Direct Evidence for Suppression of Recombination within Two Pericentric Inversions in Humans: A New Sperm-FISH Technique

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Summary

Crossover within a pericentric inversion produces reciprocal recombinant chromosomes that are duplicated/deficient for all chromatin distal to the breakpoints. In view of this fact, a new technique is presented for estimating the frequency of recombination within pericentric inversions. YAC probes were selected from within the q- and p-arm flanking regions of two human inversions, and two-color FISH analysis was performed on sperm from heterozygous inversion carriers. A total of 6,006 sperm were analyzed for chromosome 1 inversion (p31q12), and 3,168 were analyzed for chromosome 8 inversion (p23q22). Both inversions displayed suppression of crossing-over, although the amount of suppression differed between the two inversions. The recombination frequency of 13.1% recorded for chromosome 8 inversion was similar to the frequency of 11.4% previously estimated by the human/hamster–fusion method. For chromosome 1 inversion, the recombination frequency of 0.4% reported here was below the limits of detection of the fusion technique. The simplicity of the FISH technique and the ease of scoring facilitate analysis of a sample-population size much larger than previously had been possible.

Introduction

Chromosomal inversions are the result of two breaks within a single chromosome and a 180° reorientation of the chromatin between the breaks. Inversions with both breaks in one arm are called "paracentric inversions," whereas those with breaks on either side of the centromere are called "pericentric inversions." Within the human population, the frequency of pericentric inversions is 1%–2% (de la Chapelle et al. 1974; Kaiser 1984).

The cytogenetic consequences of pericentric inversions are often severe. Although heterozygous carriers usually exhibit a normal phenotype, the inverted chromosome region poses synaptic and recombinational problems during meiosis. If the inverted and noninverted chromosome segments synapse homologously by forming an inversion loop during meiotic prophase, and if an odd number of crossovers occur within the loop, the result is two chromosomes that are duplicated/deleted for the regions outside the inversion (fig. 1*A–C*). Thus, one of the recombinant chromosomes is duplicated for the flanking p-arm chromatin and is deficient for the flanking q-arm chromatin, and the reciprocal recombinant chromosome is duplicated for the flanking q arm and is deficient for the flanking p arm. Fertilization of the duplication and deficiency gametes results in an embryo that is trisomic/monosomic for the chromosome regions outside the inversion. Numerous studies have shown that mammals tolerate partial trisomy better than partial monosomy (Winsor et al. 1978; Dutrillaux et al. 1980; Daniel 1981; Kaiser 1984). Consequently, the recombinant chromosome that is both duplicated for the larger segment and deficient for the smaller segment is more likely to survive to term than is the reciprocal combination. Duplication/deficiency individuals who survive often have severely debilitating syndromes that are characteristic for the specific inversion. These aberrations may involve developmental delay, mental retardation, abnormal development of certain organ systems, and, often, early death.

Prevention of birth defects arising from pericentric inversions depends on identifying inversion carriers and providing appropriate genetic counseling. Assessment of the risk to carriers depends on knowledge of the recombination frequency within an inversion. To date, there have been two methods of estimating the frequency of recombinants: (1) direct counts of newborns with a characteristic associated syndrome (Smith et al. 1987) and (2) karyotype analysis of duplicated/deficient chromosomes at the first mitotic division in hamster oocytes

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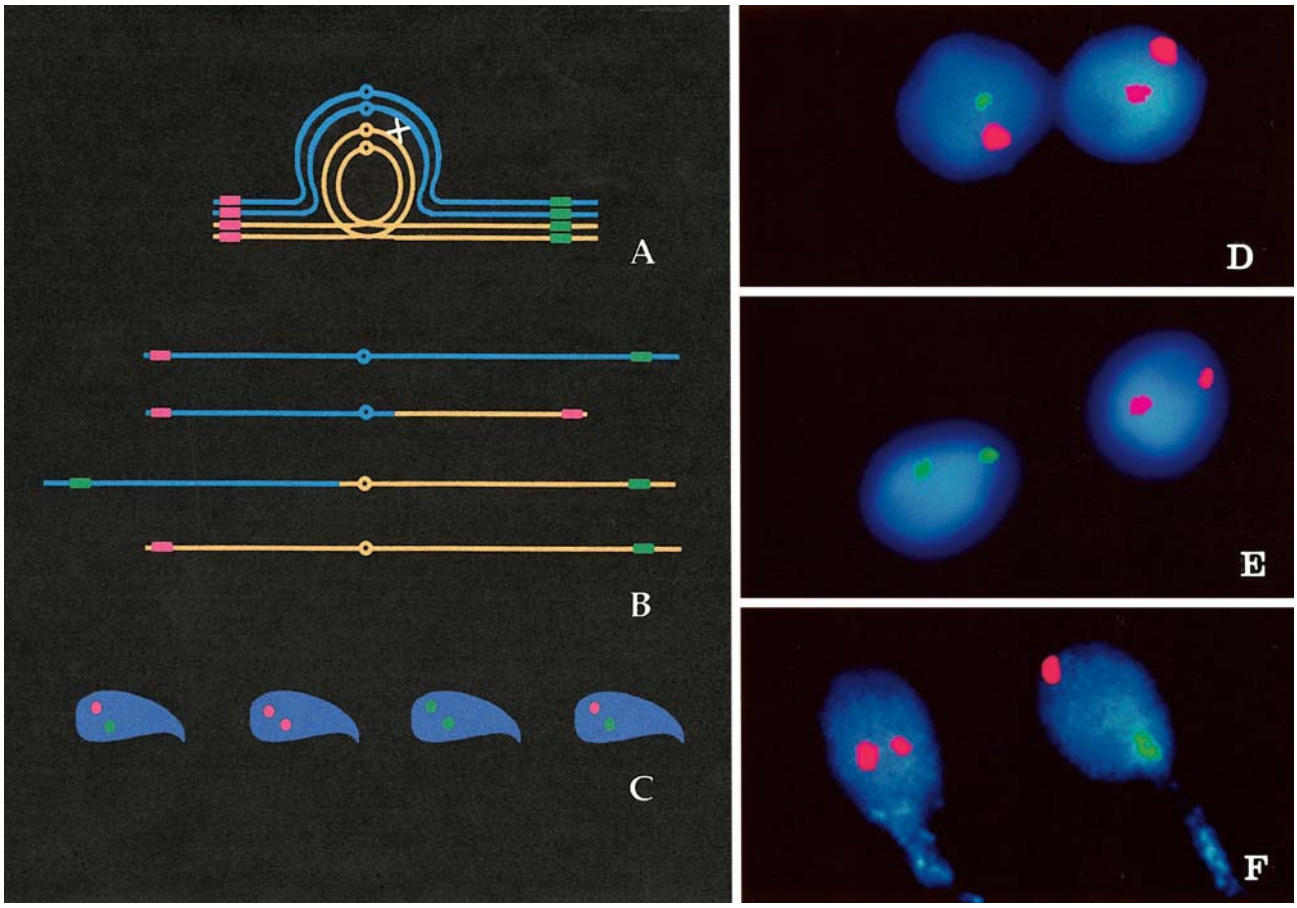

Figure 1 Recombination within a pericentric inversion. *A–C,* Diagram of a pericentric inversion and the consequences of a crossover within the inverted segment; for details, see text. *A,* Inversion loop with a crossover. *B,* Recombinant and nonrecombinant chromosomes. *C,* Nonrecombinant (*green-red*) and recombinant (*two red or two green*) sperm, by two-color FISH. *D-F,* Images of two-color sperm FISH. Analysis of a chromosome 8–inversion carrier, using YAC probes specific for the p- and q-arm regions outside the inverted segment, are shown. The pseudocolor code is green for the flanking p-arm region and is red for the flanking q-arm segment. *D,* One normal sperm (*red-green*) and one dup(q)/del(p) sperm (*two red*). *E,* One dup(p)/del(q) sperm (*two green*) and one dup(q)/del(p) sperm (*two red*). F, One normal sperm (*red-green*) and one dup(q)/del(p) sperm (*two red*).

that have been fertilized in vitro with human sperm from an inversion carrier (Balkan et al. 1983; Martin 1991, 1993; Jenderny et al. 1992; Martin et al. 1994). The former method is limited by the number of recombinant individuals who have been identified, and it can provide little or no information about rare or de novo inversions; the latter method is restricted to the small number of sperm nuclei that can be scored per experiment.

In this study we used two-color FISH of sperm to score recombination frequency within two human pericentric inversions. We differentially labeled p- and q-arm DNA markers (YACs) outside the inverted chromosome region and scored the frequency of normal versus recombinant sperm, on the basis of different FISH color combinations. To test the accuracy of our new method, we analyzed two different human pericentric inversions that previously had been studied with the human-sperm/hamster-oocyte–fusion method (Martin 1991, 1993; Martin et al. 1994): chromosome 1 inversion (p31q12) and chromosome 8 inversion (p23q22). The frequencies observed with the FISH and the fusion method were comparable. The main advantage of the two-color FISH approach is the rapidity of scoring and the larger sample sizes that are more likely to allow detection of rare events and to provide a statistically more significant analysis.

Subjects, Material, and Methods

Controls and Inversion Carriers

Sperm from two healthy, normal men were used as controls. Sperm were also obtained from a male heter-

ozygous for a pericentric chromosome 1 inversion, inv(1)(p31q12), and from a male heterozygous for a pericentric chromosome 8 inversion, inv(8)(p23q22). The breakpoints of each inversion are illustrated in figure 2. These were the same individuals whose sperm were used for previous hamster-oocyte/human-sperm analysis, and their karyotypes and family histories have been described elsewhere (Martin 1993; Martin et al. 1994).

Preparation of Sperm

Sperm samples were processed according to the method of Martin and Ko (1994) and Martin et al. (1991). Preparations for both control samples and chromosome 8–inversion samples were made from fresh sperm; those for chromosome 1–inversion samples were from both fresh and frozen samples. Slides were stored at −20°C until decondensation and hybridization and were generally used $\leqslant 5$ wk after being collected.

Decondensation of sperm was as described by Williams et al. (1993) and was performed immediately before hybridization. The slides were first fixed in 3:1 methanol: acetic acid for 10 min, dehydrated by passage through a cold ethanol series, and then swelled with 10 mM DTT for 30 min and with 10 mM lithium diiodosalicylate (LIS) for 3–4 h. The decondensation step renders the sperm chromatin accessible to probe DNA and is critical for the performance of sperm FISH—especially when YACs are used as probes. Therefore, the swelling of sperm was monitored under a phase-contrast microscope every 15 min during the last hour of LIS treatment. To obtain maximal hybridization efficiency and signal intensity of FISH, the sperm were treated with LIS until a small population of sperm heads started to burst.

Probes and FISH Signal Optimization

Human YACs from the CEPH libraries were selected on the basis of their cytogenetic map position (Bray-Ward et al. 1996). The p-arm probes were labeled by nick translation with biotin-11-dUTP, and the q-arm probes were labeled with digoxigenin-11-dUTP. Initially, six YAC probes for each inversion were screened in two steps. Each of the 12 YACs was tested with FISH on mitotic metaphase chromosomes to verify nonchimerism, and then on sperm from normal men to select YACs that produced the strongest and most-concentrated hybridization signals. YACs selected for each inversion were tested on a minimum of three control slides with sperm from normal men (~5,000 sperm per slide). For the chromosome 8 inversion, YAC 930a2 (containing the sequence-tagged site [STS] marker D8S262) was selected as the probe for the flanking p arm, and 935a12 (D8S508) was selected as the probe for the flanking q arm (fig. 2*B*). For chromosome 1 inversion, the two YAC probes 750g5 (D1S241) and 907g1 (D1S441) were pooled to provide a sufficiently strong signal for the flanking p-arm segment, and 958e1 (D1S237) was used for the q arm (fig. 2*A*).

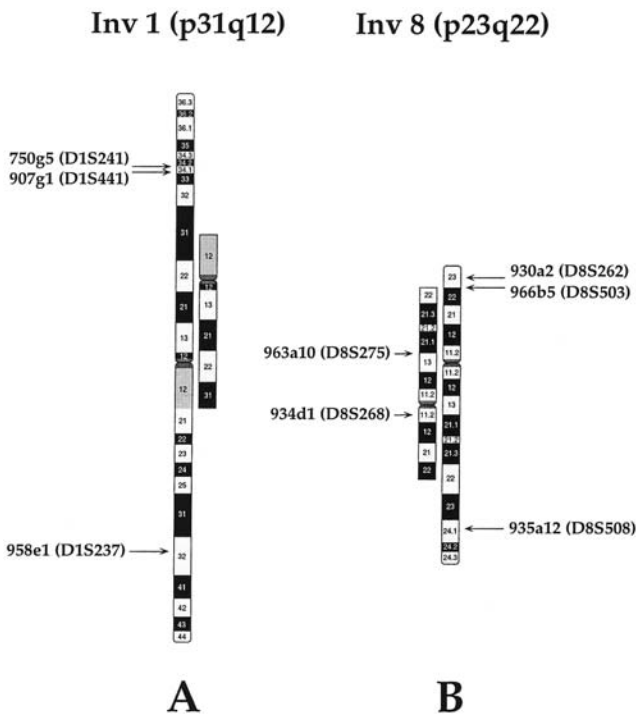

Figure 2 Schematic illustration of G-banded chromosomes in humans, depicting inversion breakpoints and presumed pachytene alignment of homologues. The localizations of YACs used as probes in the sperm FISH are indicated by the longer arrows. Also, YACs used for estimating the male recombination frequency within the chromosome 8–inversion region expected to pair homologously are indicated by the shorter arrows. *A,* Chromosome 1 inversion (p31q12). *B,* Chromosome 8 inversion (p23q22). YAC data are from Murray et al. (1994) and Bray-Ward et al. (1996).

Sperm FISH is widely used to determine frequencies of aneuploidy for specific chromosomes (Williams et al. 1993; Spriggs et al. 1995, 1996). However, the probes used in aneuploidy studies, probes that mainly are different satellite sequences, are large and thus yield strong FISH signals. Since the YAC probes are smaller than the probes generally used for sperm FISH, the decondensation and denaturation steps were especially crucial for a good FISH signal. Also, the ratio of YAC and Cot-1 DNA had to be optimized. Although increasing the amount of probe DNA resulted in a better signal, it also increased the background signal, since the YAC probes contain many repetitive sequences. Although the Cot-1 DNA reduced background, in too-high concentration it also reduced the probe signal.

FISH-Sperm Protocol

The differentially labeled probes for the p- and q-arm flanking chromatin were hybridized simultaneously. The

sperm FISH protocol of Williams et al. (1993), was used with minor modifications. The sperm DNA was dehydrated through an ethanol series, denatured at 70°C (plus 1°C for each slide processed) for 11–12 min in 70% formamide/2 × SSC, was dehydrated again through a cold-ethanol series, and then was air-dried. For each slide, 250–500 ng of DNA of each YAC probe, 8 μg of salmon sperm, and 3 μg of human Cot-1 DNA were dissolved in 6 μl of deionized formamide and were incubated at 37°C for ⩾30 min, after which 6 μl of hybridization buffer (20% dextran sulfate/4 × SSC) was added. The probe mix was denatured at 75°C for 8 min and was reannealed at 37°C for 30–60 min. The hybridization mixture was placed on the slide, in a region with a high concentration of sperm; an 18 × 18-mm coverslip was added, and the slides were incubated in a moist chamber at 37°C overnight.

After hybridization, slides were washed three times for 7 min each in 50% formamide/2 × SSC (pH 7.0) at 42°C, once for 7 min in 2 × SSC (pH 7.0), and briefly in PN buffer (0.1 M $NaH_2PO_4 \cdot H_2O$, 0.1 M Na_2HPO_4, and 0.05% Nonidet P-40, pH 8-10) at room temperature. Blocking was performed in 1 ml of PN buffer at 37°C for 20 min. The probe sequences were detected simultaneously with 5 μg avidin-FITC/ml in PNM buffer (PN buffer, 5% nonfat dry milk, and 0.02% sodium azide) and 2 μg anti-digoxigenin rhodamine/ml at 37°C for 45 min. After detection, slides were washed three times for 5 min each in PN buffer and were counterstained with 4,6-diaminino-2-phenylindole (DAPI), and antifade solution was applied.

Scoring and Analysis

The preparations were examined under a Zeiss Axioskop epifluorescence microscope equipped with filter sets optimized for DAPI, FITC, and rhodamine. The combined background signals from the FITC and rhodamine made scoring with a dual-bandpass filter difficult. Therefore, scoring was performed by shifting between the three individual filters. Sperm with one green (FITC) and one red (rhodamine) signal were classified as nonrecombinant, whereas sperm with *either* two green signals *or* two red signals were scored as recombinant. Sperm with two red signals *and* two green signals were classified as disomic/diploid. Partial nulls were used to estimate the hybridization efficiency but were not included in the data calculations. Areas where the hybridization efficiency was <98% or where large clumps of overlapping sperm occurred were not used for counting. For chromosome 1 inversion, one hybridization was used for scoring; for chromosome 8 inversion, two hybridizations were used. Representative gray-scale images were obtained by use of a cooled CCD camera coupled to a Macintosh computer, and individual images of each fluorophor were merged and pseudocolored by use of the GENE JOIN program (Office of Cooperative Research, Yale University).

Results

Our two-color FISH approach was designed to generate one green (FITC) and one red (rhodamine) signal in nonrecombinant sperm and to generate two green (no red) or two red (no green) signals in recombinant sperm. Examples of the different sperm types are depicted in figure 1*D–F*. Sperm with two green signals resulted from recombinants that produced both a duplication of flanking p-arm chromatin and a deletion of flanking q-arm chromatin, dup(p)/del(q), whereas the two-red–signal sperm resulted from recombinants that generated both a deletion of flanking p-arm chromatin and duplication of flanking q-arm chromatin, del(p)/dup(q) (fig. 1*A–C*). For a sperm to be considered recombinant, the two "same-color" signals had to be (*a*) separated from each other by at least one signal domain, (*b*) approximately equal in intensity, and (*c*) clearly positioned within an intact sperm head. FISH on sperm from chromosomally normal individuals yielded no recombinant-signal combinations.

A total of 6,006 sperm were analyzed for chromosome 1 inversion (p31q12), and 3,168 sperm were analyzed for chromosome 8 inversion (p23q22). The results are presented in table 1. For chromosome 8 inversion, the frequency of recombinants was estimated to be 13.1%. The frequency of the two types of recombinants, dup(p)/del(q) and del(p)/dup(q), was not statistically different from the expected ratio of 1:1 ($\chi^2 = 0.96$, $P > .30$). The frequency of recombinants for chromosome 1 inversion was estimated to be 0.38%. The frequency of the two

Table 1

Total Number and Frequency of Recombinant Sperm in Men Heterozygous for Pericentric Inversions in Chromosomes 1 and 8

Study and Segregation Type	No. (% of Total) of Sperm with Inversion	
	1 (p31q12)	8 (p23q22)
Present study:		
Nonrecombinant	5,966 (99.33)	2,734 (86.30)
Diploid/disomic	17 (.28)	18 (.57)
Recombinant:		
dup(p)/del(q)	15 (.25)	198 (6.25)
del(p)/dup(q)	8 (.13)	218 (6.88)
Subtotal	23 (.38)	416 (13.10)
Total	6,006	3,168
Martin (1993), Martin et al. (1994):		
Recombinant	0	18 (11.4)
Total	157	158

Note.—Aberrant sperm were not included in the data calculations; the number of aberrant sperm was 24 and 18 for chromosome 1 inversion and chromosome 8 inversion, respectively.

types of recombinants for chromosome 1 inversion did not differ significantly from a 1:1 ratio ($\chi^2 = 2.13$, $P > .20$) but was uneven (table 1). Since it is unlikely that there is genetic selection against a specific recombinant at the sperm stage, it is more probable that the slightly higher frequency of dup(p)/del(q) recombinants represents a small methodological bias due to our use of two probes for the 1p arm.

The frequency of diploid/disomic sperm was noted to be 0.28% and 0.57% for chromosome 1 inversion and chromosome 8 inversion, respectively, which is within a normal frequency range of diploidy/disomy (Spriggs et al. 1996). In addition, a few aberrant sperm (e.g., those with three FITC signals and one rhodamine signal) were detected, but, because of their rarity, they were not included in the data calculations (see the footnote to table 1).

Discussion

We have developed a new, rapid technique for estimating the recombination frequency within pericentric inversions. Except for family studies, the sole method available until now has been the human-sperm/hamster-oocyte–fusion assay, originally developed by Rudak et al. (1978). The fusion technique is difficult, time-consuming, and labor intensive, requiring karyotype analysis by a trained cytotechnician (Spriggs et al. 1996). To date, a total of five different pericentric inversions have been analyzed with the fusion method, yielding a total sample size of <600 (Balkan et al. 1983; Jenderny et al. 1992; Martin 1991, 1993; Martin et al. 1994). Although the FISH method is both faster and easier to perform, the most significant improvement is the larger sample size obtainable through rapid screening of thousands of sperm. Specifically, a preparation with a good hybridization efficiency and a well-spread population of sperm can yield >5,000 scorable sperm within an 18 × 18-mm hybridization area, and 500–600 sperm/h can be assayed by a relatively untrained individual.

We used two probes for the flanking p-arm segment for chromosome 1 inversion, to provide a sufficiently strong signal. Although the two YAC probes 750g5 and 907g1 are located only 3 cM apart and displayed only one FISH signal in initial tests on normal sperm, the chromosome 1–inversion samples included a few sperm with two distinct signals for the 1p probes plus a single 1q probe signal. These data suggest that caution must be taken when more than one YAC probe per arm is used, to avoid the scoring of "false" recombinants. A recombination event within an inversion should result in a duplication of the flanking p-arm region and should generate a recombinant sperm with two FITC signals. However, theoretically, two FITC signals could be seen in a nonrecombinant sperm if the two 1p probes produced separate signals and if the q-arm probe did not hybridize . Therefore, if two probes are used to produce a strong FISH signal, then tightly linked YACs should be selected, to avoid split signals. A high hybridization efficiency is also essential, as is avoidance of overdenaturing of the sperm DNA. Overdenaturation will generate split signals even when only one YAC is used. Finally, stringent scoring criteria must be applied (Martin and Rademaker 1995).

The absolute error is mainly dependent on the factors listed above and may, as in our study, actually be very small and insignificant. However, the relative error increases with decreasing recombination frequency. In our study, if it is assumed that the q-arm duplication that produced no split signals can be more accurately scored, the number of recombinants with del(p)/dup(q) reflects the "true" frequency of recombinants for chromosome 1 inversion and yields an estimate of 0.26%, compared with the 0.38% noted above (see table 1); thus, the absolute error is $\leqslant 0.1\%$.

Comparison with Previously Published Recombination Frequencies

The inversion carriers used in this study were chosen because sperm from these same individuals had been used in previous studies that employed the fusion technique. The close correlation between our results and the previously reported recombination frequencies for chromosome 1 inversion (p31q12) and chromosome 8 inversion (p23q22) indicates that the sperm-FISH method does generate reliable data. In fact, as suggested by the chromosome 1–inversion results, the much larger sample size possible with sperm FISH can be expected to generate more-accurate estimates of rare events than are obtainable by the fusion method. Martin et al. (1994) surveyed 157 sperm by the fusion method and found no recombinants for chromosome 1 inversion (p31q12). In comparison, with a sample size of 6,006 cells, we were able to detect a recombination frequency of ~0.4% in the same individual. Thus, the large sample size of the sperm-FISH approach allows identification of very rare recombination events undetectable in the smaller population scorable by the fusion method.

Chromosome 8 inversion (p23q22) constitutes one of the most common pericentric inversions in the human population. Besides cases reported in Canada (Aveling et al. 1977; Martin 1993; present study) and the southwestern United States, which are described below (Smith et al. 1987), the inversion has been reported in Austria (Andrle et al. 1987) and Argentina (Lovell et al. 1982). For the chromosome 8 inversion (p23q22), our estimate of 13.1% recombination within the inverted segment is close to the estimate of 11.4% obtained by the fusion assay (Martin 1993). However, in the southwestern

United States, chromosome 8 inversion (p23q22) has been documented in 393 heterozygous carriers with a common Hispanic origin. The frequency of recombinant 8 offspring from carrier parents has been estimated to be 6.2% (Smith et al. 1987). Since all the progeny are of one recombinant type (duplication of the flanking q-arm chromatin and deficiency for the flanking p-arm segment), because of the early lethality of embryos with the reciprocal type of recombinant chromosome, the actual frequency of recombination can be expected to be approximately twice that reported, or 12.4%. Although this estimate is from a larger population sample than is used by the fusion assay, and although it more closely approximates our sperm-FISH estimate, it is sex averaged. In fact, Smith et al. (1987) found that the transmission rate of the chromosome 8 inversion was slightly higher for female carriers (6.6%) than for male carriers (5.9%). When this adjustment is made, the expected recombination frequency for males is 11.8%. Interviews with the Canadian family provided no traceable relationship to the southwestern United States Hispanic population, suggesting that the Canadian case represents a separate origin of the inversion. However, both the similarity of reported breakpoints and the recombination frequency in male carriers confirm that these two apparently independent inversions spanning the 8p23q22 region are virtually identical.

The R-Band/G-Band Model and Suppression of Crossing-Over

On the basis of the cytogenetic breakpoints and assigned cytogenetic positions of the STS-linked YACs closest to the breakpoints (Bray-Ward et al. 1996), the expected recombination frequency in males is 100% for the chromosome 1 inverted interval and 63%–70% for the chromosome 8 inverted interval (Murray et al. 1994). As noted above, recombination within the inversion is almost totally suppressed in the chromosome 1 heterozygote and is reduced to 13% in the chromosome 8 heterozygote.

Ashley (1988) has suggested that it is possible to predict both the synaptic behavior and the recombinational behavior of a chromosome aberration, on the basis of its breakpoints relative to the banding pattern of mitotic metaphase chromosomes. Specifically, if both breakpoints lie in G-negative (R-) bands, synapsis will be confined to homology, and the recombination frequency will approach normal. However, if one break lies in a G-positive (G-) band, there will be nonhomologous synapsis and suppression of recombination. Moreover, once nonhomologous synapsis has started by misalignment of G- and R-band chromatin, it will continue by cascading from one homologue to the other, as long as this type of misalignment continues Ashley (1990). An examination of the G-band chromosome diagrams of both inversions in the current study suggests that G-band/R-band misalignment and nonhomologous synapsis has occurred. In the chromosome 1 inversion, G-band/R-band misalignment continues throughout the length of the inverted segment (fig. 2*A*), consistent with the observed near-total suppression of crossing-over. In the chromosome 8 inversion, G-bands are misaligned at the breakpoints, suggesting that nonhomologous synapsis is initiated (fig. 2*B*) but that R-band chromatin in band p11.2 will encounter R-band chromatin in q13. The above-mentioned hypothesis predicts that this R-band/R-band misalignment will halt nonhomologous synapsis and lead to formation of a smaller loop—one equivalent to that expected if the breaks had occurred in bands p11.2 and q13. When STS-linked YACs in bands p11.2 and q13 are used to calculate the sex-biased recombination frequency of the predicted "effective" loop, it is found to be 11.2% (fig. 2*B*) (Murray et al. 1994), amazingly close to the 13% found in the present study and almost exactly twice the frequency of dup(q) offspring of chromosome 8–inversion fathers in the Hispanic population (see Smith et al. 1987).

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