cosegregated.

In addition to establishing that both *mscd* mutations were X;4 translocations, we noted that the major constriction within the X heterochromatin of each translocation chromosome was consistently reduced in size relative to that of the wild-type X (Figure 1). This heterochromatic region, h29, corresponds to the location of the rDNA (Gatti *et al.* 1976). The altered appearance of this region suggested that changes in the rDNA are also associated with each of the translocations.

The *mscd* mutations are alleles of the *bobbed* locus: Several additional observations also suggested that the *mscd* mutations might affect the rDNA. When *mscd1* or *mscd2* males were mated to *X^X/O* females, the *X/O* sons produced had short bristles, abnormal abdomens, and reduced viability. These features are characteristic of a bobbed (bb) phenotype that results from a reduction in the number of rDNA repeats and reflects reduced protein translation owing to a deficiency in functional ribosomes (Ritossa *et al.* 1966). X chromosomes that retain fewer than 160 of the 200–250 tandem copies of the rDNA produce a bb phenotype; those retaining 40 or fewer copies are recessive lethals (Tartof and Hawley in Lindsley and Zimm 1992). The mutant phenotypes of *mscd1* and *mscd2 X/O* males were extreme, and

TABLE 1

Nondisjunction and meiotic drive in *mscd1* and *mscd2* males

Paternal X	Sex and fourth chromosomes of sperm						% XY nondisjunction	R_Y/R_X	R_{XY}/R_O
	x;4	Y;4	XY;4	O;4	X;O	Y;O			
+	1224 (46.3)	1406 (53.2)	6 (0.2)	5 (0.2)	2 (0.1)	0	0.5	1.15	1.2
mscd1	1385 (50.1)	1261 (45.7)	18 (0.7)	98 (3.5)	0	0	4.2	0.91	0.18
mscd2	1138 (51.4)	719 (32.4)	71 (3.2)	171 (7.7)	0	117 (5.3)	10.9	0.73	0.42

Results of crosses of X/y^+Y males to *y w sn*^{3}; c(4) ci ey females. The frequency of each sperm class recovered among progeny is indicated in parentheses. R_Y/R_X, which is a measure of the recovery of the Y chromosome relative to that of the X, is calculated as Y sperm/X sperm. R_{XY}/R_O measures the recovery of diplo-exceptional XY sperm relative to null-exceptional sperm that lack a sex chromosome and is calculated as XY sperm/nullo-XY sperm.

their viability reduced to about half that of wild-type *X/O* males (Table 6), whereas *mscd/Y* males were phenotypically normal. Similarly, homozygous *mscd* females had an extreme phenotype, whereas *X/X/Y mscd* females were normal. These results are consistent with the interpretation that both *mscd* mutations are strong *bobbed* alleles, deficient for rDNA function, and that this deficiency is complemented by the Y chromosome rDNA array. In agreement with this interpretation, recombination mapping localized the defect(s) responsible for both the bb phenotype and the meiotic nondisjunction to the proximal region of the X, the location of the rDNA (see materials and methods).

We asked if the *mscd* mutations affect rDNA function by testing for complementation of several *bb* alleles, including *bb*^{5}, *bb*^{l} (*bobbed lethal*), and *In(1)sc*^{4L}*sc*^{8R}. These *bb* mutations vary in severity, reflecting differences in rDNA copy number (Tartof and Hawley in Lindsley and Zimm 1992). The phenotypes of individuals heterozygous for the *mscd* and the *bb* alleles varied correspondingly. Therefore *mscd* mutations are *bona fide* alleles of *bb*.

Measurement of rDNA copy number and activity: We measured the amount of rDNA on each *mscd*-bearing chromosome relative to the wild-type parent X chromosome by performing quantitative PCR on genomic DNA isolated from *X/O* males. Copy numbers of both 18S and 28S rDNA sequences were quantified relative to the single copy β2-tubulin gene. As a control, we also measured rDNA copy number in *bb*^{5}*/O* males. The *bb*^{5} allele produces a relatively mild bb phenotype and therefore was expected to contain from 50 to 80% of the wild-type number of X rDNA repeats. Neither 18S nor 28S sequences were significantly reduced in copy number in *mscd* mutants, but were reduced in the *bb*^{5} control (Table 2). To further ensure that the primers and PCR conditions were suitable, we verified this result by Southern hybridization to genomic dot blots using the entire rDNA cistron as a probe. Densitometric scans indicated that *mscd1* and *mscd2* X chromosomes contained 107 ± 11% and 91 ± 8% rDNA relative to the wild-type progenitor X, respectively (data not shown).

These measurements also reveal that compensation is not defective in *mscd* mutants. Compensation refers to an amplification of rDNA sequences on an X chromosome that occurs in an individual lacking rDNA on the homolog (*e.g.*, in *X/O* males or in *X/In(1)sc*^{4L}*sc*^{8R} females). This amplification results from a disproportionate replication of rDNA in somatic tissues and is controlled by the compensatory response locus that maps adjacent to the rDNA (Procunier and Tartof 1978). We found the same copy number of rDNA in wild-type *X/O* males, which are presumed to have compensated, and in *mscd X/O* males.

We asked if transcription of the rDNA was altered by performing quantitative RT-PCR on total RNA isolated from testes of mutant and wild-type *X/O* and *X/Y* males, using the same primers and conditions as for the DNA measurements. For both mutants, we found a small but consistent reduction (~20%) in rRNA levels in *X/O* but not *X/Y* males. A similar reduction was observed for *bb*^{5}*/O* males, but only for the 18S transcript (Table 2). These results suggest that there may not be a strict correlation between the severity of a bobbed phenotype and levels of accumulated rDNA transcripts in the adult testis. Nonetheless, they suggest that rDNA transcription from each *mscd* X chromosome is indeed decreased.

IGS within the rDNA loci on *mscd1* and *mscd2*: Several observations suggest that functional differences exist between individual cistrons within the rDNA arrays. First, both the pairing ability of transgenes and rDNA transcription (Hayward and Glover 1988; Grimaldi *et al.* 1990) are sensitive to the number of 240-bp IGS repeats, which vary between cistrons. Second, partial reversions of bb mutations can occur by rearrangement of the rDNA without an increase in gene number (Terracol *et al.* 1990). Third, using a series of free X chromosome duplications, a functional pairing site could be mapped *within* the rDNA, suggesting that not all cistrons

TABLE 2

Quantitative PCR and RT-PCR measurement of rDNA and rRNA in *mscd1* and *mscd2* males

	Value relative to wild type			Divided by βTUB control	
Genotype	18S	28S	βTUB	18S	28S
Genomic DNA					
X + */O*	1.00 ± 0.02	1.00 ± 0.15	1.00 ± 0.08		
mscd1/*O*	0.86 ± 0.16	0.84 ± 0.14	0.81 ± 0.14	1.07	1.04
mscd2/*O*	0.85 ± 0.16	0.86 ± 0.31	0.93 ± 0.31	0.91	0.90
bb^5/*O*	0.61 ± 0.09	0.87 ± 0.06	1.13 ± 0.02	0.54	0.77
RT-PCR					
X + */O*	1.00 ± 0.07	1.00 ± 0.07	1.00 ± 0.06		
mscd1/*O*	0.88 ± 0.02	0.87 ± 0.01	1.06 ± 0.00	0.82	0.82
mscd2/*O*	1.11 ± 0.07	1.01 ± 0.03	1.24 ± 0.06	0.82	0.89
bb^5/*O*	0.80 ± 0.02	1.06 ± 0.10	0.94 ± 0.11	0.85	1.13
X + */Y*	1.00 ± 0.05	1.00 ± 0.04	1.00 ± 0.05		
mscd1/*Y*	1.13 ± 0.04	1.02 ± 0.04	1.03 ± 0.01	0.99	1.10
mscd2/*Y*	1.03 ± 0.03	1.10 ± 0.09	1.02 ± 0.15	1.08	1.01

The copy numbers of 18S and 28S rDNA genes in *mscd1* and *mscd2* males were determined relative to wild-type males by quantitative PCR. The single copy β2-tubulin gene was used as a control to adjust for differences in the amount of starting template. Numbers shown are averages of two trials plus or minus standard deviations. RT-PCR was performed using total RNA isolated from adult male testis, and amount of transcripts in mutant males was similarly determined relative to transcript levels in wild-type males.

participate equally in pairing (Park and Yamamoto 1995).

To ask if rearrangements of rDNA sequences might be responsible for the *mscd* mutations, we looked for changes in the profile of IGS repeats. IGS regions were amplified from *X*/*O* genomic DNA by PCR using a forward primer located in the 3′ end of the 28S gene and a reverse primer located in the 5′ end of the external transcribed spacer, which is located 5′ of the 18S coding sequence (Polanco *et al.* 1998). Amplified fragments were separated on agarose gels, and the mutant patterns were compared to wild type (Figure 2). The patterns of the mutants were similar to each other and differed from that of the wild-type progenitor chromosome in two respects. The relative abundance of repeat classes of >3.5 kb in length was increased in both mutants, while a 1.4-kb variant present in wild type was absent in both mutants. We cannot ascribe particular changes at this level to the mutant phenotypes, however, since the two *mscd* IGS profiles were virtually identical, and yet the mutant phenotypes were different.

The *mscd* phenotypes might instead have resulted from smaller scale changes within the IGS repeats, such as point mutations in the 240-bp subunits. Such mutations may have become fixed within the entire array by the gene conversion-mediated process of homogenization (Dover 1982). To address this possibility, we determined the sequence of the 1.2-kb IGS variant from both mutants and wild type, as well as the 1.4-kb variant present only in wild type. In addition, we determined partial sequence of the IGS repeats from the 4.2-kb variant present on all three chromosomes. The structures of each repeat sequenced are shown in Figure 2.

The 1.4-kb band, present only in the wild-type strain, contains the 3′ end of the 28S gene, seven 95-bp subunit repeats, followed by two repeats of 120 bp that are internal to the 240-bp repeat, and the conserved promoter consensus within the ETS. It lacks a 330-bp subunit repeat. The portion of the 240-bp repeat unit present lacks homology to the promoter. Given that this variant contains only a partial 240-bp repeat, it seems unlikely that its absence in the mutants can account for the phenotypes. We found no sequence difference between wild-type and *mscd* chromosomes for the 1.2-kb and 4.2-kb repeat variants. Of note, the 4.2-kb variant contains at least one 240-bp repeat 5′ to an intact 18S, indicating that both *mscd* chromosomes contain sequences that could potentially function as meiotic pairing sites.

These data suggest that the *mscd* phenotypes do not result from changes in the overall organization and representation of sequences in the rDNA loci, but rather that they might be owing to changes external to the rDNA. These may include changes in *trans*-acting factors (*e.g.*, mutations in heterochromatic genes required for rDNA function) or *cis*-acting effects (*e.g.*, position effects or mutations in *cis*-acting regulatory loci).

Complementation by rDNA transgenes: To differentiate between *trans*-acting and *cis*-acting effects of the mutants, we tested the ability of rDNA transgenes to complement. Additional copies of rDNA inserted into the euchromatin have been shown to act *in trans* to ameliorate the bb phenotype in rDNA-deficient flies and also

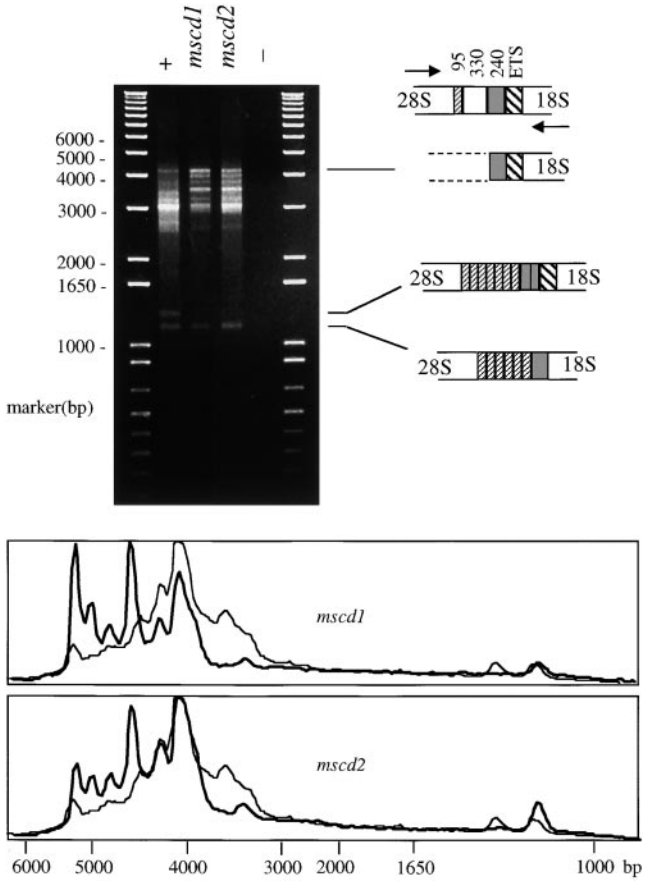

Figure 2.—Amplification of IGS regions using IGS-F and ETS-R primers and genomic DNA extracted from wild-type, *mscd1*, and *mscd2* *X*/*O* males, including a no template control. IGS length variants were compared using densitometry plots as shown in the bottom half of the figure. In each plot, the wild-type tracing is shown by the thin line and the mutant tracing by the thick line. Positions of known molecular size markers are indicated below the tracings and to the left of the gel. Diagrams on the upper right indicate the structure of the indicated 4.2-, 1.4-, and 1.2-kb bands as determined by DNA sequencing.

to confer the ability to form nucleoli (Karpen *et al.* 1988; McKee and Karpen 1990). The meiotic pairing defect in rDNA-deficient males can be complemented *in cis* by the addition of rDNA and, specifically, IGS sequences in a dose-dependent manner (McKee *et al.* 1992; Merrill *et al.* 1992; Ren *et al.* 1997). If the translocations disrupted a *cis*-acting element (*e.g.*, the rDNA itself or *cis*-acting regulators), we expected complementation of the bb phenotype by autosomal or X-linked rDNA transgenes and complementation of the meiotic defect by X-linked transgenes. If the *mscd* mutants affect *trans*-acting functions, then addition of rDNA transgenes should have no effect on the bb phenotype or the meiotic pairing.

To test for complementation of the bb phenotype, a transgene containing a complete rDNA cistron, inserted at salivary gland chromosome band 68B or 94B ([rib7], McKee and Karpen 1990), was introduced into mutant males. Males with zero, one, or two copies of the transgene were then mated to *X*^*X*/*O* females, and both XY nondisjunction and recovery of *X*/*O* sons were measured (Table 3). Measurements of nondisjunction in these crosses can be complicated by viability differences between bb and bb$^+$ individuals. Therefore, we considered only the frequency of progeny receiving *nullo-XY* sperm (*X*^*X*/*O* daughters), relative to those receiving Y-bearing sperm (*X*^*X*/*Y* daughters). Neither of these classes are bb, since both contain an intact rDNA cluster on the X^X chromosome. Thus their recoveries are unlikely to reflect differences in viability owing to the presence or absence of the transgenes.

As expected, autosomal transgenes did not alter XY disjunction in either *mscd* mutant. However, the viability of bb *X*/*O* sons was measurably affected by the presence of the transgene (Table 3). The recovery of *X*/*O* sons receiving the transgene (marked with a wild-type copy of the *rosy* gene, *ry*$^+$) was measured relative to *X*/*O* sons that did not receive the transgene (ry$^-$). This ratio was adjusted by the relative recoveries of ry$^+$ *vs.* ry bb$^+$ sisters to control for any viability differences due to the ry$^+$ marker. Sons receiving the transgene survived two to three times as well as their brothers that did not receive the transgene. The addition of the transgene also significantly decreased the *bb*-associated developmental delay (Ritossa 1976) of these *X*/*O* individuals, as measured by eclosion time of ry$^+$ *vs.* ry individuals (mean eclosion time 15.3 ± 2 *vs.* 17.1 ± 3 days). These results indicate that the bb phenotype caused by either *mscd* mutation can be rescued *in trans* by the addition of a functional rDNA cistron. Therefore, it is likely that both translocations affect rDNA function *in cis*, since the *trans*-acting components required for activity of the rDNA transgene appear to be intact.

A *cis* effect might result from a mutation in the rDNA itself or in a gene required for the activity of the heterochromatic rDNA array (*e.g.*, a function affecting the chromatin structure of the locus). We expected that in either case, the meiotic phenotype would be complemented by the addition of euchromatic rDNA transgenes *in cis*. To test this, three different transgenes were recombined onto both *mscd1* and *mscd2* chromosomes. The first transgene contained a single copy of the complete rDNA cistron, containing the 18S, 28S, and IGS sequences, including 11 copies of the 240-bp repeat subunits that contain pairing ability (*[rib7]1A1-4*; McKee and Karpen 1990). The other two constructs, *[rib7]**HJ+B and *[rib7]**211, are deletion derivatives of the complete cistron and contain 10 and 8 repeats of the 240-bp subunit of the IGS, respectively (McKee *et al.* 1992). The *[rib7]**HJ+B is deleted for all of the 28S and part of the internal transcribed spacer, while *[rib7]**211 retains only 240-bp repeats. Males bearing each *mscd* mutation were then crossed to *X*^*X y v*/*O* females. In these crosses we could detect only half of the nondisjunction events (the nullo-exceptions) be-

TABLE 3

The effects of autosomal rDNA transgenes on nondisjunction and X/O viability

Paternal genotype		Sex chromosome of sperm: T(1;4)	Y	T(1;4)+Y	0	% nullo-XY	[rib7] recovery
mscd1; ry^{506}		183	401	58	127	24.1	
mscd1; [rib7]94B ry^{506}/ry^{506}	ry	182	650	99	140	17.7	2.98
	+	612	734	125	140	16.2	
mscd1; [rib7]94B ry^{506}/[rib7]94B ry^{506}		328	381	83	98	20.5	
mscd1; ry^{506}		55	195	3	28	12.6	
mscd1; [rib7]68B ry^{506}/ry^{506}	ry	223	598	16	100	14.3	1.80
	+	514	767	36	155	16.8	
mscd1; [rib7]68B ry^{506}/[rib7]68B ry^{506}		423	586	27	78	11.7	
mscd2; ry^{506}		111	365	22	49	11.8	
mscd2; [rib7]94B ry^{506}/ry^{506}	ry	187	565	113	106	15.8	2.44
	+	508	630	114	101	13.8	
mscd2; [rib7]94B ry^{506}/[rib7]94B ry^{506}		154	158	28	31	16.4	
mscd2; ry^{506}		127	337	55	112	24.9	
mscd2; [rib7]68B ry^{506}/ry^{506}	ry	172	638	117	205	24.3	3.51
	+	667	705	135	219	23.7	
mscd1; [rib7]68B ry^{506}/[rib7]68B ry^{506}		434	53	136	181	28.5	

Crosses *mscd1/y$^+$Y; ry* or *mscd2/y$^+$Y; ry* males bearing zero, one, or two copies of an autosomally inserted transgenic rDNA construct located at polytene band 68B or 94B, to *C(1)RM, y v; ry^{506}* females. The percentage of nullo-XY is calculated as (X^X/O)/(X^X/O + X^X/Y). Each insert contains an entire rDNA cistron ([rib7]) and a wild-type copy of the *rosy* gene. The [rib7] recovery is a measurement of the effect of receiving a [rib7] insert on the viability of X/O sons and is calculated as (ry$^+$ X/O)(ry$^-$ X^X/Y)/(ry$^-$ X/O)(ry$^+$ X^X/Y).

cause the *y* mutation that allowed detection of the reciprocal class had been removed by recombination.

A significant decrease in sex chromosome nondisjunction was observed in *mscd1* males bearing the *[rib7]* HJ+B* transgene derivative (Table 4). The other transgenes had no effect. Of the three transgenes used, *[rib7]* HJ+B* has been shown to be most effective at promoting pairing of rDNA-deleted X chromosomes (McKee *et al.* 1992, 1998). The lack of an effect of the other two transgenes may reflect a difference in pairing activities of the constructs. In *mscd2* males, none of the three X-linked transgenes had an effect on sex chromosome meiotic disjunction.

Together, the results of somatic and meiotic complementation by rDNA transgenes argue that the *mscd* defects are *in cis*-acting components required for ribosome biogenesis and/or meiotic pairing. The rescue of both defects in *mscd1* suggests that it behaves as a loss-of-function mutant with respect to both activities. The *mscd2* mutation, on the other hand, differentially affects the two functions. It behaves as a loss-of-function mutation with respect to the bb phenotype, whereas XY pairing is unaffected.

Suppression by Y heterochromatin: Our results suggest that the activity of the rDNA loci on the *mscd* chromosomes is modified as a result of the translocation breakpoints. This may occur via a stable or a variegated position effect resulting from proximity to fourth chromosome sequences. Position-effect variegation (PEV) of the rDNA has been previously reported for chromosomes with breakpoints within the rDNA (Hannah-Alava 1971) and for a series of X chromosome inversions in which the rDNA is displaced to the distal end of the X (Baker 1971).

The classical test of position-effect variegation is suppression by the Y chromosome (Gowen and Gay 1933). We could not test for suppression of the *mscd* phenotypes by an intact Y chromosome, since the Y contains an rDNA locus and therefore would not allow us to differentiate between suppression and complementation. However, all Y heterochromatin is capable of suppressing variegated position effects (Dimitri and Pisano 1989); thus we could test for suppression of the bb phenotype using a Y chromosome that is completely deleted for the rDNA, *Df(Y)S12* (Gatti *et al.* 1976).

Females bearing attached-X chromosomes and *Df(Y)S12* were crossed to *mscd/Y* males, and the viability of the *X/Y* sons was compared to that of *X/O* sons produced from matings of X^X/O females and the same *mscd/Y* males. The presence of *Df(Y)S12* increased the recovery of *mscd1* and *mscd2* sons relative to their *X^X/Y* sisters (Table 5). Furthermore, these sons were phenotypically bb$^+$. These results confirm that the rDNA is subject to chromosomal position effects in both *mscd1* and *mscd2*.

Comparison of *mscd1* and *mscd2* phenotypes to rDNA-deletion phenotypes: In addition to the somatic bb phenotype, three phenotypes are associated with deletions

TABLE 4

The effects of X-linked rDNA transgenes on sex chromosome nondisjunction

Paternal X	Sex chromosome of sperm			% nullo-XY
	X	Y	O	
mscd1, y w sn^3	236	544	38	6.5
mscd1,[rib7], w sn^3	114	648	41	6.0
*mscd1,[rib7]*211, w sn*^{3}	129	619	34	5.2
*mscd1,[rib7]*HJ*$^+$*B, w sn*^{3}	254	886	29	3.2**
mscd2, y w sn^3	258	529	67	11.2
mscd2,[rib7], w sn^3	309	683	114	14.3
*mscd2,[rib7]*211, w sn*^{3}	486	959	175	15.4*
*mscd2,[rib7]*HJ*$^+$*B, w sn*^{3}	417	731	130	15.1*

Transgenic rDNA constructs containing an entire rDNA cistron (*X[rib7]*) or deletion derivatives containing only 8 (*X[rib7]*211*) or 10 (*X[rib7]*HJ+B*) IGS tandem repeats were recombined onto a *mscd1, y w sn*^{3} or *mscd2, y w sn*^{3} chromosome. Meiotic tests were carried out by crossing males bearing the recombinant X and y^+Y to *C(1)RM y v/O* females. See materials and methods for details. * $P < 0.05$ when compared to control crosses of *mscd1, y w sn*^{3} males; ** $P < 0.01$ when compared to control crosses of *mscd2, y w sn*^{3} males.

TABLE 5

Y heterochromatin increases the viability of *mscd1* and *mscd2* males

Paternal genotype	Sex chromosome of sperm			Disjunction frequency	Male recovery
	X	Y	O		
+ / y^+Y	889	642	3	0.998	1.39
mscd1/y^+Y	1015	664	49	0.972	4.34 (3.13)
mscd2/y^+Y	1470	661	207	0.912	3.82 (2.76)

Results of crosses of X/y^+Y males to *C(1)RM, y v / Df(Y)S12* y^+ females. Male recovery is a measurement of the survival of $X/Df(Y)S12\ y^+$ sons relative to X/O sons from Table 2 and is calculated as ($X/Df(Y)S12\ y^+$ sons)/(*C(1)RM, y v*/y^+ *Y* daughters)* (*C(1)RM, y v*/y^+ *Y* daughters)(X/O sons). The value in parentheses has been adjusted by dividing by the male recovery obtained from crosses involving control +/y^+ *Y* males.

of rDNA on the X chromosome: (1) the X and Y fail to pair at meiosis I (Gershenson 1933; Cooper 1964; Appels and Hilliker 1982; McKee and Lindsley 1987), (2) sperm genotypes from males bearing the rDNA-deficient X are recovered in non-Mendelian ratios (meiotic drive; Gershenson 1933; Sandler and Braver 1954; McKee and Lindsley 1987), and (3) males bearing an rDNA-deficient X and Y *mal*$^+$ are sterile (Lifschytz and Lindsley 1972; Schalet 1972; Schalet and Lefevre 1973; Rahman and Lindsley 1981; McKee and Lindsley 1987). These phenotypes result from a lack of the rDNA cistrons, and not surrounding sequences, as they can be complemented by rDNA transgenes (McKee and Karpen 1990; McKee *et al.* 1998). However, it is unclear if they result from the lack of rDNA transcription, or if rDNA sequences provide binding sites or play a structural role required for normal progression of meiosis and spermatogenesis. To discern between these alternatives, we asked if the *mscd1* and *mscd2* chromosomes also produced these phenotypes.

Cytological examination of meiosis in mutants: Deletions of the rDNA specifically disrupt XY pairing in meiosis I in males, resulting in sex chromosome nondisjunction (Gershenson 1933; Cooper 1964; Appels and Hilliker 1982; McKee and Lindsley 1987). Such deletions remove the majority of the *cis*-acting male meiotic pairing sites, IGS within the rDNA, that promote meiotic pairing between the *X* and *Y* chromosome (McKee and Karpen 1990; McKee *et al.* 1992; Merrill *et al.* 1992; Ren *et al.* 1997).

To ask if the XY nondisjunction in *mscd* males resulted from a pairing defect, we examined orcein-stained meiosis I chromosomes in testis squashes. In such preparations, chromosome pairing can be easily assayed from prophase I until anaphase I by counting the number of orcein-staining bodies within meiocytes. Normal meiocytes contain three orcein-positive bodies corresponding to the sex and major autosome bivalents and a smaller body corresponding to the fourth chromosome bivalent. The sex chromosome bivalent can be distinguished from the autosomes by position and shape; it tends to be separate from the autosomes and is more elongated. In all meiocytes from *mscd1*/*Y* and *mscd2*/*Y* males, the major autosomes appeared to be paired normally. However, in *mscd1*/*Y* males, the X and Y were not associated in 13.9% (39/281) meioses examined. The frequency of this phenotype was roughly the same at prophase (14/88, Figure 3, A and D) and prometaphase/metaphase (25/172, Figure 3, B and E), suggesting a defect in pairing rather than in cohesion. It is also notable that this frequency is higher than predicted from the genetic assays of nondisjunction (4.2%, Table 1), suggesting that some products of these aberrant meioses are eliminated prior to fertilization.

In contrast, *mscd2*/*Y* males did not exhibit the same XY pairing defect. Rather, in 224/227 meioses examined, both sex chromosome and autosome pairing appeared normal. This difference from *mscd1* males cannot be attributed to the number of free fourth chromosomes present, as one-third of the *mscd2* males also had two free fourth chromosomes. Rather, these observations suggest that, unlike *mscd1*, the meiotic pairing activity of the rDNA locus is not disrupted by *mscd2* and point to a different cause for nondisjunction.

Examination of anaphase I meiocytes of *mscd2*/*Y* males indicated that segregation rather than pairing was perturbed. Of 50 anaphase I figures observed in

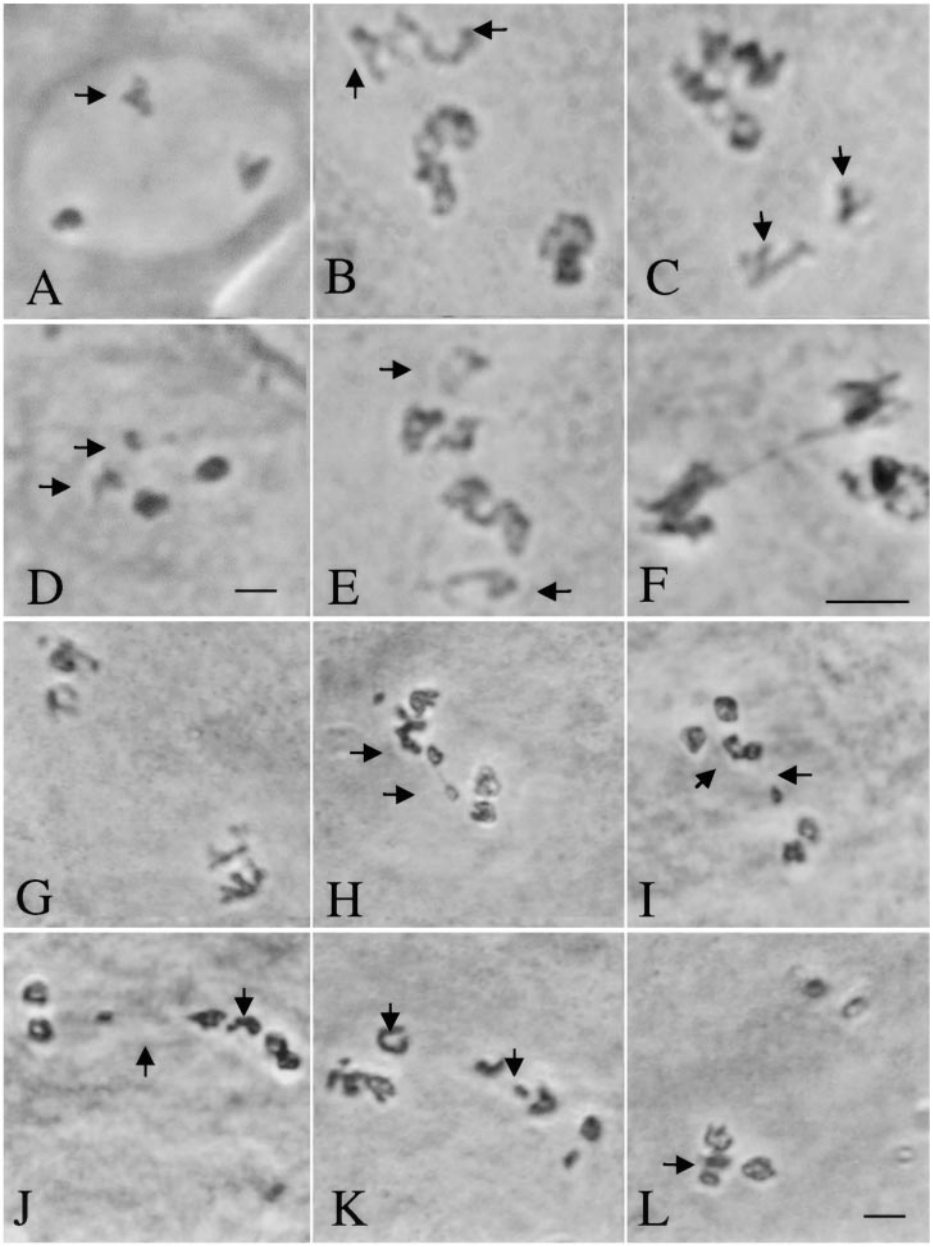

Figure 3.—Orcein-stained meiosis I chromosome squashes of *mscd* mutants. Prophase *mscd1* meiocytes with paired (A) and unpaired (D) sex chromosomes. Metaphase *mscd1* meiocytes with paired (B) and unpaired (E) sex chromosomes. Anaphase *mscd2* meiocytes showing normal sex chromosome disjunction (C) and anaphase bridge formation (F). Normal anaphase in wild type (G). Anaphase bridges involving the T(1;4) chromosome in *mscd2* meiocytes (H–K). Nondisjunction of sex chromosomes in telophase *mscd2* meiocyte (L). Arrows indicate sex chromosomes. Bar, 10 μm.

mscd2 males, 24 could be unambiguously classified as abnormal. Chromatin bridges were observed in 6 meiocytes (Figure 3, F, H–K), and in five cases it could be discerned that the bridge involved the T(1;4) chromosome. In these cells, the Y chromosome was associated with the X portion of the T(1;4), while the fourth chromosome portion of the T(1;4) was displaced toward a pole. A resulting bridge of stretched chromatin was visible between the X and four parts of the T(1;4). In 11 meiocytes, sex chromosomes were observed near the metaphase I plate, while the autosomes were at the poles (Figure 3K), and in 7 additional meiocytes, the sex bivalent was observed near one pole (Figure 3L).

Because Hoeschst staining of neuroblast chromosomes indicated that both the X and fourth chromosome centromeres are present on this translocation, it is possible that such meiosis I bridges result from dicentric behavior; that is, opposite orientation of two functional centromeres on the T(1;4) would result in a bridge as the centromeres segregate at anaphase. However, several observations suggest that kinetic activity is retained at only one centromere. First, in mitosis this translocation does not result in nondisjunction or loss, as mosaicism for X-linked markers and sexually dimorphic structures in translocation-bearing progeny would have been readily detectable but was not observed. Second, we failed to observe bridges in meiosis II. Thus, any dicentric activity would have to be confined to meiosis I. Finally, we never observed a meiosis I bridge in which the X and four halves of the translocation appeared to be leading movement to opposite spindle poles. Notably, in each meiocyte that had a bridge, the Y and free

TABLE 6

Effects of *mscd1* and *mscd2* on nondisjunction, meiotic drive, and fertility with y^+ *Y mal*$^+$

Paternal genotype	Sex chromosome of sperm				% XY nondisjunction	R_{XY}/R_O
	X	Y	XY	O		
y/y^+Y	6887	6889	6	5	0.01	1.20
	(50.0	(50.0)	(0)	(0)		
y mscd1 $/y^+Y$	301	752	21	53	6.6	0.40
	(26.7)	(66.7)	(1.9)	(4.7)		
y mscd2 $/y^+Y$	283	552	86	113	19.2	0.76
	(27.4)	(53.4)	(8.3)	(10.9)		
y/y^+ *Y mal*$^+$	2353	1558	7	47	1.4	0.15
	(59.3)	(39.3)	(0.2)	(1.2)		
y mscd1 $/y^+$ *Y mal*$^+$	471	481	8	192	17.4	0.04
	(40.9)	(41.8)	(0.7)	(16.7)		
y mscd2 $/y^+$ *Y mal*$^+$	528	857	137	544	33.0	0.25
	(25.6)	(41.5)	(6.6)	(26.3)		

Results of crosses of *Xy*$/y^+Y$ and *Xy*$/y^+$*Ymal*$^+$ males to *C(1)RM, y v*$/O$ females. The frequency of each sperm class recovered among progeny is indicated in parentheses. R_{XY}/R_O measures the recovery of diplo-exceptional XY sperm relative to nullo-exceptional sperm that lack a sex chromosome and is calculated as XY sperm/nullo-XY sperm.

4 appeared to have oriented to the same pole. In these cells, the X^D part of the T(1;4) was clearly associated with the Y, while the 4^P part was closer to the opposite pole. The Y portion of the sex bivalent was always observed closer to the pole than the X.

We suggest that these bridges are a consequence of trivalent formation between the Y, the free 4, and the T(1;4). In meioses in which the Y and free 4 orient to the same pole, any lag in separation or segregation of the sex chromosomes with respect to the fourth chromosomes would result in a bridge. The *mscd* rearrangements may cause such an asynchrony by delaying the release of the sex chromosome cohesion at anaphase, perhaps owing to decreased tension on the X-Y pairing bond as a result of trivalent formation. Asynchrony of bivalent separation at anaphase may reflect a peculiarity of meiosis I in this organism. Whereas in most eukaryotes, synchrony at anaphase initiation is maintained by a metaphase checkpoint that senses improper tension across a bivalent (Nicklas 1997), male Drosophila seem to lack this checkpoint at meiosis I. Univalent chromosomes, which perforce lack tension at metaphase I, do not detectably delay the onset of anaphase in male Drosophila (Basu *et al.* 1998).

The majority of aneuploidy caused by both *mscd1* and *mscd2* can be attributed to meiosis I nondisjunction rather than meiosis II nondisjunction or chromosome loss. In *mscd* males, the frequency of sex chromosome aneuploidy seen at meiosis II was roughly equal to the frequency of meiosis I defects observed, and *diplo-XY* and *nullo-XY* cells were approximately equal in number. Of 91 meiosis II anaphases observed in *mscd1* males, 4 were *nullo-XY* and 4 were *diplo-XY* (8.8% aneuploid). Of 64 meiosis II anaphase spreads observed in *mscd2* males, 7 were *nullo-XY* and 6 were *diplo-XY* (20.3% aneuploid). No primary meiosis II nondisjunction was observed for either mutation. These cytological observations are consistent with those of crosses of *y mscd*$/y^+Y$ males to *X^Xyv*$/O$ females in Table 6. Among progeny of such crosses we failed to observe v^+ daughters, which would have been indicative of meiosis II nondisjunction.

***mscd1* and *mscd2* cause meiotic drive:** Meiotic drive associated with rDNA deficiencies favors the recovery of sperm bearing less chromatin (Gershenson 1933; Sandler and Braver 1954; McKee 1984). The relative recoveries of sperm classes from *Df(rDNA)*$/Y$ males are nullo-XY > X > Y > diplo-XY. In addition, a direct correlation has been reported between the "strength" of the drive (the greater the difference from Mendelian expectations) and the frequency of sex chromosome nondisjunction (McKee and Lindsley 1987). We observe both of these properties in *mscd1* and *mscd2* mutant males. This is evident in Table 1 by comparison of the recovery of the Y chromosome relative to the X chromosome, as measured by the ratio Ry/Rx of Y-bearing progeny (*y w sn*$/y^+Y$ males) to X-bearing progeny (*y w sn*/*mscd* females). Among progeny of *mscd1* males this ratio is reduced to 0.91, and among the progeny of *mscd2* males, which have a higher frequency of XY nondisjunction, the ratio is further reduced to 0.73. It is unlikely that these numbers reflect viability differences, because in all crosses the progeny receiving Y-bearing sperm are genetically identical, and all progeny considered for this calculation are bb^+. Furthermore, neither *mscd1* nor *mscd2* decrease viability in heterozygous individuals. In crosses in which either *mscd* is contributed maternally, we find no reduction in viability of heterozygous *mscd*/+ daughters compared to +/+ sisters (data not shown).

Meiotic drive is also evident from the relative recovery of diplo- *vs.* nullo-exceptional progeny (Tables 1 and 6). This ratio, R_{XY}/R_O, is affected to a greater degree by drive owing to the greater difference in chromatin content between the two sperm genotypes compared (*XY vs. nullo-XY*). In Table 1, the recovery of *X/O* (nullo-exceptional) males can be compared to that *of X/X/Y* (diplo-exceptional) females, and in Table 6 the recovery of *X^X/O* (nullo-exceptional) females can be compared to that of *X/Y* (diplo-exceptional) males. In both crosses, the recovery of nullo-XY sperm exceeds that of diplo-*XY* sperm, despite the fact that the corresponding zygotic genotypes differ. This argues that the differential recoveries of sperm genotypes are not due to differences in zygotic viabilities, but rather reflect the frequencies of fertilization by the different sperm classes.

To gain insight into the mechanism(s) of meiotic drive, we looked for cytological evidence of sperm elimination by examining orcein-stained preparations of postmeiotic spermatids. Our observations suggest that there may be more than one mechanism of sperm elimination in operation and that the effects of each mechanism may differ quantitatively between the two mutants. At the light microscope level, *mscd1* males exhibit a sperm differentiation defect apparent in late stages of maturation. Individual cysts, each containing 64 spermatids, can be separated in testis squashes such that related spermatids can be examined. In 29/35 cysts from *mscd1* males, as many as 10 spermatid nuclei per cyst that failed to properly elongate were observed (mean abnormal spermatids, 3.1/cyst; Figure 3), producing a round spermatid phenotype very similar to that reported for the male sterile mutation *ms(2)46C* (see Figure 6C in Castrillon *et al.* 1993). In *mscd2* males, a similar defect was observed, but at a much lower frequency. Only 7/35 bundles of spermatids examined contained abnormal sperm nuclei (mean, 0.9/cyst); the remainder had only normal-appearing sperm with elongated heads. We saw no defect in spermatid individualization in *mscd* mutants, as reported for rDNA-deficient X chromosomes (Peacock 1965; Peacock *et al.* 1975), although we cannot rule out the possibility that the nuclear phenotype we observed represents a less severe consequence of a similar defect.

The proportion of cytologically abnormal spermatids we observed is insufficient to completely account for the differential sperm recoveries from *mscd* mutants, indicating that sperm are also being eliminated by postdifferentiation events. These may include differential sperm function, transfer, or posttransfer utilization. Such postdifferentiation mechanisms of meiotic drive have been reported in association with both sex chromosome nondisjunction and the differential transmission of autosomal translocations (Peacock *et al.* 1975; Tokuyasu *et al.* 1977; Dernburg *et al.* 1996).

Unlike rDNA deletions, *mscd* males bearing *Ymal*$^+$ are fertile: As a rule, deletions of rDNA that result in elevated frequencies of sex chromosome nondisjunction cause sterility in males bearing *Ymal*$^+$, a Y chromosome that carries a duplication of the proximal X material (Rahman and Lindsley 1981; McKee and Lindsley 1987). The reason for the *Ymal*$^+$ synthetic sterility with rDNA deletions is unknown. However, it can be suppressed by insertions of rDNA transgenes that partially restore XY pairing (McKee *et al.* 1998), suggesting that it is a consequence of pairing failure. It has been hypothesized that the sterility may be mechanistically related to meiotic drive and may represent an extreme form of drive in which all spermatids are rendered dysfunctional (McKee *et al.* 1998).

Both *mscd2/Ymal*$^+$ and *mscd1/Ymal*$^+$ males are fertile, but nondisjunction and drive are increased relative to males bearing a y^+ Y (Table 6), which demonstrates that these mutants are qualitatively different from rDNA deletions.

***mscd/Ybb*$^-$ males are semisterile:** An additional phenomenon associated with X chromosome rDNA deletions is the ability to magnify or increase the copy number of rDNA repeats in the presence of a Y chromosome that is also deficient for rDNA (Y*bb*$^-$; Ritossa 1968; Tartof 1973, 1974). It is unknown if magnification occurs in response to rDNA copy number reduction or if transcriptional repression of rDNA might also cause magnification. To address this question, we also tested if the *mscd* chromosomes could magnify their rDNA under such conditions.

Magnification occurs at a relatively high frequency (1–10%) in males bearing *Ybb*$^-$ (Ritossa 1968) by unequal sister chromatid exchange (Tartof 1973, 1974; Endow *et al.* 1984) or by excision and reintegration of circular rDNA molecules (Ritossa 1972). To test for magnification in *mscd* males, we mated *mscd/B*^{s}Y bb$^-$ males to y *In(1)sc*^{4R}*s*^{c8L}*/M-5, B* females and scored for reversion to bb$^+$ phenotypes among the *mscd/In(1)sc*^{4R}*s*^{c8L} progeny. Surprisingly, the fecundity of *mscd/Ybb*$^-$ males was drastically reduced. Only 12/60 *mscd1* and 12/59 *mscd2* males were fertile and produced on average 13.5 and 22.8 progeny per fertile male, respectively. In comparison, control crosses of 20 *y car/B*^{s}*Ybb*$^-$ males mated to the same females produced 58.8 progeny per male. Of the *mscd/In(1)sc*^{4R}*s*^{c8L} progeny that could be scored for magnification, 10/22 from *mscd1* and 30/67 from *mscd2* fathers appeared bb$^+$. These bb$^+$ progeny were produced in clusters; all *mscd/In(1)sc*^{4R}*s*^{c8L} progeny from any given pair mating were bb or bb$^+$. Thus, these bb$^+$ daughters are unlikely to have arisen from meiotic magnification events. The nature of these reversion events is currently under investigation.

To investigate the synthetic sterility, we examined live and fixed squashes of testes of *mscd/B*^{s}*Y bb*$^-$ males. Only 13/30 *mscd1* and 7/18 *mscd2I* males had motile sperm. Fixation and orcein staining of these preparations revealed that even in males that had motile sperm, most mature sperm bundles had abnormal morphologies. A

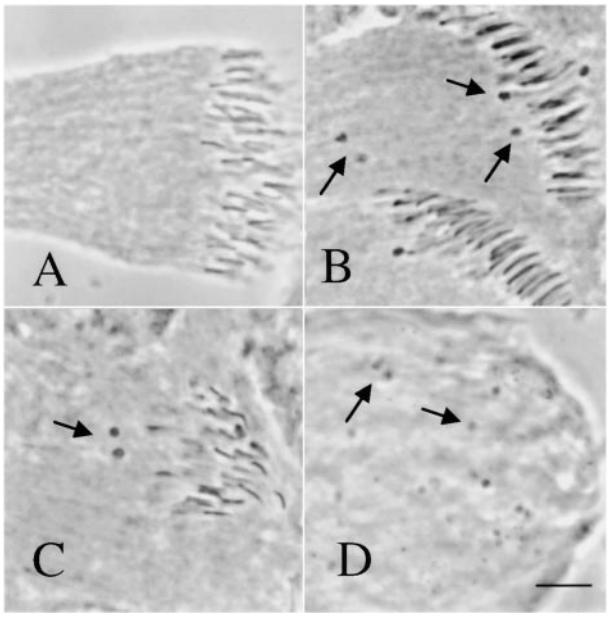

Figure 4.—Orcein-stained bundles of mature sperm from (A) wild-type and mutant (B) *mscd1* and (C) *mscd2* males, showing a defect in sperm head maturation. (D) sperm bundle from an *mscd1*/*Ybb*$^-$ male. Arrows indicate abnormal spermatid. Bar, 10 μm.

wide range of phenotypes was observed, from nearly normal bundles with 1–10 sperm nuclei of abnormal size or shape, to entire cysts of abnormal sperm with round heads (Figure 4D). Examination of orcein-stained meiocytes in these same males, however, revealed no appreciable differences from *mscd*/*Y* males in the frequency of sex chromosome pairing. Sex chromosomes were paired in 20/22 meioses in *mscd1*/*Ybb*$^-$ males and 21/21 *mscd2*/*Ybb*$^-$. These observations suggest that *Ybb*$^-$ enhances the sperm maturation defects in *mscd* males, but not necessarily as a consequence of altering meiosis I sex chromosome behavior.

DISCUSSION

Position effects on the rDNA: We have isolated two X;4 translocations that cause sex chromosome nondisjunction at meiosis I in male Drosophila. Both produce a similar bb phenotype characteristic of a reduction in ribosome biogenesis. In *mscd* males, decreased rDNA transcription results from a chromosomal position effect induced by juxtaposition of the fourth chromosome to the X heterochromatin. Multiple lines of evidence support this conclusion. First, neither copy number nor gross organization of the rDNA is detectably altered by the rearrangements. Thus, the phenotypes do not result from a reduction in rDNA sequences, but rather a change in their activity. We demonstrate directly that the amount of rRNA product from each rearrangement is reduced. Second, the bb phenotypes resulting from the *mscd* rearrangements are suppressed by addition of rDNA *in trans*. This indicates that *trans*-acting factors required for rDNA transcription are intact and suggests that the *rDNA* locus on each *T(1;4)* has been affected *in cis*. In the case of *mscd1*, meiotic pairing can also be partially restored by an X-linked transgene, indicating that the *trans*-acting factors required for meiotic rDNA function are also intact in this rearrangement. Finally, the bb phenotypes are suppressed by the addition of Y heterochromatin that lacks rDNA, which suggests that the position effect is variegated rather than stable, as suppression by the Y chromosome is the classical test of PEV (Gowen and Gay 1933).

Euchromatic rDNA transgenes have been shown to function in nucleolar formation and ribosomal biogenesis and in sex chromosome pairing (Karpen *et al.* 1988; McKee and Karpen 1990). These studies indicate that, unlike other heterochromatic genes (Wakimoto and Hearn 1990; Eberl *et al.* 1993), the function of a single rDNA cistron does not require proximity to heterochromatin. Our data suggest, however, that there may be a specialized environment required for activity of the endogenous repetitive heterochromatic rDNA locus on the X chromosome. We hypothesize that translocation of the fourth chromosome to the X disrupts this environment, resulting in a decrease in transcription. Our results also suggest that the different activities of the rDNA may have different sensitivities to such disruptions. Although both *mscd* translocations are similar in nature, they have cytologically different X chromosome breakpoints and are likely to have different fourth chromosome breakpoints as well. The location of the breakpoint and/or the nature of the sequences between the breakpoint and the rDNA may be important determinants of chromatin structure at the rDNA locus. That both *mscd* mutations affect rDNA transcription, but only one perturbs meiotic pairing, argues that there are different chromatin requirements for rDNA transcription *vs.* pairing.

How might such a disruption occur? Spreading effect models of PEV suggest that a disruptive change in chromatin structure may be propagated from a rearrangement breakpoint (Zuckerkandl 1974; Tartoff *et al.* 1984). The distance over which PEV spreads can be quite large, almost 2 Mb from the euchromatic-heterochromatic breakpoint in the case of a variegating allele of the *Notched* locus (Demerec 1940). Our results can be explained by propagation of a disruptive effect over an even greater distance. However, our observations are unusual in that this disruption would have to occur across heterochromatic sequences and is caused by juxtaposition of X heterochromatin to the fourth chromosome, which itself has been characterized as partially heterochromatic by cytological criteria (Hochman 1976).

In the case of *mscd2*, a spreading model would require that chromatin structure can be perturbed across the X centromere. In this translocation, nearly the entire

fourth chromosome is appended to the right arm of the X, and the X centromeric heterochromatin appears to be intact. The rDNA is located in its normal position on the left arm. Thus, the rDNA locus is affected by a breakpoint on the opposite side of the centromere. To our knowledge, PEV across a centromere has not been reported previously. We speculate that the X centromere itself has also been inactivated in this rearrangement, as we do not see evidence for dicentric behavior in mitosis or meiosis. While it is not clear that the mechanism of centromere inactivation is related to transcriptional inactivation of the rDNA, there is precedence for PEV of centromeres in other organisms. In the fission yeast *Saccharomyces pombe*, genes placed within centromeric sequences variegate, and mutations that affect transcriptional silencing of such centromere-localized genes also affect chromosome segregation (Allshire *et al.* 1994, 1995; Javerzat *et al.* 1999). It is interesting to consider PEV as a general means of centromere inactivation in dicentric chromosomes. There are numerous cases of dicentric chromosomes reported in mammals in which both centromeres appear to retain centromeric satellite DNA, yet only one retains kinetic activity (*e.g.*, Earnshaw and Midgeon 1985; Earnshaw *et al.* 1989; Sullivan and Schwartz 1996; Fisher *et al.* 1997). In some dicentric cell lines, the choice of active centromere can switch over generations (Wandall 1994), not unlike the manner in which gene expression variegates as a result of a position effect. The translocation *mscd2* may provide a useful tool to test the possibility of centromeric PEV in Drosophila by observing its kinetic behavior in genetic backgrounds with various amounts of heterochromatin or other modifiers of PEV.

An alternative model for the mechanism of PEV on the rDNA is that the *mscd* rearrangements disrupt the localization of the rDNA to their proper nuclear compartment, resulting in improper expression. A similar model for PEV has been proposed for the heterochromatic *light* gene. Among rearrangements that cause variegation of *light*, distal euchromatic breakpoints are recovered more frequently than proximal ones (Wakimoto and Hearn 1990). This distance-dependent effect has been explained by postulating that variegation is related to the ability of these genes to interact with proximal heterochromatic sequences. The further removed from proximal heterochromatin, the more likely that these genes will be affected by PEV (Wakimoto and Hearn 1990). Studies on *brown*^{dominant} in Drosophila and the lymphocyte-specific transcriptional repressor *Ikaros* in mouse directly demonstrate that changes in gene activity can occur by movement of a gene to a different domain within the nucleus (Henikoff *et al.* 1995; Brown *et al.* 1997).

The model of gene misregulation resulting from nuclear mislocalization is particularly attractive for explaining PEV of the rDNA, since the nucleolar organizer can be recognized as a discrete compartment cytologically. Moreover, no regions appear to play a central role in organizing other nuclear structures, such as gems, coiled bodies, and the perinuclear compartment (for review, see Lamond and Earnshaw 1998). Changes in the association of nucleoli with these other nuclear structures seen during carcinogenesis have been proposed to be related to reprogramming of gene expression during transformation (Busch 1981). The localization model for PEV of the rDNA is straightforward to test, because it predicts that the organization or positioning of the nucleolus within the nucleus will be abnormal in *mscd/O* individuals. By this model, we might predict that the differential effects of *mscsd1* and *mscd2* on transcription and pairing would be reflected in tissue-specific differences in nuclear positioning.

Comparison of *mscd* mutations to rDNA deletions: Phenotypes associated with rDNA deletions have been well described, including growth retardation and bristle size reduction (bobbed), disruption of sex chromosome pairing, meiotic drive, synthetic sterility with certain sex chromosome translocations, and the ability to change rDNA copy number in the presence of an rDNA-deficient Y (magnification). With the exception of magnification, it has been established that the addition of rDNA transgenes can suppress each of these phenotypes (McKee and Karpen 1990; McKee *et al.* 1998). This demonstrates that some property of rDNA is related to each of these phenotypes. Because rDNA sequences are retained in *mscd* mutations, but are transcriptionally repressed, we could begin to ask about the relationship of each of these phenotypes to rDNA transcription.

It has been previously reported that the severities of different bb mutant phenotypes are correlated to the rate of rRNA sythesis (Weinmann 1972; Shermoen and Kiefer 1975). Other studies, however, reach the opposite conclusion (Terracol and Prud'homme 1981). While we found rRNA levels were decreased in *mscd* mutants, the magnitude of the decreases was relatively small ($\sim$20%) and similar to that observed in *bb*^{5} flies, which have a much milder bb phenotype. However, because we measured total transcript levels in adult testis, we cannot rule out the possibility that there may be a simple correlation between the severity of bb phenotypes and the levels of functional transcripts in other tissues or at other developmental stages.

Previous studies have suggested that XY pairing may be related to rDNA transcription. This hypothesis is based on observations that transgenes consisting of only IGS and promoter sequences are capable of promoting pairing (Ren *et al.* 1997). While our data do not argue against a role of transcription in pairing, they suggest that rDNA transcription levels and pairing ability also may not be simply correlated. We found both decreased rRNA and decreased meiotic XY pairing ability in *mscd1* males, yet a similar rRNA decrease was observed in *mscd2* and *bb*^{5} males, in which XY pairing frequencies are wild type.

The presence of 240-bp IGS repeats on *mscd* chromosomes indicates that these sequences in and of themselves are insufficient to mediate efficient meiotic pairing. Rather, there may be additional required constraints for the chromatin environment that are disrupted by the *mscd1* rearrangement. It may be that the IGS/promoter regions need to assume an open chromatin configuration conducive to pairing. This configuration may be disrupted by PEV in the same way that a transcriptionally competent chromatin structure is altered in somatic cells.

Several lines of evidence suggest a connection between the failure of XY pairing and the differential sperm dysfunction that results in meiotic drive. However, a disruption of pairing does not seem to be a prerequisite for drive (for recent discussion, see Robbins 1999). Our data provide another case of meiotic drive in the absence of a pairing defect. In *mscd2* males, sex chromosomes pair efficiently, yet meiotic drive is still observed. The mechanism of drive in *mscd2* males differs from that in *mscd1* males in which XY pairing is disrupted. Most of the sperm elimination in *mscd2* males occurs after sperm maturation, while a significantly higher proportion of sperm is eliminated during the maturation stages in *mscd1* males. This suggests that different meiotic defects may activate different pathways to sperm elimination. Sperm maturation defects may result when the X and Y chromosomes do not pair, while postdifferentiation selective processes may be activated by disjunctional defects or by differences in sperm chromatin content.

It has been suggested that male sterility of rDNA-deficient chromosomes in combination with $Ymal^{+}$ may be an extreme manifestation of meiotic drive (McKee *et al.* 1998). While we observe drive in *mscd* males, they are fertile with $Ymal^{+}$. Our results raise the possibility that this synthetic sterility is dependent on deletion of the rDNA sequences rather than their inactivation.

Last, we could not readily test if magnification occurs in *mscd* mutants in the presence of Ybb^{-} because *mscd*/Ybb^{-} males exhibited a synthetic sterility. The spermatogenesis phenotype in these males was similar to, albeit more extreme than, that of *mscd*/*Y* males. A cursory examination of meioses in these sterile males failed to reveal any dramatic effect of Ybb^{-} on chromosome behavior. These observations suggest that sperm dysfunction may be triggered by abnormalities in rDNA metabolism independently of its role in meiotic pairing.

The paucity of X-linked male meiotic mutants: Despite screening over 5000 mutagenized, male-fertile X chromosomes, we did not identify any X-linked genes encoding *trans*-acting factors required for meiotic sex chromosome transmission. The failure to recover these types of mutants may in part reflect the limited size of our screen, which was not large enough to achieve saturation. A previous screen (Baker and Carpenter 1972) identified two male meiotic mutations that mapped within the X euchromatin; however, these mutants and 18 others were unstable and were eventually lost. The majority of mutations isolated in our screen exhibited a similar instability. The reasons for this phenomenon are unclear, but may be attributed to a high reversion frequency or to a rapid accumulation of second site suppressors (Baker and Carpenter 1972). It is also possible that these "unstable mutations" represent reversible, epigenetic changes as a consequence of mutagenesis, rather than true gene disruptions. Such epigenetic changes have been proposed to cause chromosome instability in mammalian cells exposed to ionizing radiation or alkylating agents (Murnane 1996).

Alternatively, our negative results may reflect a strong selection against the X localization of genes involved in male meiosis. The X chromosome is hypothesized to be precociously inactivated in the primary spermatocyte in Drosophila, on the basis of observations of male-specific sterility of X-autosome translocations (Lifschytz and Lindsley 1972; Lifschytz and Hareven 1977). Inactivation of the X chromosome during spermatogenesis would result in improper regulation of both autosomal and X genetic material on X;A translocations, resulting in male sterility (for discussion see Lindsley and Tokuyasu 1980). X-inactivation in primary spermatocytes may preclude the activity of X-linked genes during periods critical for the proper execution of meiotic events. This suggests that it may be more fruitful to screen autosomal male meiotic mutations to identify the *trans*-acting products involved in sex chromosome pairing.

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