

Detection of nondisjunction and recombination in meiotic and postmeiotic cells from XY^{Sxr} [XY,Tp(Y)1Ct] mice using multicolor fluorescence *in situ* hybridization

(aneuploidy/spermatids/sperm/sex reversal/pseudoautosomal region)

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ABSTRACT Current meiotic dogma holds that synapsis is required for recombination and that recombination is required for proper disjunction. The mouse chromosome aberration XY^{Sxr} [sex reversal; redesignated XY,Tp(Y)1Ct] appears to challenge this assumption, for although chromosomes X and Y often fail to synapse and recombine, there is no dramatic increase in aneuploid progeny. An explanation of this conundrum might be that X–Y univalent spermatocytes do not survive. The phenotype of sex reversal is generated by the “obligatory” crossover between the X and Y chromosomes, which always occurs proximal to a duplicated copy of the testis-determining gene *Sry* and transfers one copy from one chromatid of the Y chromosome to one chromatid of the X. Animals that inherit an X chromosome with the *Sry* gene are chromosomally female but phenotypically male. We have used fluorescence *in situ* hybridization (FISH) to visualize probes for the X and Y chromosomes and for the *Sry* sequence and chromosome 8 to track the fate of both recombinant and nonrecombinant chromosomes through metaphases I and II into spermatids and sperm. In the 219 gametes examined by multicolor FISH, the frequency of aneuploid products (XY or “O”) was low (3.7%) despite a high frequency (66%) of X–Y separation at metaphase I. In balanced gametes, X and Y recombinant chromosomes slightly exceeded nonrecombinants. Both of these observations support the earlier proposal that asynapsis and nondisjunction in primary spermatocytes lead to their developmental arrest and degeneration.

Aneuploidy arising from meiotic missegregation is the primary cause of numerical chromosome abnormalities. Two general approaches have been developed to directly measure frequency of aneuploidy in spermatozoa: (i) cytogenetic analysis of the sperm's whole chromosome complement following penetration of sperm from a xenotropic species into zona-free hamster oocytes (1–4) and (ii) *in situ* hybridization to sperm cells (5). The first technique is tedious, technically difficult, and limited by the number of hamster oocytes that can be fertilized for any single analysis. Until now, *in situ* hybridization studies, whether isotopic (6) or nonisotopic (5, 7), have been limited to the detection of one or at most two chromosome markers, thus severely limiting utility of this procedure. However, the recent introduction of multicolor *in situ* hybridization methods (8, 9) has greatly increased the potential usefulness of *in situ* hybridization for studying aneuploidy in spermatozoa.

Meiotic synapsis is generally considered necessary to bring homologous DNA into close enough register for recombination to occur. Normal disjunction at anaphase I depends upon the production of at least one chiasma (the cytological

consequence of meiotic recombination) per bivalent (pair of synapsed chromosomes) to facilitate orientation of autosomal homologues or the X and Y chromosomes to opposite poles. Lack of recombination (chiasma formation) between homologues of a bivalent is a significant cause of meiotic I nondisjunction (10–12). The sex chromosomes of male mammals have a single chiasma, which is highly localized within the “pseudoautosomal” region (13–15). Dependence on this single recombination event vs. multiple events in most autosomal bivalents may account for the fact that paternally derived aneuploidy resulting from recombination failure is highest for the sex chromosomes (16).

Cytogenetic detection of recombination after resolution of chiasmata requires a double polymorphism: one that distinguishes between donor and recipient chromosomes and another that identifies the exchanged segment. Current *in situ* techniques cannot detect allelic differences because point mutations, restriction fragment length polymorphisms (RFLPs), and other small molecular changes are insufficient to lead to differential probe hybridization. However, the XY^{Sxr} rearrangement [redesignated XY,Tp(Y)1Ct] is detectable by *in situ* methods. This rearrangement places a second copy of *Sry*, the testis-determining sequence (17), in the distal pseudoautosomal segment of the Y chromosome in addition to the copy in the normal proximal location. Evans *et al.* (18) showed that each “obligatory” recombination event transfers one copy of *Sry* (plus additional duplicated chromatin) from the distal end of one chromatid of the Y chromosome to one chromatid of the X, as a result of 50% recombination between *Sry* and other X- or Y-linked genes. The recombinant X chromosome gives rise to mice that are chromosomal [XX,Tp(Y)1Ct] females but develop as phenotypic, though sterile, males (19). Thus, the X and Y chromosomes themselves constitute a “chromosomal” polymorphism, while the presence or absence of the distal *Sry* sequence on the X or Y chromosome provides an “exchange” polymorphism. This makes XY^{Sxr} carrier males an ideal test system for studying recombination events by *in situ* hybridization.

Specific probes are available to tag the X and Y chromosomes and the *Sry* gene, so recombinant vs. nonrecombinant X- and Y-bearing cells can be detected not only in cells in metaphases I and II but also in interphase spermatids and sperm. Gametes without an X or Y signal might be chromosomal aneuploids—i.e., lacking sex chromosomes—or they might be merely unlabeled by the probe because of hybridization inefficiencies. Gametes with both an X and Y signal might be either aneuploid or diploid. To distinguish between these alternatives, a control autosomal probe for chromosome 8 was also used.

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Abbreviations: FISH, fluorescence *in situ* hybridization; DAPI, 4',6-diamidino-2-phenylindole.

XY^{Sxr} carrier males also provide an excellent opportunity to follow the production and fate of aneuploid gametes, since X and Y univalency at metaphase I is 60–90% in these animals (18). These sex chromosome univalents can be expected to segregate randomly at anaphase, producing XY and “O” gametes (1:1 segregation) as well as gametes containing a nonrecombinant X or Y (1:1 segregation). However, Evans *et al.* (18) suggest that spermatocytes with X–Y univalents at metaphase I never reach metaphase II.

If univalent spermatocytes do proceed through metaphase II, their products will increase the frequency of aneuploid and nonrecombinant sperm in XY^{Sxr} males over that in normal males. If univalent spermatocytes do not proceed into metaphase II, XY^{Sxr} males and normal males will have similar frequencies of aneuploid (rare) and nonrecombinant (50%) sperm.

MATERIALS AND METHODS

Chromosome Preparations. XY^{Sxr} males were killed by cervical dislocation. Spleen culture and preparation of mitotic metaphase spreads were as described by Boyle *et al.* (20). For meiotic preparations testicular cells were minced, and the testicular suspension containing meiotic cells, spermatozoa, and Sertoli cells was hypotonically treated and fixed (21). No special procedure to “permeabilize” the sperm or to decondense the highly compact sperm nuclei was used.

Probes. Four probes were used in this study: a 4.2-kb chromosome X-specific repeat, designated 68-36 (22); a chromosome Y-specific repeat, pY353 (23); a chromosome 8-specific repeat (24); and a 14-kb single-copy sequence, L7.4.1, that encompasses *Sry*, the putative testes-determining gene (17). The X-specific repeat hybridizes to approximately band XA3 (22); the Y-specific repeat hybridizes along nearly the entire length of the Y chromosome (23); and the chromosome 8-specific repeat hybridizes to band 8A4 (24) (Fig. 1A). A single copy of the *Sry* gene is normally located at the proximal end of the Y chromosome; however, a second copy is present distal to the pseudoautosomal region in XY^{Sxr} carrier males (Fig. 1A). The position of the X- and Y-specific repeats and the *Sry* sequences before and after recombination are diagrammed in Fig. 2.

Probe Labeling and *In Situ* Hybridization. The DNA probes were combinatorially labeled by nick-translation as described by Ried *et al.* (9). The X chromosome repeat was labeled with 45 μ M biotin-11-dUTP, the Y chromosome repeat, with 20 μ M of fluorescein isothiocyanate (FITC)-labeled dUTP; the chromosome 8-repeat, with 20 μ M FITC-dUTP and 25 μ M digoxigenin-11-dUTP; and the *Sry* sequence, with 40 μ M of digoxigenin-11-dUTP. The nick-translation solution contained unlabeled dTTP at 10 μ M for the *Sry* and Y probes and at 5 μ M for the 8 and X probes; unlabeled dATP, dCTP, and dGTP were also included at 40 μ M each.

For *in situ* hybridization, 80 ng of the *Sry*-containing plasmid probe and 40 ng of each of the repeat-sequence clones were precipitated in the presence of 5 μ g of salmon sperm DNA and 5 μ g of yeast tRNA. The probes were resuspended in 10 μ l of hybridization solution [50% formamide/2 \times SSC (1 \times = 0.15 M NaCl/0.015 M sodium citrate)/10% dextran sulfate], denatured at 76°C for 5 min, and immediately applied to slides; which were denatured separately at 80°C for 2 min in 70% formamide/2 \times SSC and dehydrated by passage through an ethanol series (70%, 90%, and 100%, 5 min each).

The biotinylated probe sequences were detected with the infrared fluorochrome Cy5 conjugated to streptavidin (Jackson ImmunoResearch); the digoxigenin-labeled sequences were detected with an anti-digoxigenin IgG Fab fragment conjugated to Cy3 (Jackson ImmunoResearch). The FITC-tagged probes were labeled directly by nick-translation and

did not require any secondary immunological detection step (25, 26). DAPI (4',6-diamidino-2-phenylindole) was used as a counterstain for chromosomes and nuclei.

A cooled CCD (charged coupled device) camera (PM512, Photometrics, Tucson, AZ) coupled to a Macintosh computer was used to visualize the different fluorophores as independent grey-scale images with filter sets optimized for DAPI, fluorescein, rhodamine, and Cy5 fluorophores. By using computer software developed by Timothy Rand in the laboratory of D.C.W., the grey-scale images were pseudocolored and merged. This software, termed GENE JOIN, is available for a fee through the Office of Cooperative Research, Yale University, 246 Church Street, New Haven, CT 06510. Photographs were taken with Kodak 100 HC color-slide films. Details of the image-acquisition and image-processing protocols and of the filter sets used are described elsewhere (9). Analysis for the presence or absence of each probe was performed on the original unmerged grey-scale images.

RESULTS

To unequivocally detect both nondisjunction and recombination in XY^{Sxr} mice, it was necessary to use four probes: an X-specific repeat, a Y-specific repeat, an autosomal chromosome control (a chromosome 8-specific repeat), and the Y-located testis-determining gene *Sry* that recombines with the X chromosome in XY^{Sxr} mice. The location and appearance of each probe on mitotic metaphase chromosomes are depicted in Fig. 1A.

In XY^{Sxr} males, both genetic and cytogenetic evidence suggests that each obligatory crossover between the sex chromosomes transfers a copy of the distal *Sry* from one chromatid of the Y chromosome to one chromatid of the X chromosome. As discussed above, in cells in which this single XY chiasma occurs as expected, there will be equal proportions of sperm containing nonrecombinant X, nonrecombinant Y, recombinant X (carrying *Sry*), and recombinant Y (carrying only the normal testis-determining locus, not the second *Sry* locus) (Fig. 2). If, on the other hand, the X and Y chromosomes fail to undergo the obligatory recombination event, their meiotic products will have equal frequencies of XY and O gametes (without a sex chromosome), and gametes containing a single nonrecombinant X or Y chromosome. By determining the actual frequency of X and Y univalents in metaphase I, we can predict the frequency of sex-aneuploid (XY and O) gametes and calculate the expected increase in frequency of nonrecombinant gametes due to nondisjunction.

The frequency of X and Y univalents in XY^{Sxr} males has been reported to vary between animals from 60% to 90% (18). In the two males analyzed in this study, the frequency of X and Y univalents at metaphase I (scored by conventional light microscopy) was 63% and 65% ($n = 239$ and 266, respectively). Since these univalents resulted from an achiasmatic XY pair, they represent a failure of recombination. If they were to continue through the remaining stages of meiosis, all of their meiotic products would be nonrecombinants, whether they were euploid or aneuploid (XY or O). The 63–65% of univalent metaphase I cells in XY^{Sxr} males would be expected to increase by this percentage the relative number of nonrecombination gametes (over the frequencies expected from normal males). If metaphase I univalents were to continue through meiosis, the predicted gamete frequencies of XY^{Sxr} males in this study would be 16.25% each for XY and O, 25% each for nonrecombinant X and Y, and 8.75% each for recombinant X and Y.

However, genetic and cytogenetic evidence suggests that cells exhibiting XY univalency at metaphase I drop out of the progression and never form meiotic products. If this were the case, XY^{Sxr} males would form equal frequencies of recom-

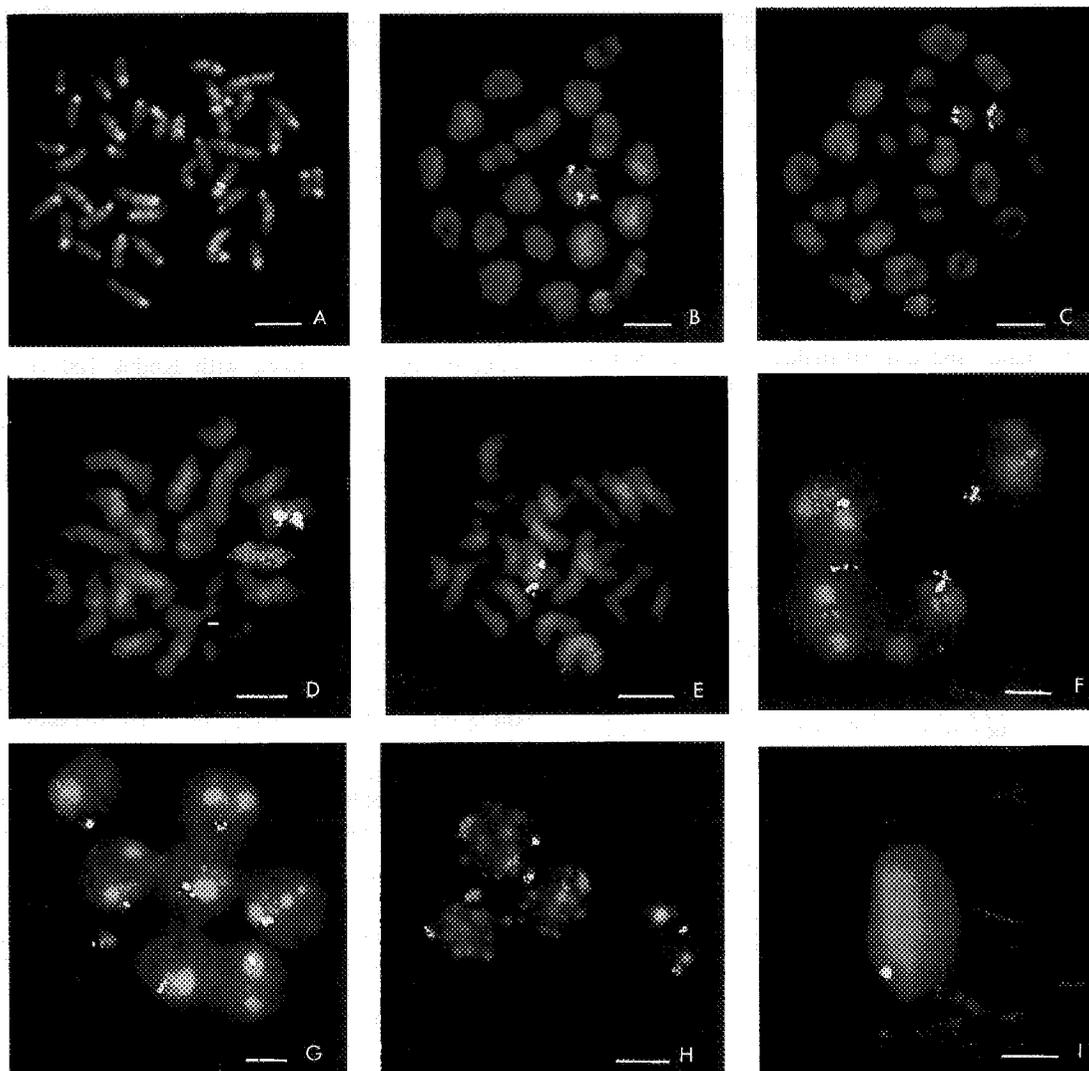


FIG. 1. Multicolor fluorescence *in situ* hybridization (FISH) analysis of mitotic and meiotic cells, spermatids, and sperm. Descriptions of probes and labeling conditions are given in Table 1. The color code for pseudocoloring in all photographs is yellow for chromosome 8 repeat, fuchsia for the X chromosome repeat, green for the Y chromosome repeat, reddish orange for the *Sry* probe, and blue for counterstaining of chromatin. (A) Mitotic metaphase spread. (B) Meiotic metaphase I in which recombination and chiasma formation lead to an XY bivalent. (C) Failure of recombination leads to a meiotic metaphase I with X and Y univalents. (D) Metaphase II cell containing a recombinant Y chromosome (two centromeric *Sry* signals but only one distal signal). (E) A metaphase II cell containing a recombinant X chromosome with a single distally located *Sry* signal. (F) Four spermatids: nonrecombinant Y, upper left; recombinant X, upper right; nonrecombinant X, lower left; and recombinant X, lower right. (G) Seven spermatids and a sperm: nonrecombinant X, upper left; recombinant X, upper right; nonrecombinant Y, middle left; nonrecombinant X, middle center; recombinant Y, middle right; nonrecombinant X, lower left; recombinant Y, lower center; nonrecombinant X, lower right. (H) Four spermatids: two recombinant Y spermatids, lower left and lower right; two XY spermatids, upper middle and center. The nonrecombinant Y spermatids have both a proximal and distal *Sry* signal. (I) An O sperm containing a chromosome 8 repeat signal but not an X or Y signal. (Bars A-I = 5 μ m.)

binant and nonrecombinant X and Y gametes (25% in each class).

Meiotic Metaphase I and II Chromosomes. The autosomal marker Chromosome 8, identified by the yellow hybridization signal of the chromosome 8 repeat, were always present as a bivalent at metaphase I (Fig. 1 B and C). When the "obligatory crossover" occurred in the pseudoautosomal regions (at their distal ends), the X and Y chromosomes were joined distally in a heteromorphic bivalent at metaphase I. Paired *Sry* signals (red), one per sister chromatid, appeared at the proximal end of the Y chromosome (the normal testis-determining locus). The extra rearrangement-derived *Sry* signals documented the exchange event: one was transferred onto the distal end of the recombinant X chromatid, and the other remained in its original location on the distal end of the nonrecombinant Y chromatid (Fig. 1B).

When the X and Y chromosomes failed to recombine, they were seen as separate univalents on the metaphase I plate. The Y bore two pairs of *Sry* signals, one per sister chromatid, on both proximal and distal ends, representing the normal and rearrangement-derived testis determining loci (Fig. 1C). In no metaphase I cell ($n = 7$) in which there was physical separation of the X and Y chromosomes was an *Sry* signal observed on a univalent X. This is consistent with the assumption that univalents are the result of failure of X-Y recombination.

After the reductional division at anaphase I, the haploid set of metaphase II chromosomes are characterized by abysmal morphology, with sister chromatids held together only at their proximal ends in the kinetochore regions. Although the X and Y chromosomes can be identified by C-banding or DAPI staining but generally not by G-banding, FISH labeling with X- and Y-specific repeats greatly facilitates their iden-

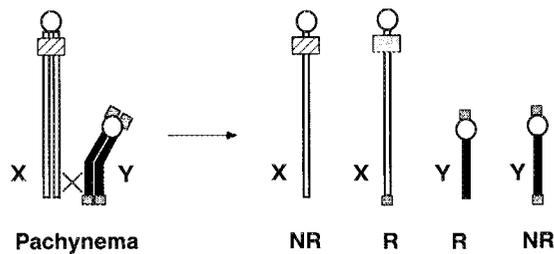


FIG. 2. Diagram of recombination between chromosomes X and Y of XY^{Sxr} males and the resulting chromatids, which give rise to four types of spermatids and sperm. □, X-specific repeat (fuchsia in Fig. 1); ■, Y-specific repeat (green in Fig. 1); ⊞, *Sry* probe (reddish orange in Fig. 1). Chromosomes 8 are not shown. R, recombinant; NR, nonrecombinant.

tification. Each metaphase II cell had either an X or Y chromosome, each with one recombinant and one nonrecombinant sister chromatid (Fig. 1 *D* and *E*). The chromosome 8 repeat probe hybridized to the subcentromeric region on each sister chromatid of the chromosome 8 in every metaphase II cell (Fig. 1 *D* and *E*).

As previously mentioned, most spermatocytes with X and Y univalents at metaphase I are thought to abort before metaphase II (18). In our unlabeled metaphase I sample ($n = 505$), 64% of the cells contained univalents. Had this class of secondary spermatocytes survived until metaphase II, they should have produced four different spermatocyte genotypes: (i) Y carriers with two sets of *Sry* signals on each chromatid, (ii) X carriers with no *Sry* signal on either chromatid, (iii) XY carriers with two sets of *Sry* signals on the Y chromosome and none on the X, and (iv) Os with no X or Y chromosomes. In the small sample of metaphase II cells imaged ($n = 6$), none of these genotypes were observed. Therefore, we conclude that the univalent-bearing spermatocytes did not reach metaphase II.

Spermatids and Sperm. After normal disjunction at anaphase II, the resulting spermatozoa (spermatids and sperm) should each contain a haploid set of autosomes plus either an X or Y chromosome. (The X-specific, Y-specific, and chromosome 8-specific probes give somewhat dispersed signals on spermatid nuclei; on the more compact sperm nuclei, the signals appear more cohesive.)

All haploid spermatids and sperm examined contained a single chromosome 8 repeat signal, indicating a high fidelity of normal disjunction of this autosomal bivalent. The X- and Y-specific repeat probes identified X- vs. Y-bearing spermatids and sperm. Previous genetic and cytogenetic data indicated that the sex pair's single obligatory recombination event always occurs proximal to the rearranged *Sry* sequence in XY^{Sxr} carrier males (18) and involves only two of the four chromatids. Therefore, we expect each recombinant XY bivalent to produce the four types of gametes diagrammed in Fig. 2 with equal frequency.

If spermatocytes with X and Y univalents survive, their products will increase the proportions of nonrecombinant X or Y gametes and aneuploid XY and O gametes in the proportions discussed above. If spermatocytes with X and Y univalents do not survive, all gametes produced will derive from spermatocytes with recombinant XY bivalents. The gamete pool will contain equal frequencies of recombinant X, nonrecombinant X, recombinant Y, and nonrecombinant Y, and the frequencies of sex-aneuploids (XY and O) will be minimal.

The Y probe (represented in green in Fig. 1) "paints" virtually the entire length of the Y chromosome, so the *Sry* signals are easily localized at one (for example, right middle spermatid in Fig. 1*G*) or both ends (for example, left middle spermatid in Fig. 1*G*) of the Y signal even in interphase

spermatids and sperms. In contrast, the X repeat only labels a subcentromeric region near the proximal end of the X chromosome, and the *Sry* sequence resulting from recombination lies quite distal on the X. Since these are interphase nuclei, the intervening X chromatin is not identifiable. However, the interval between the proximal X probe and the *Sry* probe in those cells in which the latter was present appeared to be fairly constant and remained proportional relative to the size of the cell (lower right spermatid in Fig. 1*F*, for example). Many sperm and spermatids contained only an X label, without an *Sry* signal (for example, upper right and lower left in Fig. 1*F* and upper left in Fig. 1*G*); this result identified them as nonrecombinant X gametes.

Data collected separately from two mice (Table 1) show approximately equal numbers of X- and Y-bearing gametes (spermatids and sperm). While the number of recombinant gametes observed was slightly higher than the number of nonrecombinants, the frequency was not significantly different from a 1:1 distribution.

Several nondisjunction gametes were observed—for example, XY gametes (Fig. 1*H*, upper and middle spermatids) and an O sperm (Fig. 1*I*). Since a gamete without an X or Y signal might be nullisomic for the X or Y chromosome or merely inaccessible to the probe or detection solution, the chromosome 8-specific repeat was essential to distinguish between these two alternatives. In all presumptive O sperm, a chromosome 8 repeat signal was clearly visible, marking them as true products of XY nondisjunction. Moreover, in two cases, XY and O gametes were observed in the same field of view, as might be expected if they were the reciprocal products of the same nondisjunction event. All 5 XY gametes contained two *Sry* hybridization signals—one at each end of the painted Y. This was the configuration expected if these gametes arose from failure to recombine (as in Fig. 1*H*).

DISCUSSION

Molecular analysis of sperm cells provides an excellent means of addressing a myriad of questions regarding frequency of aneuploidy and recombination during meiosis. By scoring tens of thousands of sperm labeled with one or two chromosome-specific markers, aneuploidy frequency in sperm can be compared with paternally derived aneuploidy in abortuses or live births. However, scoring recombination frequency in sperm requires a minimum of four differently labeled markers and multicolor fluorochrome detection technology that is beyond the capacity of most laboratories. Current limitations in imaging software also prohibit the use of this procedure for scoring myriads of cells. Nevertheless, we have demonstrated that analysis of spermatids and sperm can be readily achieved by using sample sizes as large as or larger than those that can be scored practically from metaphase II cells and provide equivalent information.

The high incidence of X-Y separation at metaphase I (60–90%) previously reported for XY^{Sxr} carrier males (18) was again observed in the current study. X-Y univalency might be due to failure of the sex chromosomes to synapse or recombine at meiotic prophase, or, as suggested by Lyon *et al.* (27), it might be the result of premature bivalent separation

Table 1. Types and frequencies of gametes

	X		Y		XY	"O"
	NR	R	R	NR		
Mouse 1*	34	36	39	38	4	2
Mouse 2†	13	21	14	16	1	1

NR, nonrecombinant; R, recombinant.

* $\chi^2 = 0.395$, $P = 0.80-95$.

† $\chi^2 = 2.37$, $P = 0.5$.

and precocious disjunction. The current study does not support the hypothesis of precocious disjunction of recombinant chromosomes because our data show that X-Y univalents at metaphase I are nonrecombinant chromosomes (no evidence of transfer of the distal *Sry* sequence from one chromatid of the Y chromosome to one chromatid of the X).

Evans *et al.* (18) also reported seeing only recombinant X and Y chromosomes (sample size unreported) at metaphase II when the distal transposed sex-reversal fragment was detected by G-banding procedures, a technically difficult task. They concluded that the missing nonrecombinant metaphase II class could be explained if cells with X-Y univalents at metaphase I aborted before metaphase II. If this hypothesis is correct, the frequency of recombinant and nonrecombinant X and Y gametes should be equal. However, if more cells without X-Y exchange events survive than heretofore suspected, there should have been a large population (16.25% each in the current study) of XY and O gametes, and the frequency of nonrecombinant gametes (both X and Y) should have exceeded the recombinant types by this same frequency. Neither of these two predicted possibilities was observed. The frequency of XY gametes was only 2% and the frequency of O gametes was even less (1.4%). Moreover, recombinant X and Y gametes actually slightly exceeded X and Y nonrecombinant gametes. Therefore, our gamete data support the conclusion of Evans *et al.* (18) that in XY^{Sxr} mice, cells with X-Y separation at metaphase I generally do not survive until metaphase II. This conclusion is also consistent with several reports summarized by Beechey (28) of other male mice with 100% X-Y univalents at metaphase I and an absence of metaphase II and later-stage germ cells.

X-Y univalents might be due either to lack of synapsis between the X and Y chromosomes at pachynema of meiotic prophase or to failure to recombine. Since Chandley and Speed (29) found only a slight increase in the frequency of X-Y separation at metaphase I over frequency of X-Y asynapsis at pachynema in a meiotic study of four XY^{Sxr} carrier males, synaptic failure seems the more likely explanation. The observed X-Y asynapsis in XY^{Sxr} males might offer a clue to both the high univalency attrition rate and the fact that apparently a small percentage of X-Y univalents do survive to produce both XY and O gametes. Miklos (30) pointed out a direct correlation between pairing failure and gametic loss in males, an observation that has been confirmed in many subsequent studies (31, 32). In an electron microscopic study of meiotic prophase in XY^{Sxr} carrier males, Chandley and Speed (29) observed a high frequency of unpaired sex chromosomes, which under Miklos' hypothesis (30) could account for the high attrition rate. However, they also observed "fold-back" pairing of the Y chromosome and, in a smaller number of cells, of the X chromosome. Speed has suggested (31), fold-back pairing might "rescue" meocytes from death normally associated with synaptic failure. Therefore, the surviving nondisjunction products we observed might be those in which fold-back pairing "rescued" X-Y univalents. Since Chandley and Speed observed more fold-backs in Y chromosomes than in X chromosomes, this might also account for the slightly higher frequency we observed of nonrecombinant Y gametes than X gametes.

In XY^{Sxr} in which the *Sry* rearrangement allows detection of recombination, we have shown that multicolor labeling techniques can provide as much information from interphase spermatids and sperm as can be derived from the most favorable G-banded metaphase II cells, which represent a much smaller percentage of the population of spermatocytes and gametes.

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