Genetic Analysis of *Stellate* Elements of *Drosophila melanogaster*

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ABSTRACT

Repeated elements are remarkably important for male meiosis and spermiogenesis in *Drosophila melanogaster*. Pairing of the *X* and *Y* chromosomes is mediated by the ribosomal RNA genes of the *Y* chromosome and *X* chromosome heterochromatin, spermiogenesis depends on the fertility factors of the *Y* chromosome. Intriguingly, a peculiar genetic system of interaction between the *Y*-linked *crystal* locus and the *X*-linked *Stellate* elements seem to be also involved in male meiosis and spermiogenesis. Deletion of the *crystal* element of the *Y*, via an interaction with the *Stellate* elements of the *X*, causes meiotic abnormalities, gamete-genotype dependent failure of sperm development (meiotic drive), and deposition of protein crystals in spermatocytes. The current hypothesis is that the meiotic abnormalities observed in cry^- males is due to an induced overexpression of the normally repressed *Ste* elements. An implication of this hypothesis is that the strength of the abnormalities would depend on the amount of the *Ste* copies. To test this point we have genetically and cytologically examined the relationship of *Ste* copy number and organization to meiotic behavior in cry^- males. We found that heterochromatic as well as euchromatic *Ste* repeats are functional and that the abnormality in chromosome condensation and the frequency of nondisjunction are related to *Ste* copy number. Moreover, we found that meiosis is disrupted after synapsis and that *cry*-induced meiotic drive is probably not mediated by *Ste*.

IN 1961, MEYER *et al.* examined live preparations of *X*/O male testes of *Drosophila melanogaster* by phase contrast microscopy and discovered the presence of needle- or star-shaped crystalline aggregates in the nuclei and cytoplasm of primary spermatocytes. Later, it was also observed that *X*/O males display abnormal condensation of meiotic chromosomes, a high level of meiotic chromosome nondisjunction, altered distribution of meiotic organelles and meiotic drive (LIFSCHYTZ and HAREVEN 1977; HARDY *et al.* 1984).

Two loci are involved in producing this set of abnormalities: the *crystal* (*cry*) (PIMPINELLI *et al.* 1986) or *Su(Ste)* (LIVAK 1990) locus and the *Stellate* (*Ste*) locus (HARDY *et al.* 1984). *crystal* is located on the long arm of the *Y* chromosome at position h11–h13 of the standard mitotic heterochromatic map, and it is the only locus on the entire *Y* chromosome responsible for this phenomenon (HARDY *et al.* 1984). *Stellate* is located on the *X* chromosome at position 47.5 on the genetic map (HARDY *et al.* 1984) and it has been reported to be at 12F1-2 of the polytene chromosome map (LOVETT *et al.* 1980). *Stellate* determines the shape of the crystals. In Ste^+ males the crystals are needle-shaped and in *Ste* they are star-shaped (HARDY *et al.* 1984).

Molecular analysis has shown that both the *X* and *Y* loci are composed of arrays of partially homologous, tandemly repeated sequences (LIVAK 1984). In some laboratory strains, *crystal* contains about 80 copies of a repeat that yields an 800-bp fragment upon *Cfo*I-digestion (LIVAK 1984). In natural populations, samples with as many as 240 copies have been found (LYCKEGAARD and CLARK 1989). *crystal* has been genetically characterized in some detail. Its most important genetic property is that its function seems to require only a critical number of subunits but not physical integrity (HARDY *et al.* 1984; S. BONACCORSI, S. PIMPINELLI and M. GATTI, unpublished). That is, breakpoints within the locus have no detectable effect, while partial deficiencies in combination with either *Ste* or Ste^+ show reduced effects on all of the correlated abnormalities.

The *Stellate* locus contains two classes of repeats giving 950- and 1150-bp fragments after *Cfo*I digestion. The repeats are present in about 200 copies in *Ste*, and are an order of magnitude less abundant in Ste^+ (LIVAK 1984). These sequences contain two introns, and are abundantly and correctly transcribed only in testes of cry^- males; the 750-bp poly(A^+) transcript is virtually absent in *X*/*Y* males. Sequence analysis of cDNAs has identified an ORF with the capacity to encode a 19,500-dalton protein with striking homology to the β-subunit of casein-kinase II (LIVAK 1990). Recently, it has been shown that some 1150-bp class *Ste* sequences are also located in the *X* heterochromatin and that the heterochromatic copies analyzed retain an intact open reading frame (SHEVELYOV 1992).

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One model for the interaction between *cry* and *Ste* proposes that the suppression of *Stellate* in normal *X/Y* males depends on efficient competition between *Ste* and *cry* sequences for limiting transcription factors. Such factors, in the absence of *crystal*, would be available for binding to *Ste* and would induce transcription, resulting in abundant production of the stellate protein (LIVAK 1990). Moreover, since the interaction between *Ste* and *cry* results in their mutual inactivation, it has been suggested that these two loci could represent an intriguing type of selfish genetic system (LIVAK 1990). A more formal model of this interaction has also been proposed, namely that *Ste* arose as an inducer of meiotic drive, and that *cry* evolved to suppress that drive (HURST 1992).

To test the validity of these models, and to understand the nature of the *Ste-cry* interaction and the role of each locus, it is important to study the effects of varied doses of each of these repeated elements. In this paper we report the results of basic molecular, cytological and genetic analyses of *Stellate*: (1) *in situ* hybridization to polytene and mitotic chromosomes locates *Ste* at 12E1-2 on the polytene chromosome map, with another array containing two *Ste* restriction-pattern variants located in the constriction between the C and D blocks of the *X* heterochromatin; (2) despite several attempts, using a variety of strategies, we have been unable to obtain a viable, complete deletion of the euchromatic *Stellate* sequences, but we have found substantial variation of *Ste* copy number in natural populations, including strains entirely lacking one or another of the three major *Ste* restriction-pattern variants; (3) genetic analysis shows that, as for *crystal*, there is a direct correlation between *Ste* copy number and nondisjunction, and that the heterochromatic *Ste* copies are functional. Surprisingly, unlike for *cry*, the level of meiotic drive is largely independent of *Ste* copy number, suggesting that *cry* alone, and not a *cry-Ste* interaction, causes the meiotic drive; and (4) meiotic cytology shows a correlation of the severity of visible anomalies to *Ste* copy number, and suggests that the cause of the disjunctional defect is post-synaptic.

MATERIALS AND METHODS

Drosophila strains and culture conditions: $B^S cry^1 Yy^+$ (kindly provided by PEDRO RIPOLL, and abbreviated as $cry^1 Y$ in the text) carries all of the fertility factors and the *bobbed*$^+$ (ribosomal DNA) locus but elicits the formation of needle or star-shaped crystals in primary spermatocytes in the presence of a *Ste*$^+$ or *Ste X* chromosome respectively (P. RIPOLL, personal communication). The $cry^1 Y$ chromosome was derived from a cry^+ chromosome, also used in our experiments, denoted here as $B^S cry^+ Yy^+$ or simply $cry^+ Y$.

R301.2 is an M strain containing a ry^+-marked transposase$^-$ *P* element inserted into the polytene 12D region of the *X* chromosome (SPRADLING and RUBIN 1983).

C(1)DX,y f; ry^{506} Δ *2-3(99B)* is a compound-*X* stock, homozygous for the Δ *2-3* element that constitutively produces P transposase (ROBERTSON and ENGELS 1989).

Dp(1;f)LJ9 carries a *Ste*$^+$ allele in a free duplication of the 12A6-10;13A2-5 polytene region of the *X* chromosome (HARDY *et al.* 1984).

Df(1)garnet-lethal is an *X* chromosome deleted for the 11F10;12F1 polytene region (WARING *et al.* 1983).

Df(1)KA9 is an *X* chromosome carrying a deficiency with breakpoints in the 12E1;12F1 polytene regions.

y w f is an *X* chromosome used to measure the effect of different *Ste*$^+$ alleles on sex chromosome nondisjunction.

C(2)EN (NOVITSKI *et al.* 1981) is a compound second chromosome used for detecting autosomal nondisjunction.

FM6/FM7c is a balancer stock used to select *X*-linked lethals after dysgenic crosses. The *FM6* chromosome used carries an EMS-induced early lethal.

Wild-type strains: Single wild-type males from the Umeå collection or collected from different places in southern Italy were crossed to compound-*X* females. Their male offspring were then crossed to compound-*X* females from the same stock to generate strains in which all males carry the same *X* chromosome. These stocks had been maintained for at least 40 generations before being used for the present work, but a single male from each line was used to restart each stock just prior to these experiments.

For more complete information on these chromosomes and markers see LINDSLEY and ZIMM (1992). Cultures were maintained at 24° on standard cornmeal-sucrose-yeast-agar medium containing propionic acid and Nipagin as mold inhibitors.

DNA plasmids and fragments: pSX1.3 is a 1360-bp *Ste* sequence cloned in the *Xba*I site of pBr322 (LIVAK 1990). rp49 contains a DNA fragment of the ribosomal protein 49 (rp49) gene (O'CONNEL and ROSBASH 1984). The plasmids used in the present work were prepared by cesium chloride centrifugation (SAMBROOK *et al.* 1989). Probes were separated from plasmid DNA by digestion with restriction enzymes and electroelution from agarose gels.

Genomic DNA samples: Large scale preparations of Drosophila DNA from frozen adults were performed following the protocol of BENDER *et al.* (1983); DNA from small numbers of flies was prepared according to LIVAK (1984).

DNA blot analysis: Restriction enzyme digestions were done following manufacturer's instructions; agarose gel electrophoresis and blotting to nitrocellulose filters were according to SAMBROOK *et al.* (1989). DNA probes were ^{32}P-labeled by oligo-priming (FEINBERG and VOGELSTEIN 1983). The standard hybridization conditions were 4 × SET/10 × Denhardt's solution/0.1% sodium dodecyl sulfate (SDS)/0.1% sodium phosphate overnight at 65° (1 × SET is 0.15 M NaCl/0.02 M Tris, pH 7.8/1 m EDTA). After two 15-min washes with 1 × SET/0.1% SDS/0.1% sodium phosphate and two 15-min washes with 0.2 × SET/0.1% SDS/0.1% phosphate at 65°, filters were autoradiographed using Kodak X-Omat film and DuPont Lightning Plus intensifying screens.

DNA slot blot analysis: The procedure used for slot blot hybridization was that of LYCKEGAARD and CLARK (1989). Genomic DNA was blotted on Hybond-N$^+$ membranes (Amersham Corp.). Aliquots of 1, 0.1 and 0.05 μg of denatured DNA from each strain were applied to 24 slots per membrane. Salmon sperm DNA was added at 1 μg per slot. Each determination was done four times; there were two separate experiments, each with duplicate membranes for each genotype. Films were exposed for different times, and the autoradiographs were quantified by computing the peak areas with a scanning laser densitometer (LKB Ultroscan XL). Copy numbers were calculated using only those data points where there was a linear response to both DNA dilution and film exposure.

Cytology and *in situ* hybridization: Mitotic chromosome preparations and acetic orcein and Hoechst staining were as described in GATTI and PIMPINELLI (1983).

Radioactive *in situ* hybridizations to mitotic and polytene chromosomes from third instar larvae were performed according to PARDUE (1986). The DNA *Stellate* fragment insert in the pSX1.3 plasmid was ^{3}H-labeled by random-priming (FEINBERG and VOGELSTEIN 1983). Non-radioactive *in situ* hybridization was done using *Ste* DNA biotinylated by nick translation, and biotin labeled DNA was detected using fluorescein isothiocyanate (FITC)-conjugated avidin.

The testis material for indirect immunofluorescence was prepared according to PISANO *et al.* (1993). The slides were then treated following the procedure described by CENCI *et al.* (1994). After three washes in phosphate-buffered saline (PBS), they were incubated for 45 min with an anti-α-tubulin monoclonal antibody (Amersham Corp.) diluted 1:50 in PBS, washed three times in PBS and incubated for 45 min with the secondary antibody [5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS)-conjugated goat antimouse IgGs from Boehringer Mannheim] diluted 1:15 in PBS. After washing in PBS and air drying, the slides were stained with Hoechst 33258 according to GATTI and PIMPINELLI (1983). Digital images for both nonradioactive *in situ* hybridization and immunofluorescence analysis of meiosis were obtained using a computer-controlled Zeiss Axioscope epifluorescence microscope equipped with a cooled charge coupled device (CCD) camera (Photometrics). FITC and 4′,6-diamidino-2-phenylindole dihydrochloride fluorescence, and FLUOS and Hoechst fluorescence, detected by specific filter combinations, were recorded separately as gray scale images and then digitally pseudocolored and merged.

Estimation of meiotic parameters: Although nondisjunction of the sex chromosomes yields *XY* and nullo (O) sperm, and meiotic drive is defined as unequal production of reciprocal sperm types, the observed frequencies of each of the four sex-chromosome gamete types are affected by both disjunction and drive. To allow separate evaluation of the frequency of disjunctional failures and the level of meiotic drive we have followed the model of MCKEE (1984) and MCKEE and LINDSLEY (1987). Thus, if the probability of disjunction of the *X* and *Y* chromosomes is P_{XY} and the recoveries of the *X* and *Y* chromosomes are R_X and R_Y, respectively, the frequencies of the four sex-chromosome gamete types are: $[X] = 1/2P_{XY}R_X$; $[Y] = 1/2P_{XY}R_Y$; $[XY] = 1/2(1 - P_{XY})R_XR_Y$; and $[O] = 1/2(1 - P_{XY})$. Each cross yields three independent observations, and there are three parameters. Hence, for any one cross these equations have unique solutions. Those solutions are: $P_{XY} = 1/1(1 + ([XY][O])/([X][Y]))$; $R_X = ([X][XY])/([Y][O])$; and $R_Y = ([Y][XY])/([X][O])$. Two things should be noted. First, these parameters are defined as meiotic end points rather than meiotic processes. For example, P_{XY} is the probability of disjunction, not the probability of pairing, of the *X* and *Y* chromosomes. Second, should it happen that one of the four progeny classes is not recovered in a cross, the parameters are indeterminate.

Similar parameters may be used to describe autosomal behavior. In our experiments, we followed the second chromosomes by crossing males to *C(2)EN*-bearing females. All ova produced by these females are disomic or nullisomic for the second chromosome. Thus, all normal haplo-2 sperm yield aneuploid, lethal zygotes, as do exceptional nullo-2 and diplo-2 sperm that happen to fertilize eggs of the noncomplementary karyotype. Since all survivors are necessarily nondisjunctional, scoring of survivors only would not permit estimation of the frequency of autosomal nondisjunction, but counting the number of lethal eggs does. If nullo-2 and diplo-2 ova are equally frequent, for P_2 = the probability of disjunction of the second chromosomes and R_2 = the probability of survival of a sperm bearing a second chromosome, we have: $[22] = 1/2(1 - P_2)R_2^2$; $[O] = 1/2(1 - P_2)$; and [dead zygotes] $= P_2R_2 + 1/2(1 - P_2)R_2^2 + 1/2(1 - P_2)$. The three observed numbers (two of which are independent) again give unique solutions for the parameters. They are: $P_2 = ([\text{dead eggs}] - [22] - [O])/4[O][22]/[O] - [22] - [O] + [\text{dead eggs}])$ and $R_2 = [22]/[O]$. There are two sources of error in the calculated values. First, there will be some eggs, for example non-fertilized eggs and third chromosome aneuploids, that fail to hatch for reasons other than aneuploidy for the second chromosome. Counting these as second chromosome aneuploid zygotes causes an overestimate of P_2. Second, unequal production of nullo-2 and diplo-2 ova by *C(2)EN* females causes an error in estimation of R_2. For example, excess production of nullo-2 eggs would cause underestimation of R_2 in all of the crosses. This error, however, whatever its direction, should be consistent among the crosses since the same *C(2)EN* stock was used throughout.

Statistical analysis: The effect of *Stellate* copy number on fertility was assessed using conventional linear regression, while maximum likelihood procedures (BISHOP 1975) were used to evaluate the relationship of nondisjunction and meiotic drive to *Stellate* copy number and to each other. Maximum likelihood estimates were numerically approximated using the computer program MLIKELY.PAS (version 2.2, 1992 by L. G. ROBBINS).[2] In general, ln maximum likelihoods ($\ln L_{max}$) were found for three hypotheses:

H_1: All parameters differ among the crosses in the set.

H_2: One parameter is constant, and all other parameters differ among the crosses.

H_3: One parameter is correlated to *Stellate* copy number (parameter = $m \times$ *Ste* copy number $+b$), and all other parameters differ among the crosses.

Note that under H_1 the maximum likelihood estimates of the parameters are the same as the exact solutions for the individual crosses, while under H_2 and H_3 the number of parameters is less than the number of independent observations and the maximum likelihood estimates are the minimum variance unbiased averages derived from all of the data.

These hypotheses were then compared using the *G* statistic: $G = 2 \times (\ln L_{\max}H_i - \ln L_{\max}H_j)$ which is distributed approximately as χ^2 with degrees of freedom equal to the difference in the number of parameters under the two hypotheses. Comparison of H_2 with H_1 indicates whether there is statistically significant variation in a parameter, or whether the crosses are homogeneous with respect to that parameter; a large value of *G* indicating that the parameter in question does differ among the crosses. Comparison of H_3 with H_2 asks whether the posited correlation is statistically significant; the higher the value of *G*, the tighter the coupling of that parameter to *Ste* copy number. Finally, comparison of H_3 and H_1 tests whether other sources of variation remain after the correlation is taken into account; a *G* value near zero indicating that the correlation is sufficient to explain most of the observed variation.

In several instances the same basic procedure was used to examine other hypotheses: that one parameter is correlated with another (*e.g.*, $R_X = m \times P_{XY} + b$); or that a parameter is the same for each tested genotype in two different crosses (*e.g.*,

[2] MLIKELY.PAS is a Pascal program written for TurboPascal 4.0 (Borland International) that, using user-supplied data and a set of equations describing an hypothesis, finds maximum-likelihood estimates of the parameters of those equations, the value of the ln likelihood, and provides a Chi-square goodness of fit test of the model. The source code for the program along with basic instructions and examples are available without charge from L. G. ROBBINS for noncommerical use.

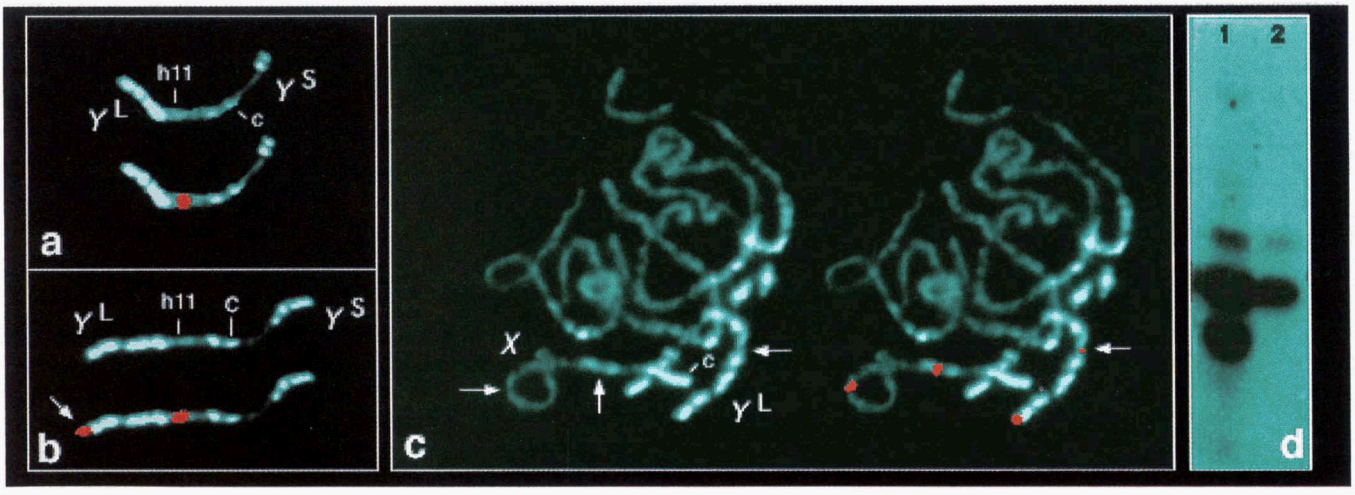


FIGURE 1.—Localization of *Ste*-like sequences on different *Y* chromosomes. (a–c) Non-radioactive *in situ* hybridization with a *Ste* probe on Hoechst-33258 stained mitotic chromosomes. (a) a wild-type *Ore-R Y* chromosome, (b) the cry^+Y chromosome and (c) its cry^1Y chromosome derivative. Note that there is a strong signal in region *h11* in both the *Ore-R* and cry^+Y chromosomes, but that the signal is very weak (arrow) in the cry^1Y chromosome because of a deletion of the *h11* region (arrow). In addition, the cry^+Y (arrow) and its derivative show a second signal in the *X*-derived heterochromatin transposed to the tip of the long arm. The presence of a heterochromatic *Ste* cluster in the *X* chromosome is also clearly evident in (c) where the *X* chromosome, besides the euchromatic signal, shows an additional signal in the heterochromatin (the arrows point the region where the signals are located in the correspondent merged metaphase). (d) *Cfo*I digests of DNA from $301.2/cry^+Y$ (lane 1) and $301.2/cry^1$ *Y* (lane 2) males probed with a ^{32}P-labeled *Ste* fragment. DNA was extracted from five males for each genotype. The arrow indicates the *Y*-specific 800-bp fragment that is completely absent in the cry^1Y chromosome.

that P_{XY} is the same for Altamura-1/cry^1Y males etc. among second chromosome regular and second chromosome exceptional progeny). Where these methods were used to compare data from our crosses with those of HARDY *et al.* (1984), the observed numbers in the earlier study, rather than any calculated measures, were used.

RESULTS

Characterization of cry^1Y, a *Y* chromosome defective for the *crystal* locus: For genetic analysis of the *X*-linked *Stellate* locus in the absence of *crystal* sequences, we used the cry^1Y chromosome. This chromosome carries all of the fertility factors and a normal *bobbed* locus but elicits crystal formation in primary spermatocytes. Southern blot comparison of *Cfo*I digests of genomic DNA from males carrying the original cry^+Y and those of males carrying its cry^1Y derivative, reveals that cry^1Y completely lacks the *Y*-specific 800-bp fragment (Figure 1d). We also did high resolution non-radioactive *in situ* hybridization analysis with the *Stellate* probe on mitotic chromosomes of males carrying a wild-type *Y* chromosome, the cry^+Y and the cry^1Y. *In situ* hybridization was followed by Hoechst 33258 staining that longitudinally differentiates the *Y* chromosome into 25 regions (GATTI and PIMPINELLI 1983). Thus, we were able to simultaneously assess the presence of *Y* chromosome rearrangements and the precise location of the signal. As shown in Figure 1a, the wild-type *Y* chromosome exhibits a strong signal in region h11. The cry^+Y chromosome exhibits two signals (Figure 1b), one in region h11 and the other in the B^SX heterochromatic block appended to the tip of the long arm (GATTI and PIMPINELLI 1983). The cry^1Y chromosome, that upon Hoechst staining appears to have a complete deletion of region h11, nevertheless exhibits a very weak residual hybridization signal at the site of the deficiency in addition to the much stronger signal in the B^S heterochromatic block (Figure 1c). Since the Southern blots indicate that the cry^1Y chromosome is devoid of 800 bp *Cfo*I-fragment-containing repeats, the weak signal observed after *in situ* hybridization suggests the presence of additional *X*-like *Ste* sequences proximal to the 800-bp cluster. Moreover, the presence of a strong signal in the B^S *X*-derived heterochromatic block confirms that the *X* chromosome carries a heterochromatic cluster of *Ste* sequences (SHEVELYOV 1992). The location of this cluster was determined by *in situ* hybridization to a normal *X* chromosome which, besides the euchromatic signal, exhibits another strong signal in the constriction between the C and D heterochromatic blocks defined by COOPER (1959) (Figure 1c).

Characterization of the *R301.2 X* chromosome carrying a *Ste* allele: To obtain deletions of the *Stellate* locus we employed the *R301.2 Ste X* chromosome carrying a marked *P* element inserted into region 12D (SPRADLING and RUBIN 1983). This chromosome was examined for the location of the *Ste* sequences and the *P* element. As shown in Figure 2, a–c, *in situ* hybridization to polytene chromosomes with the *Ste* probe revealed that the *Ste* sequences map to region 12E of the *R301.2 X* chromosome instead of region 12F1-2 as previously reported (LOVETT *et al.* 1980). It is also worthy to note the labeled ectopic threads between the 12E and 11D

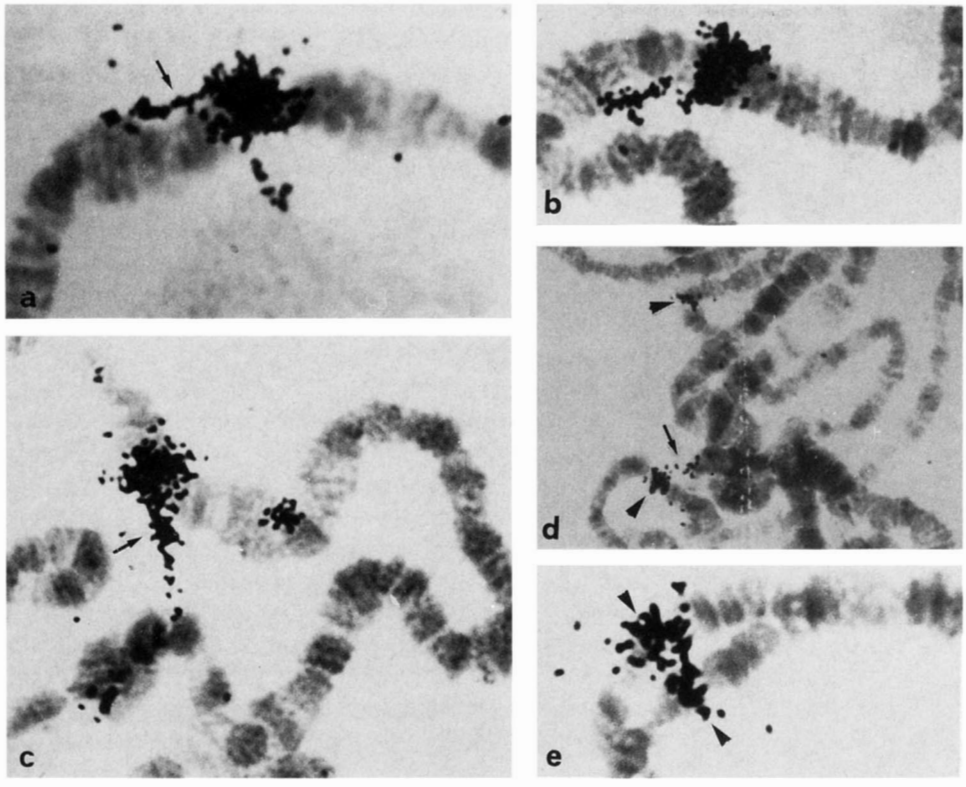


FIGURE 2.—*In situ* hybridization of ^{3}H-labeled *Ste* DNA to polytene chromosomes of *301.2* (a–c) and *301.2/y w Ste$^+$ f* (d and e) larvae. The arrows indicate the labeled ectopic threads between the 12E and 11D regions (a and b) and the chromocenter (c and d). In (d) and (e) note the heterozygosity of labeling in region 12E (arrowheads) reflecting the different numbers of *Ste* sequences in the two *X* chromosomes.

regions (a and b) and the chromocenter (c and d). This localization was confirmed by examining the *in situ* hybridization pattern of a *y w f X* chromosome (Figure 2, d and e). Moreover, the intensity of the signal on the *Ste R301.2 X* chromosome is much stronger than that observed on the *Ste$^+$ y w f* chromosome, confirming the relationship between crystal morphology and *Ste* copy number. The localization of the *Ste* sequences in the 12E polytene region has been further refined by high resolution non-radioactive *in situ* hybridization, that unambiguously places the signal in 12E1-2 (Figure 3a). In addition, high resolution *in situ* hybridization with a *P* element probe reveals that the *R301.2 X* chromosome actually carries a second *P* insertion that colocalizes with the *Ste* sequences at 12E1-2 (Figure 3b). The *in situ* hybridization on mitotic chromosomes shows that also the *R301.2 X* chromosome carries heterochromatic *Ste* copies (Figure 3c).

A search for a *Stellate* deletion: Starting with the *R301.2 X* chromosome, we first attempted to obtain *Ste* alleles with different numbers of copies using a strategy that does not depend on having precise knowledge of *Stellate*'s location. Since this chromosome carries a *Ste* allele, males with this *X* and the *cry^{1}Y* chromosome are completely sterile. *R301.2/cry^{1}Y* males were x-ray-treated (4000 rad) and then mated to fresh, virgin compound-*X* females each 5 days for a total of 20 days. All of these males will be sterile except for those in which sperm can be produced from gonial cells where part or all of the *Stellate* locus had been removed. Of the 1,700 treated males, none gave progeny.

For our second attempt to obtain *Ste* alleles with different numbers of copies, we tried to mobilize the marked *P* element inserted at the 12E1-2 polytene region of the *R301.2* chromosome. We did two types of experiments. In the first, we constructed *R301.2/cry^{1}Y* males carrying a third chromosome bearing the defective Δ *2-3 P ry$^+$* transposon that constitutively produces P transposase. This combination can produce deletions by imprecise excision of the insert (TSUBOTA and SCHEDL 1986; COOLEY *et al.* 1988). These males will be sterile unless there are gonial cells in which mobilization of the *P* element removes part or all of the *Ste* sequences. Of the 7,000 males tested in this experiment, none produced progeny.

The second *P*-mobilization experiment, was designed to avoid the possibility of selection against fertile cells in a *cry$^-$* testis. *R301.2/cry$^+$Y*; Δ *2-3* males were crossed to compound-*X/cry^{1}Y* females and their *R301.2/cry^1* sons were tested for fertility. Of 4,000 males tested, 2 were fertile. One of them, however, carried an attached-*XYL* chromosome, derived from an exchange between the *R301.2 X* and *cry$^+$Y* chromosomes, and thus carried *cry$^+$*. The *X* chromosome of the other fertile male was a *Ste$^+$* revertant that produced small needle-shaped crystals in *cry$^-$* spermatocytes. Unfortunately, this chromosome reverted to the original phenotype within a few generations precluding further analysis.

The previous experiments were done to search for viable deletions of the *Stellate* locus. We also tried a third strategy to obtain deletions of *Ste* without selection for viability. To this end, the procedure shown in Figure 4

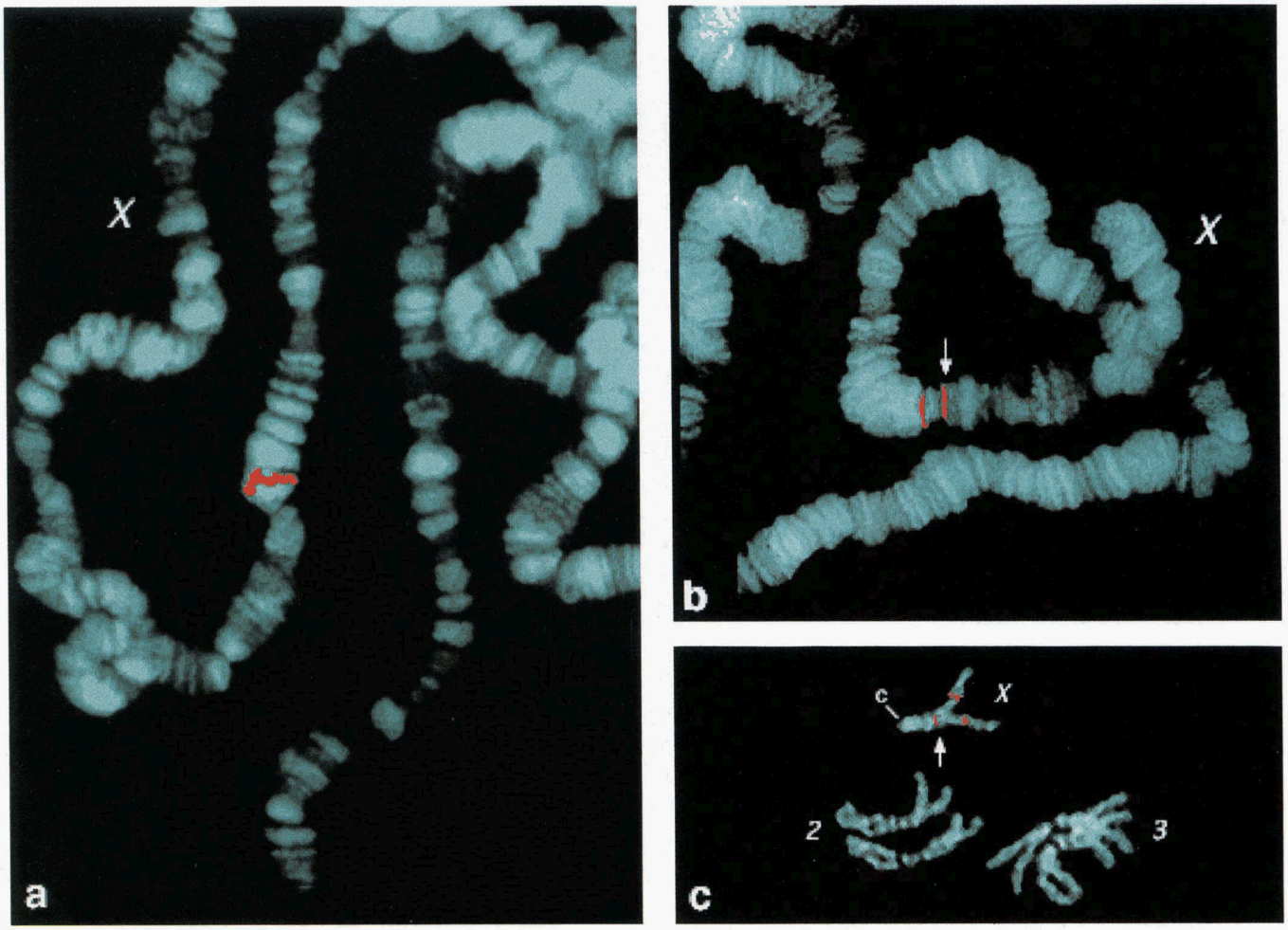
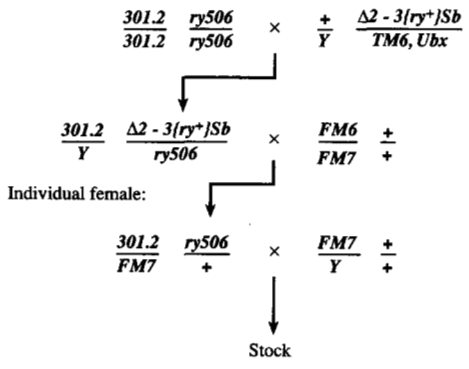


FIGURE 3.—High resolution non-radioactive *in situ* hybridization with biotinylated *Ste* (a) and *P* element (b) probes to the polytene *301.2 Ste X* chromosome. The signal in (a) clearly indicates that the *Ste* sequences are localized in the 12E1-2 region. It is also clear that in this *X* chromosome there is one *P* element insertion in 12D and another in 12E1-2. *Ste* hybridization to the mitotic *301.2 Ste X* chromosome is shown in (c). Note that in this chromosome the heterochromatic signal (arrow) is less intense than the euchromatic signal.

was followed. Out of 2922 tested chromosomes, 31 carried newly induced lethal mutations. These chromosomes were then tested for complementation with *Dp(1;f)LJ9*. The 14 lethals covered by the duplication were retained and the 17 lethals not covered by *Dp(1;f)LJ9* were discarded. Two nearly contiguous deficiencies, *Df(1;f)g-l* and *Df(1)KA9*, were used to locate the lethals with respect to *Ste*. *Df(1)g-l* is completely deleted for the *Ste* locus and extends proximally, while *Df(1)KA9* retains some *Ste* sequences and extends distally (data not shown). Eight of the lethals complemented only *Df(1)g-l* and are therefore located distal to *Ste*. Four of the lethals complemented only *Df(1)KA9* and are therefore located proximal to (most of) the *Ste* cluster. Two of the lethals were lost before analysis. All of the lethals were tested for *Ste* function by constructing *lethal/cry*^{1}*Y/Dp(1;f)LJ9* males and testing them for fertility and for crystal shape in primary spermatocytes. All eight of the distally-located lethals maintained the *Ste* phenotype while all four proximally located lethals were *Ste*$^{+}$ revertants. Cytological analysis of polytene chromosomes revealed that none of the distal lethals had visible rearrangements (data not shown), but the proximal lethals were deletions with proximal breakpoints at different locations within region 13 and the same distal breakpoint in 12E-1 corresponding to the position of one of the two original *P* insertions. One of these deletions is shown in Figure 5c. To test if these were complete deletions of the *Stellate* locus, we analyzed two of them: *Df(1)12.1* and *Df(1)12.4*. Heterozygotes carrying *Df(1)12.1* or *Df(1)12.4* with a *y w Ste*$^{+}$ *f X* chromosome were constructed and their polytene chromosomes were examined following *in situ* hybridization with the *Ste* probe. Both *Df(1)12.1* (Figure 5, b and d) and *Df(1)12.4* (not shown) are partial deletions of the *Stellate* sequences.

To investigate the nature of the induced rearrangements with respect to the *Ste* sequences, DNA extracted from *R301.2*, *Df(1)12.1* and *Df(1)12.4* in combination with *Df(1)g-l* was used for *Cfo*I restriction analysis. As



The resulting lethals were localized with respect to Stellate by crossing

$\frac{301.2}{FM7}$ females × $\frac{X\text{-}Y,\ Df(1)g\text{-}1,\ y\ f\ B}{Dp(1;f)Lj9}$ or $\frac{X\text{-}Y,\ Df(1)KA9,\ y}{Dp(1;f)Lj9}$ males

FIGURE 4.—Crossing scheme for inducing deletions of *Ste* sequences by *P* mobilization. The *Ste 301.2* chromosome bearing two *P* elements and the transposase-producing Δ*2-3* chromosome were brought together in the second generation, and the presence of new lethal mutations in the *Ste 301.2* chromosomes was signalled by the failure of the third generation to yield non-*FM7* sons. The third generation's offspring were kept as balanced stocks, and the location of the lethals with respect to *Ste* was tested by complementation tests with two deficiencies and a duplication.

shown in Figure 5a, both deficiencies (lanes 2 and 3) have few 950 bp repeats as compared to the *R301.2* chromosome (lane 1). This analysis of the *12.1* and *12.4* chromosomes provides some new information about the organization of the repeats within the *X* chromosome; the 950-bp sequences are mainly, if not exclusively, clustered in the euchromatin. Conversely, many or all of the 1150-bp sequences must be in the heterochromatic cluster.

Genetic analysis of *Stellate* in natural populations: Our failure to obtain viable deletions of the euchromatic *Ste* cluster induced us to examine the variability of this locus in natural populations. We analyzed 463 *X* chromosomes extracted from males collected from several locations in southern Italy and 18 *X* chromosomes from wild-type stocks from the Umeå stock center. We assessed the presence and the shape of crystals in spermatocytes of X/cry^1Y males as well as in *X*/O males. As reported in Table 1, in all of the populations that we examined the frequency of Ste^+ alleles (89%) vastly exceeds that of *Ste* alleles. In one case, males failed to show any kind of crystals, but the cytological analysis of their mitotic chromosomes revealed that they carried an attached *X-Y* chromosome.

One hundred randomly selected Ste^+ and *Ste* stocks were also analyzed by Southern blots after *Cfo*I digestion. As shown in Figure 6, there are three major classes of *Ste* repeats. Under our experimental conditions (see MATERIALS AND METHODS), the 1150-bp fragment can be resolved into two bands of 1100 and 1150 bp. Both of these fragments are present in nearly all Ste^+ and *Ste* alleles, and show little quantitative variation. Two exceptions were found, and both are Ste^+: the Altamura-66 and 61 variants lack the 1150- and 1100-bp fragments respectively (Figure 7). The third repeat class, 950 bp long, exhibits great variation in copy number and is invariably present and abundant in *Ste* alleles. We have, however, found one Ste^+ *X* chromosome (*W-12*) that completely lacks the 950-bp sequences; in Southern blots of DNA from females or *X*/O males a 950-bp signal is not visible even after long exposure (Figure 7). Interestingly, Southern blots of DNA from *W-12/Y* males show a weak 950-bp signal. This confirms our inference from *in situ* hybridization that the *Y* chromosome carries some *X*-like *Ste* sequences and indicates that these copies share the restriction pattern of the 950-bp *X*-linked repeats.

We performed *in situ* hybridization experiments with the *Ste* probe on polytene chromosomes of some of these stocks, including the three that lack one of the repeat classes. First of all, we observed that the intensity of the signal in the 12E1-2 euchromatic region was directly correlated with the quantity of the 950-bp sequences previously determined by Southern blot analysis, thus confirming their euchromatic location (data not shown). A special case was presented by the *W-12 X* chromosome that was devoid of 950-bp sequences in Southern blots, yet showed a very weak signal in region 12E1-2 (Figure 8). This result can be explained in two ways: either the 12E1-2 region of this chromosome contains one, and only one, 1150- or 1100-bp sequence, or an incomplete 950-bp sequence is still present. In either case, these observations confirm the heterochromatic location of most or all of the 1150- and 1100-bp copies.

***Ste* copy number and chromosome behavior:** To analyze the relationship of the number of *Ste* repeats to the meiotic phenotypes, we chose nineteen Ste^+ alleles containing from 15 to 52 *Ste* copies as estimated by slot blots. The three chromosomes that entirely lack one or another repeat class were included in this sample. Most of the variation in copy number in the other chromosomes is variation in the number of copies of the 950-bp repeat (Figure 6).

We analyzed fertility and sex chromosome behavior in males carrying each Ste^+ allele in combination with the cry^1Y chromosome. The derivation of the disjunctional parameter P_{XY} (the probability of disjunction of the *X* and *Y*) and the drive parameters R_X (the recovery of *X*-bearing sperm) and R_Y (the recovery of *Y*-bearing sperm), and the statistical methods used have been described in MATERIALS AND METHODS. Inspection of the data (Table 2) and statistical analysis (Figure 9a) show a clear correlation between fertility and the total number of *Stellate* copies. The frequency of sex chromosome nondisjunction (Figure 9b) is also tightly correlated with *Ste* copy number. The correlation explains most of the variation in disjunction, and disjunction from the cry^1Y chromosome would be expected to be entirely normal for chromosomes that have 12 or fewer *Ste* copies.

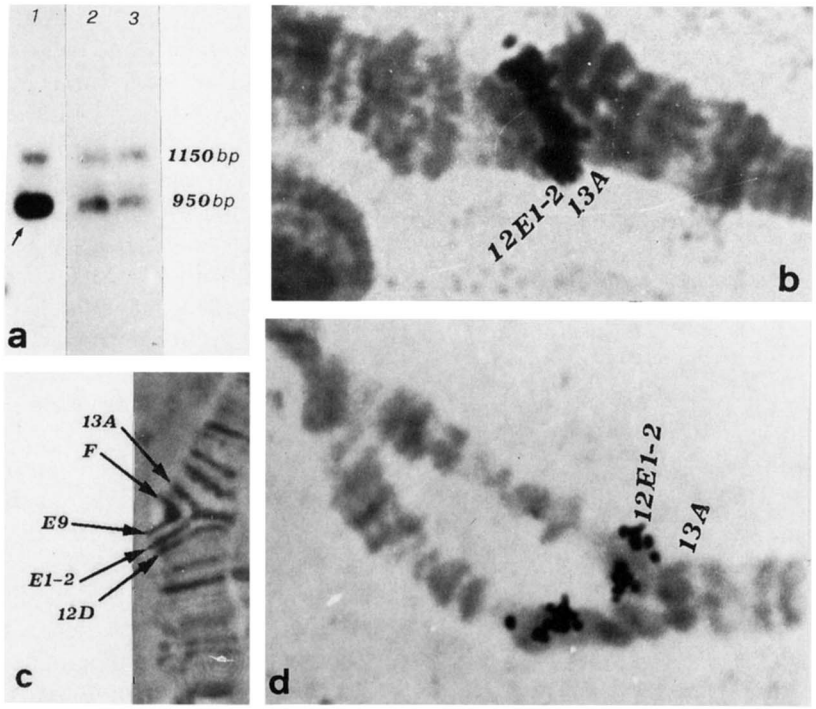


FIGURE 5.—(a) Southern blot analysis of *Cfo*I digested DNA from *301.2* (lane 1), *Df(1)R12.1/Df(1)g-l* (lane 2) and *Df(1)R12.4/Df(1)g-l* (lane 3) females probed with a *Ste* fragment. DNA was estracted from five females for each genotype. The arrow indicates the 950-bp fragment virtually absent in the *R12.1* and *R12.4* deficiencies. (c) Lacto-acetic orcein-stained polytene *X* chromosomes from a heterozygous *Df(1)R12.1/y w Ste*$^+$ *f* larva. The deficiency extends from 12E1-2 to 13A1. (b and d) *in situ* hybridization by a *Ste* probe to polytene chromosomes of the same heterozygous genotype. Note that the signal on the deficiency carrying chromosome is approximately as intense as that present on the *Ste*$^+$ chromosome.

TABLE 1

Meiotic crystal phenotypes of *X* chromosomes from natural populations

Population	No. of *X* chromosomes tested	Crystal phenotype	
		Ste$^+$	Ste
Gandoli	15	15	0
Frascati	58	49	9
Guastameroli	34	26	8
Sammichele	18	16	2
Valenzano	23	21	2
Salve	60	53	7
Altamura	174	164	10
Giovinazzo	25	20	5
Foggia	32	32	0
Ariano Irpino	24	19	5
Umea stocks	18	14	4
Total	481	429	52

These effects on fertility and disjunction are independent of the proportions of the different repeat classes. For example, the *Altamura-1* stock, that carries almost exclusively heterochromatic *Ste* sequences, shows a frequency of nondisjunction comparable to that of strains that carry the euchromatic cluster but have a similar total number of *Ste* sequences.

One interesting point is that males carrying *Ste*$^+$ *X* chromosomes with a copy number around fifty are almost sterile; *Salve-1* (52 *Ste* copies) produces only 0.78 progeny per male. Males with somewhat higher copy numbers still show needle shaped (*Ste*$^+$-morphology) crystals but are completely sterile. Thus the borderline for fertility would seem to be around 50–60 *Ste* copies. However, as we show in Figure 1, the *cry*^{1}*Y* chromosome carries an additional block of heterochromatic *Ste* sequences in its long arm that are derived from the *X* chromosome. These sequences, if functional, could contribute to the severity of the meiotic phenotypes, shifting the border for fertility by 15–25 additional copies. Conversely, although disjunction from the *cry*^{1}*Y* should be regular for *X* chromosomes having 12 or fewer copies of *Ste*, if the heterochromatic cluster in *cry*^{1}*Y* is functional, the disjunctional process might tolerate as many as 27–37 copies.

Table 2 also reveals the presence of meiotic drive and the presence of a small, but statistically significant, amount of variation among the crosses. However, the strength of the drive is only weakly correlated to *Ste* copy number (Figure 10, a and b), and most of the observed variation is unrelated to *Ste* copy number. Moreover, unless there is a spectacular non-linearity of response, much of the drive would remain even at *Ste* copy number = 0. Discounting the B^S block of *Ste* copies, the *Y*-intercepts are $R_X = 0.71$ compared to an average R_X of 0.49, and $R_Y = 0.27$ compared to an average R_Y of 0.16. If the B^S cluster has as many as 30 copies, the intercepts would still be $R_X = 0.86$ and $R_Y = 0.35$.

Because an unknown part of the apparent variation in drive could result from errors in copy number measurement, we also asked whether recoveries of the *X* and *Y* chromosomes are correlated to disjunction–for which the likelihood methods take sampling errors into account. The results (Figure 10, c and d) are nearly identical to those described above; only a small part of the variation of drive is correlated with variation in disjunction. These *X* chromosomes came from several different populations, and it appears that most of the variation in drive is related to differences that do not reside at *Ste*.

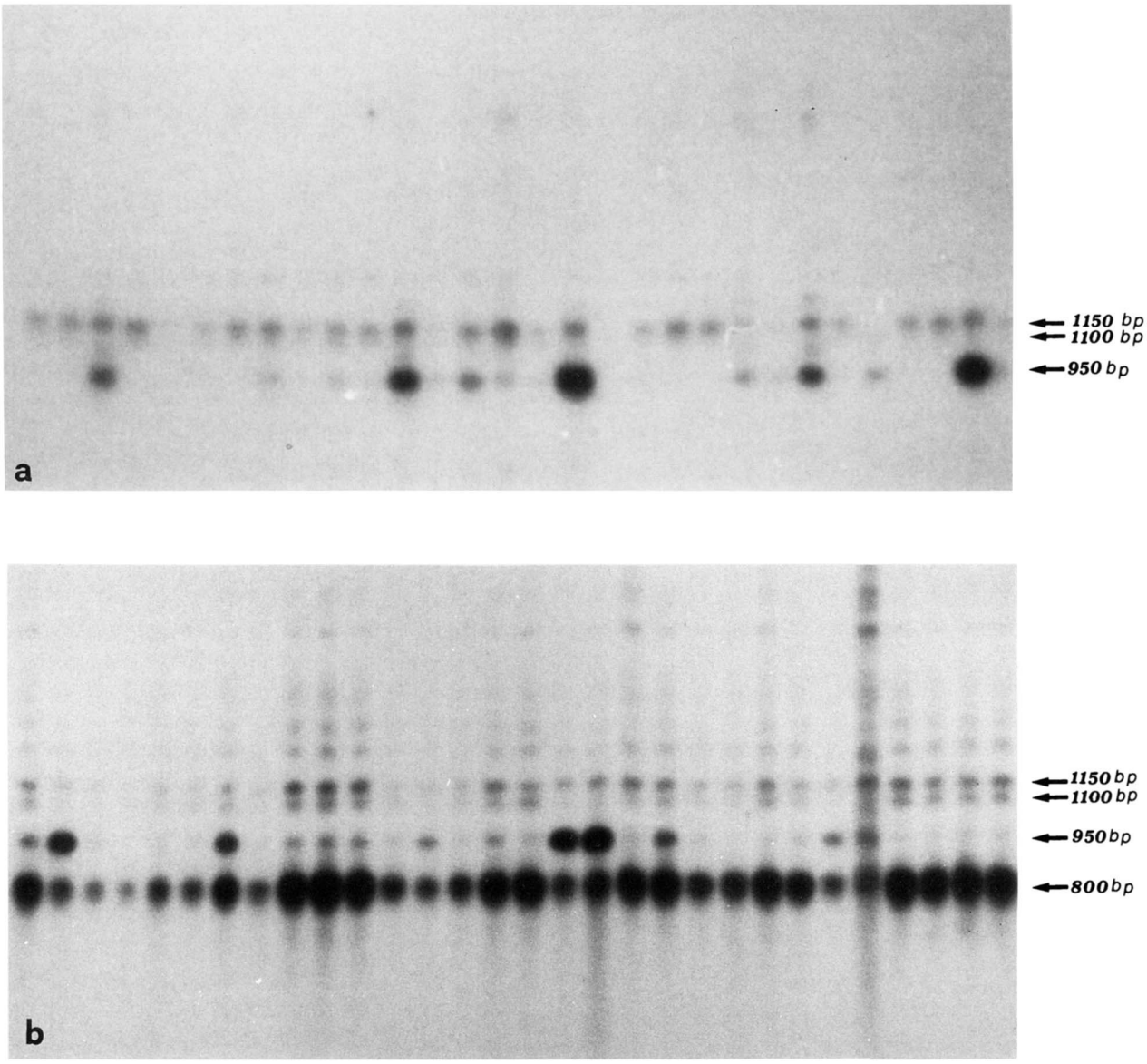


FIGURE 6.—*Ste* sequence variation in natural populations. *Cfo*I digested DNA from single *X*/O (a) and *X*/*Y* (b) males probed with a ^{32}P-labeled *Ste* DNA fragment. The comparison of these two types of males shows that there are three major 1100-, 1150- and 950-bp *Ste* fragments on the *X* chromosome and an 800-bp *Y*-specific fragment. In addition, many weak signals, identifying other *Ste* fragments of higher molecular weight, are visible in *X*/*Y* males (b) most of which are absent in *X*/O males (a). This suggests that, besides the main 800-bp cluster, there are other differently arranged *Ste* sequences in the *Y* chromosome.

However it should be noted that the comparisons of the natural *X* chromosomes were not carried out in an isogenic background to exclude some of *Ste* unrelated variation could reflect uncontrolled modifiers. In any case, cry^1 seems to cause meiotic drive, but its strength is hardly related to the state of the *Ste* locus and is largely uncoupled from disjunction.

The effects of *Ste* copy number on autosomal behavior were also assessed. Males carrying five different Ste^+ alleles in combination with the cry^1Y were crossed to females carrying the compound second chromosome *C(2)EN* with the results shown in Table 3. These crosses show the same basic pattern reported by HARDY *et al.* (1984) for Ste^+ combined with synthetic, translocation-generated *cry* deficiencies: (1) numerous diplo-*2* and nullo-*2* sperm are recovered; (2) sex-chromosome disjunction is even more disrupted among these sperm than among autosomally regular sperm (comparison of the recovery of different sex-chromosome genotypes in these samples with those of Table 2 yields $G = 29.2$ with 4 d.f.); (3) sex-chromosome meiotic drive is also apparent in the autosomally exceptional sample. Compared to the data of Table 2, there is no difference in the effect of the *Y* chromosome on sperm survival ($G = 3.6$, 4 d.f.), but a weaker effect of the *X* chromosome ($G = 9.8$, 4 d.f.); (4) there is an excess recovery of nullo-*2* sperm which, although it could have other causes, we presume reflects autosomal meiotic drive; and (5) disjunction of

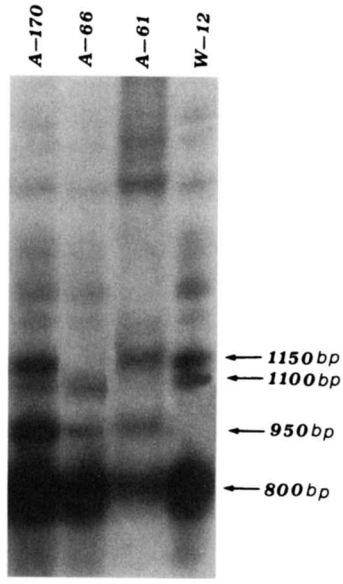


FIGURE 7.—*Cfo*I-digested DNA from males carrying a normal *Y* chromosome and different *X* chromosomes carrying specific *Ste* repeat classes. The three main fragments are all present in Altamura-170 (A-170) males, while Altamura-66 (A-66), Altamura-61 (A-61) and W-12 males lack the 1150-, 1100- and 950-bp fragments, respectively. Note that the *Y*-specific 800-bp fragment is present in all of the genotypes.

the sex chromosomes and autosomes is not only nonindependent in frequency, but is biased toward disjunction to opposite poles. This is most clearly indicated by the excess of *0;22* over *0;0* sperm, where drive would cause a disparity in the opposite direction if disjunction were independent. An *a priori* Chi-square test against a 1:1 expectation yields $\chi^2 = 15.3$ with 5 d.f. Disjunction of the *X* and second chromosomes yields an excess of low chromatin content *X*;nullo-*2* sperm, accounting for the reduced impact of the *X* chromosome on sperm survival in these crosses.

These data also permit us to examine the effect of *Ste* copy number on autosomal behavior. Disjunction (P_2) decreases with *Ste* copy number. Although all of the calculated values of P_2 are overestimates (see MATERIALS AND METHODS), the variation in P_2 is highly significant, it is strongly correlated to *Ste* copy number, and the correlation explains all but an insignificant fraction of the variation. Second chromosome drive, as for sex-chromosome drive, appears to behave differently; there is neither significant variation nor a correlation with *Ste* copy number.

Cytological analysis of meiosis in different *Ste* alleles: To cytologically assess the relationship of *Ste* copy number and the meiotic phenotype, we performed an analysis of meiotic cells of males carrying the cry^1Y chromosome in combination with the *Altamura-1* and *Salve-1* Ste^+ *X* chromosomes and the *Ste 301.2 X* chromosome containing 16, 52 and 300 *Ste* copies, respectively. These three combinations induce different crystal phenotypes: small and rare needle-shaped crystals in Ste^+ *Altamura-1*, long and frequent needle-shaped crystals in Ste^+ *Salve-1* and star-shaped crystal aggregates in *Ste 301.2 X* (data not shown). The meiotic analysis was carried out by classical orcein staining of meiotic chromosomes and by immunological staining of meiotic spindles with antitubulin antibodies combined with Hoechst fluorescence staining of chromatin. With orcein staining, the morphology of meiotic chromosomes in Ste^+ *Altamura-1* males appears normal (Figure 11a) while in Ste^+ *Salve-1* males the chromosomes are often undercondensed. In both types of males, however, meiotic pairing appears to be normal. In *Ste 301.2* males, the chromosomes are so severely affected that we never observed a normal meiotic metaphase; the chromosomes are grossly undercondensed, and in many cases they appear to be fragmented (Figure 11b).

The immunostaining results are reported in Figures 12 and 13. *Altamura-1*/cry^1Y males show apparently normal meiotic spindles (Figure 12o) and chromosome pairing, but there are some instances of altered chromatin segregation (Figure 12p). Chromosome pairing and meiotic spindle morphology are also apparently unaffected in *Salve-1*/cry^1Y males, although there are obvious defects in chromosome segregation in both meiotic divisions (Figures 12, e–h, and 13e). Chromosome pairing and spindle morphology are not consistently affected in males carrying the *Ste 301.2 X* chromosome, even though they give extremely defective chromatin distribution in virtually every meiotic figure (Figures 12, i–n, and 13, a–d).

We also analyzed meiosis in males carrying these three *X* chromosomes that had no *Y* chromosome rather than the cry^1Y. The absence of the *Y* chromosome did not significantly affect the phenotypes except in the case of *Ste 301.2/0* males in which the morphology of the spindles was strongly altered (Figure 12q). The bridges of chromatin, always visible at anaphase (Figure 12r), appear to eventually produce a distortion of the spindles. Two possibilities could explain the difference between *Ste 301.2*/cry^1Y and *Ste 301.2/0* males. The residual 950-bp class *Ste* sequences still present in the cry^1Y (Figure 1) might suppress the *X*-linked *Ste* allele, or the *X*-heterochromatic *Ste* sequences transferred to the tip of the long arm of the cry^1Y may have a *Ste*-suppressing effect. To distinguish between these two possibilities, we tested the *Ste 301.2 X* chromosome in combination with the B^SYy^+ *G18* chromosome. *G18* carries the same *X*-derived heterochromatic block on the tip of the long arm, but is completely deficient for the *crystal* region (C. PISANO and M. GATTI, personal communication). The cytological phenotype of *Ste 301.2/G18* testes is identical to that observed in *Ste 301.2/0* testes (data not shown). Thus, the stronger phenotype observed in *X*/O males is caused by removal of the few *X*-like *Ste* sequences that remain in the h11 region of the cry^1Y, and *X*-heterochromatin derived *Ste* sequences do not acquire *cry*-like suppressive activity when transferred to the *Y* chromosome.

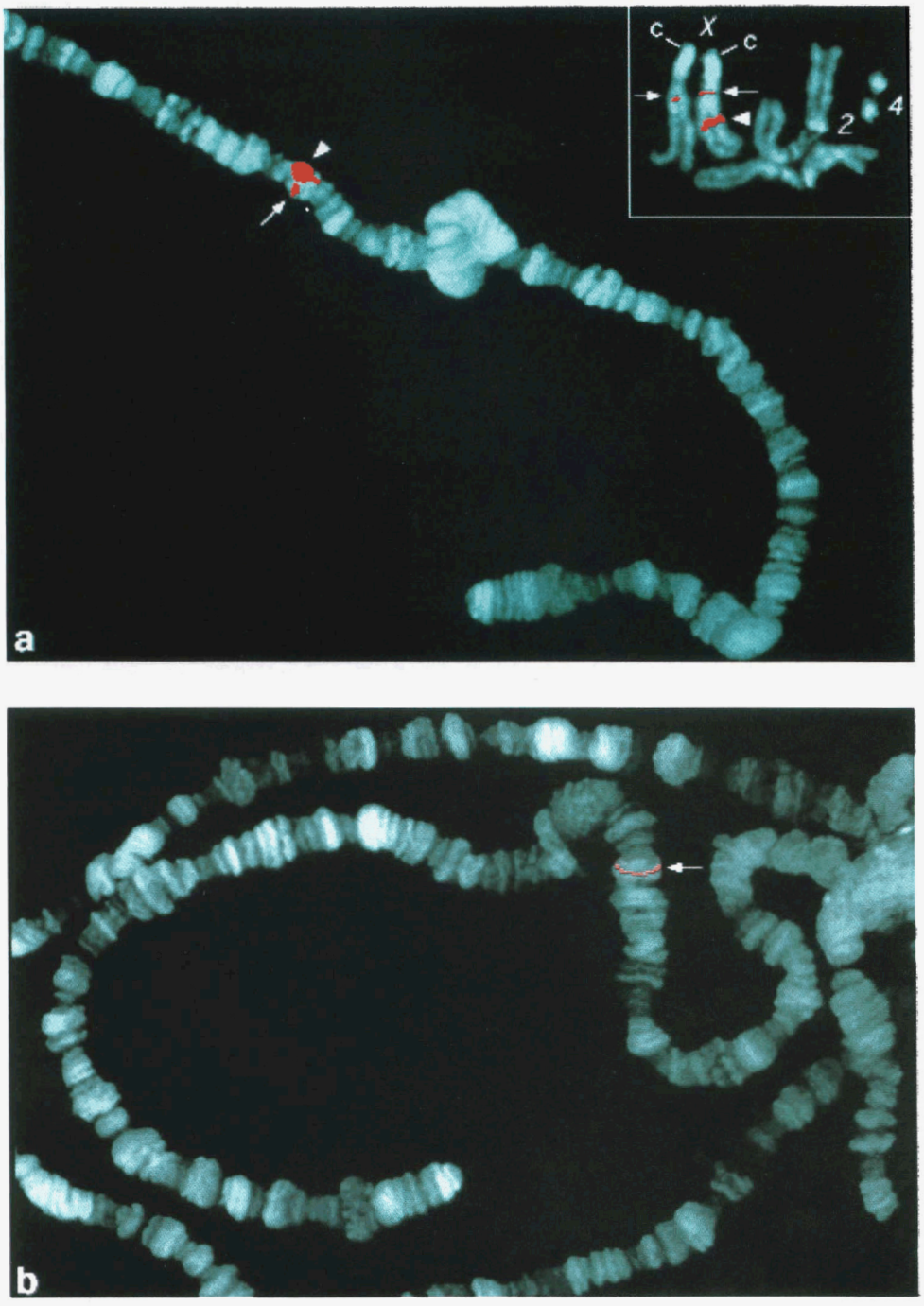


FIGURE 8.—Non-radioactive *in situ* hybridization of *Ste* sequences to the polytene *W-12 X* chromosome (a) in combination with the *Ste 301.2 X* chromosome and (b) in homozygotes. Note the very weak signal in the 12E1-2 region of the *W-12* chromosome, especially compared to the signal on the *301.2* chromosome. The differences between the two *X* chromosomes are also very clear from the insert in (a) where the *in situ* hybridization to a partial mitotic metaphase is shown. Both the *X* chromosomes show a signal in the heterochromatin (arrows) while a euchromatic signal is present only in the *301.2 Ste X* chromosome (arrowhead).

DISCUSSION

Organization of the *Stellate* sequences: The *Stellate* sequences of the *X* chromosome fall into three classes based on *Cfo*I fragment sizes of 1150, 1100 and 950 bp. Two types of evidence indicate that the three classes form separate clusters: (1) partial deletions of the 12E1-2 *Stellate* region invariably reduced the amount of only the 950-bp repeat type, and (2) we found variants completely lacking a single repeat type in chromosomes taken from natural populations. The three repeat clusters cytologically map at two loci: one is in the 12E1-2 polytene region and mostly, if not exclusively, contains the 950-bp class repeats. The other locus maps between the C and D heterochromatic blocks of the *X* chromosome and contains both the 1150- and 1100-bp repeats. Most of the variation of *Ste* copy number in natural populations is variation in number of copies of the 950-bp class. One possible explanation is that the variability is driven by meiotic unequal crossing over and that the predominantly euchromatic 950-bp repeat would be more subject to this phenomenon than the other two classes located in the recombinational refractory heterochromatin.

Meiotic properties of the *Ste-cry* interaction: Genetically, we found that *Stellate* shows a high degree of variability in natural populations. Although, based on crystal

TABLE 2

Progeny produced by crosses of males carrying *X* chromosomes with different *Ste* copy numbers in combination with the *cry*^{1}*Y* chromosome to *y w f* females

Chromosome	No. of *Ste* copies	No. of males	Sperm genotype				Progeny/ Male	Disjunction P_{XY}	Drive	
			X	*Y*	*XY*	O			R_X	R_Y
W-12	15	14	1206	667	4	64	138.6	0.98	0.34	0.19
Altamura-1	16	34	1567	966	11	44	76.1	0.98	0.64	0.39
Fairfield-11	16	17	1261	354	4	102	101.2	0.97	0.37	0.10
Salve-3	20	17	1369	705	12	96	128.4	0.97	0.49	0.25
Salve-2	21	21	1720	374	11	187	109.1	0.95	0.52	0.11
Altamura-46	22	19	1219	343	25	216	94.9	0.90	0.64	0.18
Altamura-66	22	22	1645	576	39	312	116.9	0.90	0.60	0.21
y w f	29	17	1186	529	19	260	117.3	0.92	0.40	0.18
Altamura-40	30	25	1400	425	34	294	86.1	0.89	0.62	0.19
Altamura-61	34	33	1506	447	29	476	74.5	0.87	0.45	0.13
Valenzano-2	40	24	243	91	15	134	20.1	0.77	0.55	0.20
Sammichele	42	34	233	57	6	105	11.8	0.82	0.48	0.12
Giovinazzo	45	24	333	125	14	183	27.3	0.80	0.45	0.17
Altamura-4	45	35	467	175	20	255	26.2	0.80	0.46	0.17
Altamura-22	45	21	373	94	1	226	33.1	0.93	0.13	0.03
Salve-4	50	30	120	29	3	85	7.9	0.79	0.38	0.09
Altamura-36	50	33	184	41	4	132	10.9	0.79	0.37	0.08
Gandoli-6	50	34	183	58	7	140	11.4	0.77	0.40	0.13
Salve-1[a]	52	54	15	10	0	17	0.8	—	—	—

[a] Although 40% of the offspring of *Salve-1/cry*^{1}*Y* males are exceptional for the sex chromosomes, the meiotic parameters P_{XY}, R_X and R_Y are indeterminate because of the absence of the *XY* class.

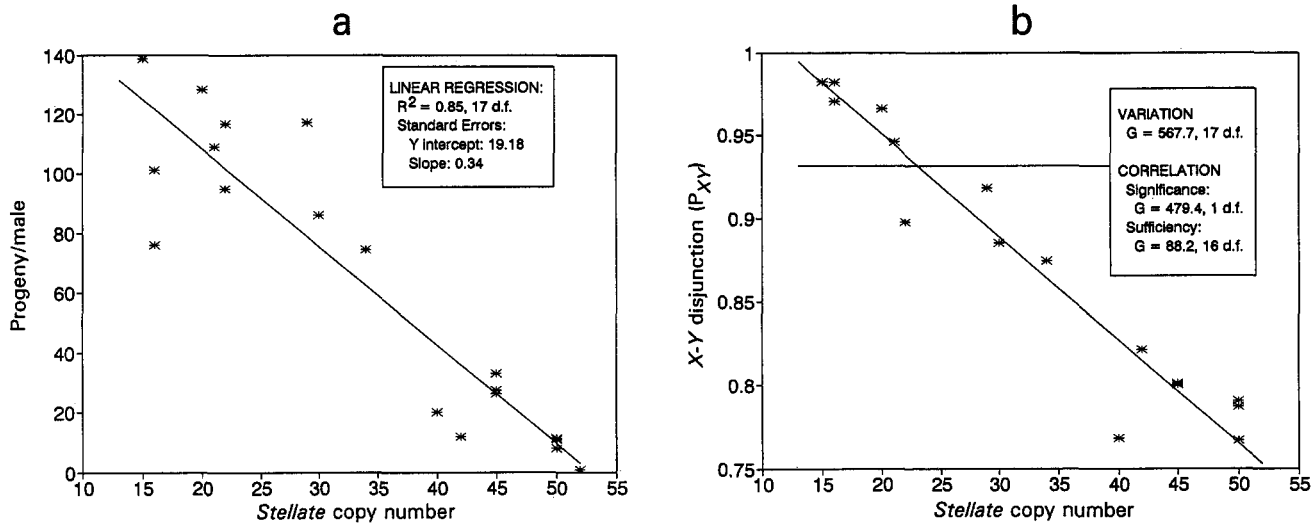


FIGURE 9.—Relationship of male fertility (a) and sex-chromosome disjunction (b) to *Ste* copy number. The data of Table 2 were analyzed by linear regression to generate panel (a) and by the maximum-likelihood procedures outlined in MATERIALS AND METHODS to generate panel (b). Both of these parameters are strongly correlated with *Ste* copy number.

shape alone, only a *Ste*$^+$ and a *Ste* allele can be distinguished, we found many variants among *Ste*$^+$ *X* chromosomes that, in combination with the *cry*^{1}*Y*, produce different degrees of fertility and meiotic chromosome nondisjunction. These *Ste*$^+$ variants contain different numbers of *Ste* copies, and both fertility and the frequency of sex chromosome nondisjunction are closely correlated to *Ste* copy number. Meiotic drive also occurs in *Ste*$^+$ males, but the degree of drive is, for the most part, independent of the level of nondisjunction and independent of *Ste* copy number. The *Ste-cry* interaction is, therefore, a system in which sex chromosome disjunction and drive are uncoupled, in contrast to deficiencies of the *X-Y* pairing sites of the *X* heterochromatin, subsequently identified as the ribosomal DNA (MCKEE and KARPEN 1990), that cause strongly correlated nondisjunction and meiotic drive (MCKEE and LINDSLEY 1987). Both the nondisjunction and drive, however, are cellular rather than sex chromosome-specific problems, since autosomal nondisjunction (also correlated to *Ste* copy number) and drive (also little affected by *Ste* copy number) are observed. Moreover, the nondisjunction is likely to reflect a post-synaptic effect of the *Ste-cry* interaction, rather than an effect on pairing

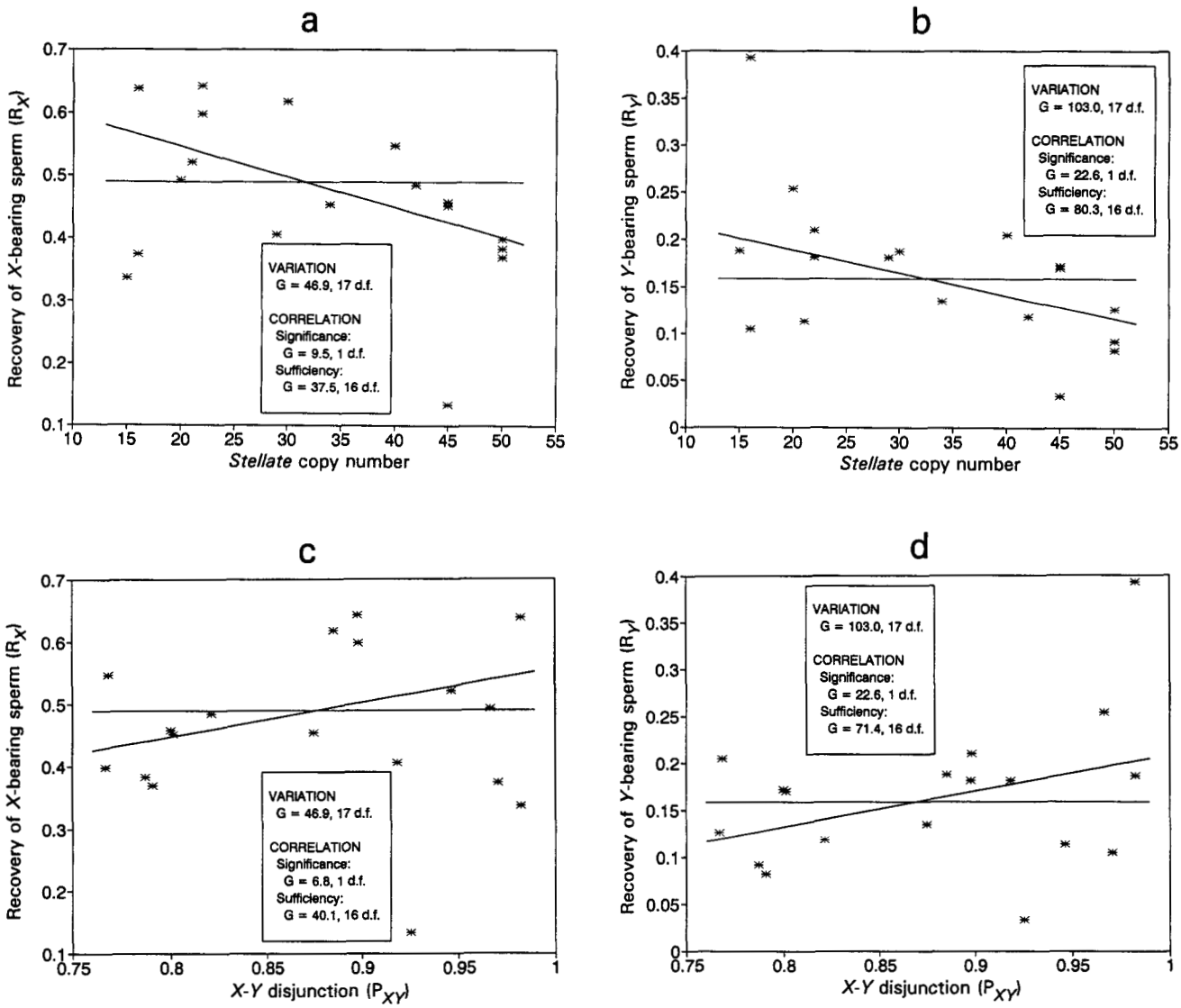


FIGURE 10.—Relationship of *X* chromosome meiotic drive (a and c) and *Y* chromosome meiotic drive (b and d) to *Ste* copy number and sex-chromosome disjunction. Maximum-likelihood analysis of meiotic drive from the data of Table 2 indicates that there is only weak correlation with either *Ste* copy number (a and b) or disjunctional behavior (c and d), and that most of the small amount of variation observed for recovery of the *X* and *Y* chromosomes is unrelated to either of these parameters.

per se. Sex-chromosome nondisjunction is more severe among autosomal exceptions, doubly nondisjunctional sperm show an excess of sex chromosome-from-autosome disjunction, and, at the cytological level, *X-Y* pairing in cry^1Y males carrying different Ste^+ variants seems to be normal, even in males that evince drastic disjunctional problems and are nearly sterile.

The disjunctional defect could even be ascribed to nonspecific interference of the crystal-forming protein with the progression of meiosis, but one would have difficulty explaining the meiotic drive in this fashion. We have not been able to get a viable, complete deficiency of *Ste*, but we have tested variants with as few as 15 copies; they show little if any diminution in the severity of drive. Moreover, even projecting what weak correlation there may be of drive with *Ste* copy number to the situation of no *Ste* copies would still leave substantial drive. *cry* has been described as a suppressor of *Stellate*, and given the name *Su(Ste)*, because cry^+ males do not form crystalline inclusions. At a formal level, however, we do not find *Ste*-caused drive that is suppressible by cry^+, but a drive phenotype that can be ascribed directly to *cry*.

If, as has been suggested (HURST 1992), there is some relic *SD*-like drive element that is suppressed by cry^+, it is probably not *Ste*. Intriguingly, MCKEE (1987) has shown that physically uncoupling a site located only a short distance from *Ste* from the *X-Y* pairing elements causes the meiotic drive seen in translocations such as $T(1;4)B^S$. He has proposed that this locus is also involved in the drive that occurs in pairing-element deficiencies. Perhaps this locus, as *Ste*, interacts with *cry*. In any case, the differences between the disjunctional and drive effects produced by the *Ste-cry* interaction and that produced by ribosomal DNA deficiencies suggest that

TABLE 3

Progeny produced by crosses of males carrying *X* chromosomes with different *Ste* copy numbers in combination with the *cry^{1}Y* chromosome to females carrying *C(2)EN*

Chromosome[a]	Total eggs	Sperm genotype of survivors					Disjunction		Drive		
			X	*Y*	*XY*	O	P_{XY}	P_2	R_X	R_Y	R_2
Altamura-1 (16)[b]	1000	*2/2*	4	4	0	7	—	0.90	—	—	0.62
		0	26	11	0	2					
Salve-3 (20)	1333	*2/2*	12	4	2	14	0.76	0.88	1.00	0.21	0.82
		0	39	6	2	1					
y w f (29)	642	*2/2*	5	0	0	5	0.77	0.88	1.00	0.10	0.55
		0	25	3	1	4					
Sammichele (42)	1158	*2/2*	6	3	2	19	0.83	0.80	0.59	0.15	0.54
		0	62	16	2	22					
Salve-4 (50)	479	*2/2*	4	1	0	11	0.65	0.78	0.93	0.18	0.62
		0	27	5	3	7					
							G statistic; degrees of freedom				
Variation:							1.58; 3	31.2; 4	1.74; 3	1.69; 3	7.73; 4
Correlation											
Significance:							0.84; 1	28.9; 1	0.66; 1	0.10; 1	2.76; 1
Sufficiency:							0.75; 2	2.20; 3	1.07; 2	1.59; 2	4.97; 3

[a] *Ste* copy numbers are shown in parentheses.
[b] Because of the absence of the *XY* class in this cross, the sex-chromosome meiotic parameters are indeterminate.

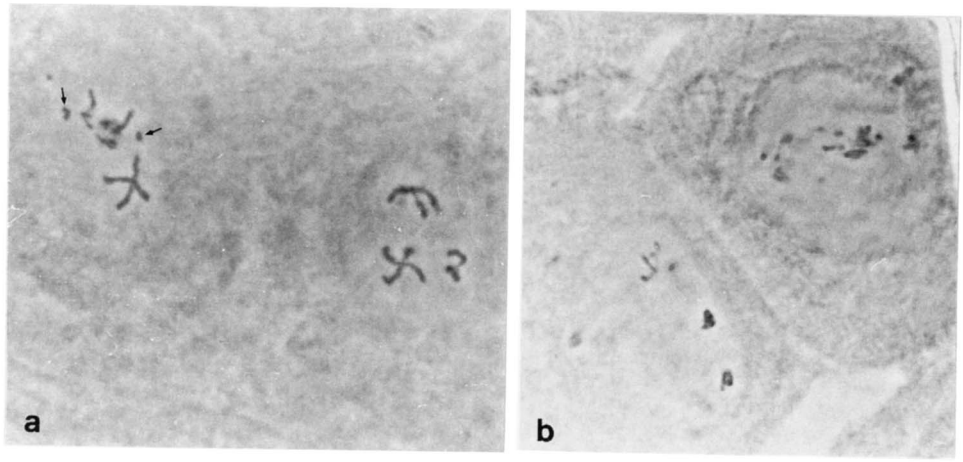


FIGURE 11.—Orcein stained meiotic chromosomes from *Ste$^+$ Altamura-1/cry^{1}Y* (a) and *Ste 301.2/cry^{1}Y* (b) males. Note the normal morphology of the chromosomes and the nondisjunction of the fourth chromosomes (arrows) in *Ste$^+$ Altamura-1* males (a), and the abnormal chromosome condensation and segregation in *Ste 301.2* males (b).

detailed analysis of this system, and of its interactions with paring-site deficiencies, will provide useful information about the regulation of male meiosis.

The cytological analysis of meiosis in *cry$^-$* males carrying *Ste* alleles strongly suggests that the sterility of these males is due to enhancement of the same kind of chromosome abnormalities observed in *Ste$^+$/cry$^-$* males. In *Ste/cry$^-$* testes the defects in chromosome condensation and segregation are much more severe than in *Ste$^+$/cry$^-$* testes, and they are present in every meiotic figure. Interestingly, in *Ste*/O males we also observed distortion of the meiotic spindles. This additional defect is probably due to mechanical entanglement of decondensed chromosomes that, interfering with normal centromere segregation, causes distortion of an otherwise normal spindle.

Function of the heterochromatic *Stellate* copies: An important issue is the functional equivalence of the euchromatic and heterochromatic *Stellate* sequences. Molecular analysis by SHEVELYOV (1992) indicates that the 1150-bp class *Ste* sequences located in *X* heterochromatin have intact open reading frames. Our data suggest that these copies are actually functional and are, as for the euchromatic copies, derepressed in the absence of the *crystal* locus. In particular, *X* chromosomes lacking the euchromatic 950-bp cluster promote nondisjunction proportionate to the number of remaining 1150- or 1100-bp heterochromatic copies when tested in combination with *cry^{1}Y*.

Southern blot analysis of genomes with *X*-heterochromatic deletions indicate that the 1150- and 1100-bp copies are almost exclusively heterochromatic. The same experiments also provided strong evidence that the euchromatic *Ste* sequences are not underreplicated in polytene chromosomes. In this context, if the extremely weak hybridization signal in the 12E1-2 polytene region of the *W-12* deficiency chromosome indicates the complete absence of functional *Ste* sequences in this

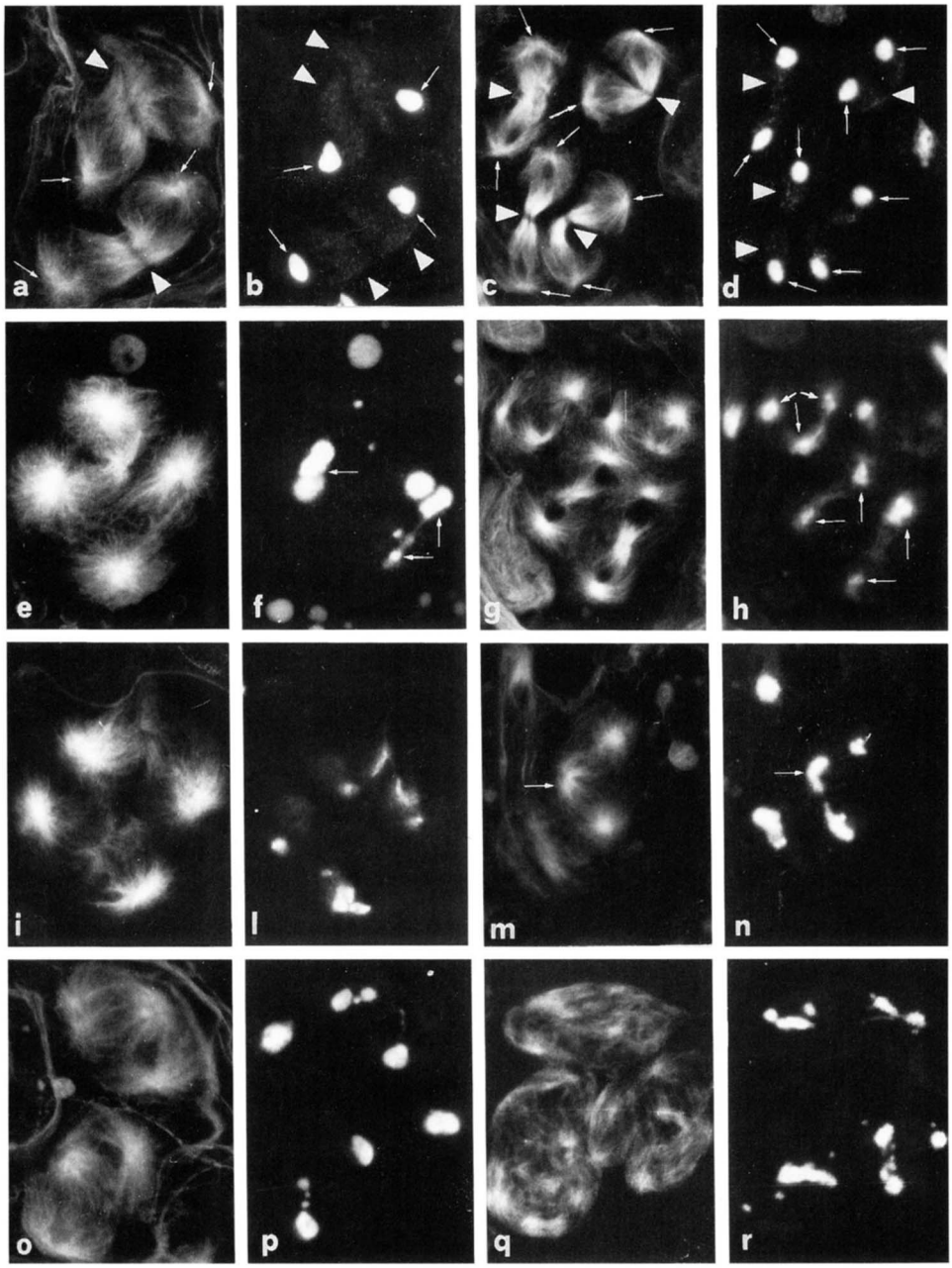


FIGURE 12.—Testis preparations from males of different genetic constitutions immunostained with anti-α tubulin (a, c, e, g, i, m, o and q) and with Hoechst 33258 (b, d, f, h, l, n, p and r). (a and b) Meiosis I and (c and d) meiosis II telophases of a wild-type male. Note in (a) and (c) the fully developed spindles with regular asters (arrows) and prominent midbodies (arrowheads). In (b) and (d) the regular segregation of chromosomes into two equal-size daughter nuclei (arrows) can be observed together with the lining up of the mitochondria along the midbody (arrowheads). (e and f) Meiosis I and (g and h) meiosis II telophases of a *Salve1/cry*^{1}*Y* male, showing (e and g) normal spindles but (f and h) partially decondensed chromosomes that segregate irregularly (arrows). (i and l) Meiosis I and (m and n) meiosis II telophases of a *301.2/cry*^{1}*Y* male. The morphology of the spindles does not appear to be substantially affected (i and m), but chromosome segregation is quite defective (l and n) and there are frequent anaphase bridges. See an example in (m) and (n) where chromatin lagging in a zone corresponding to the midbody is clearly visible (arrows). (o and p) Meiosis I telophase of an *Altamura-1/cry*^{1}*Y* male. Note the normal morphology of the spindles (o) and the irregular segregation of the chromosomes. (q and r) Extremely aberrant spindle morphology (q) and severely impaired chromosome segregation (r) in *301.2/0* males.

region, all of the 1100- and 1150-bp copies would have to be both heterochromatic and functional. To assess this, we are attempting to clone the residual euchromatic *Ste* element of the *W-12 X* chromosome.

We also show that the *X* heterochromatic *Ste* cluster transferred to the tip of the $B^{S}Yy^{+}$ chromosome does not suppress *Ste* expression. Thus, although these copies function like the euchromatic *X*-linked copies, they do not function like the analogs located at *cry*. Thus, the suppressive effect of the *cry* sequences is probably caused by differences in their molecular organization (LIVAK 1984, 1990; BALAKIREVA *et al.* 1992), rather than their *Y* chromosome location.

The problem of the biological role of *Stellate*: In this report we have also described our attempts to obtain an *X* chromosome completely devoid of *Stellate* sequences in order to elucidate its possible functional role. Despite the various experimental strategies used, we could not obtain viable complete deletions of the euchromatic *Stellate* locus but only a viable partial revertant. The possibility that the failure of obtaining fertile revertants because of the presence of an abundant amount of the heterochromatic *Ste* sequences, is ruled out from the fertility of the four lethal partial deficiencies of the *Ste* sequences in the 12E region that we recovered. Thus, also if for the *P*-mediated mutagenesis is possible that we induced only one side deletions, with the x-rays we should have induced viable complete deficiencies unless the number that we observed were below the probability of their induction. Other possibility is that *Stellate* itself

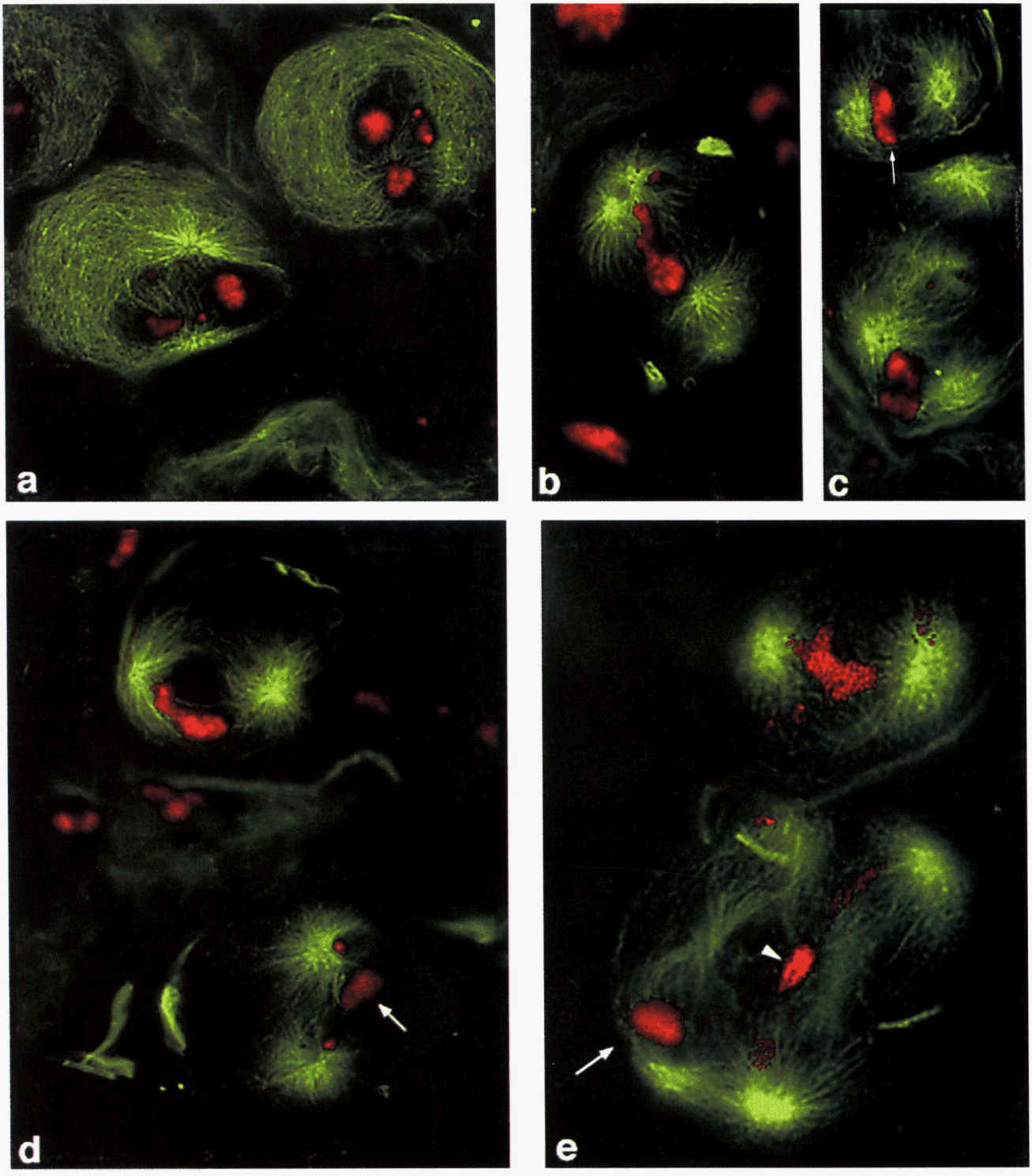


FIGURE 13.—Pseudocolored and merged digital images of *301.2/cry^{1}Y* (a–d) and *Salve-1/cry^{1}Y* (e) male testis preparations immunostained with antitubulin antibody (green) and Hoechst 33258 (red). (a) Prometaphase nuclei showing normal chromatin condensation and normal development and migration of asters. (b–d) Ana-telophase figures exhibiting severely impaired chromatin segregation. In (b) virtually all the chromosomes are lagging between the poles; in (c) all the chromosomes have migrated to one pole (arrow); in (d) only the fourth chromosomes have disjoined and the rest of chromatin is lagging in the middle of the spindle (arrow). The same type of meiotic defects can be observed in *Salve-1/cry^{1}Y* male testes (e) which show both migration of most of the chromosomes to one pole (arrow) as well as lagging chromatin (arrowhead).

has an essential function or alternatively, *Stellate* could be dispensable, but have an essential sequence so tightly linked that complete removal of the *Stellate* cluster is lethal. Intriguingly, a transfer RNA has been mapped in the 12DE region (HAYASHI *et al.* 1980). If this gene is located within the 950-bp cluster, it could explain our

inability to generate complete deletions of the euchromatic *Ste* cluster. The present data are not definitive, but continued molecular analysis of the *W12* deficiency may be. If the remnant *Stellate* sequence(s) in *W12* are rearranged and inactive, we will know that *Stellate* itself does not have an essential function.

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LITERATURE CITED

BALAKIREVA, M. D., Y. Y. SHEVELYOV, D. I. NURMINSKY, K. J. LIVAK and V. A. GVOZDEV, 1992 Structural organization and diversification of *Y*-linked sequences comprising *Su(Ste)* genes in *Drosophila melanogaster*. Nucleic Acids Res. **20:** 3731–3736.

BENDER, W. P., P. SPIERER and D. S. HOGNESS, 1983 Chromosomal walking and jumping to isolate DNA from *ace* and *rosy* loci and the *bithorax* complex in *Drosophila melanogaster*. J. Mol. Biol. **168:** 17–33.

BISHOP, Y. M. M., 1975 *Discrete Multivariate Analysis: Theory and Practice*. MIT Press, Cambridge.

CENCI, G., S. BONACCORSI, C. PISANO, F. VERNI' and M. GATTI, 1994 A cytological dissection of *Drosophila melanogaster* spermatogenesis. J. Cell Sci. (in press).

COOLEY, L., C. BERG and A. SPRADLING, 1988 Controlling P element insertional mutagenesis. Trends Genet. **4:** 254–258.

COOPER, K. W., 1959 Cytogenetic analysis of major heterochromatic elements (especially *Xh* and *Y*) in *Drosophila melanogaster* and the theory of "heterochromatin." Chromosoma **10:** 535–588.

FEINBERG, A. P., and B. VOGELSTEIN, 1983 A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:** 6–13.

GATTI, M., and S. PIMPINELLI, 1983 Cytological and genetic analysis of the *Y* chromosome of *Drosophila melanogaster*. I. Organization of the fertility factors. Chromosoma **88:** 349–373.

HARDY, R. W., D. L. LINDSLEY, K. J. LIVAK, B. LEWIS, L. SIVERSTEN *et al.*, 1984 Cytogenetic analysis of a segment of the *Y* chromosome of *Drosophila melanogaster*. Genetics **107:** 591–610.

HAYASHI, S., I. C. GILLAM, A. D. DELANEY, R. DUNN, G. M. TENER *et al.*, 1980 Hybridization of the tRNAs of *Drosophila melanogaster* to polytene chromosomes. Chromosoma **76:** 65–85.

HURST, D. L., 1992 Is *Stellate* a relict meiotic driver? Genetics **130:** 229–230.

LIFSCHYTZ, E., and D. HAREVEN, 1977 Gene expression and the control of spermatid morphogenesis in *Drosophila melanogaster*. Dev. Biol. **58:** 276–94.

LINDSLEY, D. L., and G. ZIMM, 1992 The genome of *Drosophila melanogaster*. Academic Press, New York.

LIVAK, K. J. 1984 Organization and mapping of a sequence on the *Drosophila melanogaster X* and *Y* chromosomes that is transcribed during spermatogenesis. Genetics **107:** 611–34.

LIVAK, K. J. 1990 Detailed structure of the *Drosophila melanogaster Stellate* genes and their transcripts. Genetics **124:** 303 16.

LOVETT, J., T. C. KAUFMAN and A. P. MAHOWALD, 1980 A locus on the *X* chromosome apparently controlled by the *Y* chromosome during spermatogenesis in *Drosophila melanogaster*. Eur. J. Cell Biol. **22:** 49.

LYCKEGAARD, E. M. S., and A. G. CLARK, 1989 Ribosomal DNA and *Stellate* gene copy number variation on the *Y* chromosome of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA **86:** 1944–1948.

MCKEE, B., 1984 Sex chromosome meiotic drive in *Drosophila melanogaster* males. Genetics **106:** 403–422.

MCKEE, B., 1987 *X-4* translocations and meiotic drive in *Drosophila melanogaster* males: role of sex chromosome pairing. Genetics **116:** 409–413.

MCKEE, B. D., and G. H. KARPEN, 1990 Drosophila ribosomal RNA genes function as an *X-Y* pairing site during male meiosis. Cell **61:** 61–72.

MCKEE, B., and D. LINDSLEY, 1987 Inseparability of *X*-heterochromatic functions responsible for *X:Y* pairing, meiotic drive and male fertility in *Drosophila melanogaster*. Genetics **116:** 399–407.

MEYER, G. F., O. HESS and W. BEERMANN, 1961 Phasenspezifische Funktionsstrukturen in Spermatocytenkernen von *Drosophila melanogaster* und ihre Abhangigkeit vom *Y* Chromosom. Chromosoma **12:** 676–716.

NOVITSKI, E., D. GRACE and C. STROMMEN, 1981 The entire compound autosomes of *Drosophila melanogaster*. Genetics **98:** 257–273.

O'CONNELL, P., and M. ROSBACH, 1984 Sequence, structure and codon preference of the Drosophila ribosomal protein 49 gene. Nucleic Acids Res. **12:** 5495–5513.

PARDUE, M. L., 1986 *In situ* hybridization to DNA of chromosomes, pp. 11–137 in *Drosophila: A Practical Approach*, edited by D. B. ROBERTS. IRL Press, Oxford.

PIMPINELLI, S., S. BONACCORSI, M. GATTI and L. SANDLER, 1986 The peculiar genetic organization of Drosophila heterochromatin. Trends Genet. **2:** 17–20.

PISANO, C., S. BONACCORSI and M. GATTI, 1993 The *kl-3* loop of the *Y* chromosome of *Drosophila melanogaster* binds a tektin-like protein. Genetics **133:** 569–579.

ROBERTSON, H. M., and W. R. ENGELS, 1989 Modified P elements that mimic the P cytotype in *Drosophila melanogaster*. Genetics **123:** 815–824.

SAMBROOK, J., E. F. FRITSCH and T. MANIATIS, 1989 *Molecular Cloning: A Laboratory Manual*, Ed. 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

SHEVELYOV, Y. Y., 1992 Copies of a *Stellate* gene variant are located in the *X* heterochromatin of *Drosophila melanogaster* and are probably expressed. Genetics **132:** 1033–1037.

SPRADLING, A. C., and G. M. RUBIN, 1983 The effect of chromosomal position on the expression of the Drosophila xanthine dehydrogenase gene. Cell **34:** 47–57

TSUBOTA, S., and P. SHEDL, 1986 Hybrid dysgenesis-induced revertants of insertions at the 5′ end of the *rudimentary* gene in *Drosophila melanogaster*: transposon-induced control mutations. Genetics **114:** 165–182

WARING, G. L., J. P. DIORIO and S. HENNEN, 1983 Isolation of germ line-dependent female-sterile mutation that affects yolk specific sequestration and chorion formation in *Drosophila*. Dev. Biol. **100:** 452–463.

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