

Meiotic Recombination and Spatial Proximity in the Etiology of the Recurrent t(11;22)

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Although balanced translocations are among the most common human chromosomal aberrations, the constitutional t(11;22)(q23;q11) is the only known recurrent non-Robertsonian translocation. Evidence indicates that de novo formation of the t(11;22) occurs during meiosis. To test the hypothesis that spatial proximity of chromosomes 11 and 22 in meiotic prophase oocytes and spermatocytes plays a role in the rearrangement, the positions of the 11q23 and 22q11 translocation breakpoints were examined. Fluorescence in situ hybridization with use of DNA probes for these sites demonstrates that 11q23 is closer to 22q11 in meiosis than to a control at 6q26. Although chromosome 21p11, another control, often lies as close to 11q23 as does 22q11 during meiosis, chromosome 21 rarely rearranges with 11q23, and the DNA sequence of chromosome 21 appears to be less susceptible than 22q11 to double-strand breaks (DSBs). It has been suggested that the rearrangement recurs as a result of the palindromic AT-rich repeats at both 11q23 and 22q11, which extrude hairpin structures that are susceptible to DSBs. To determine whether the DSBs at these sites coincide with normal hotspots of meiotic recombination, immunocytochemical mapping of MLH1, a protein involved in crossing over, was employed. The results indicate that the translocation breakpoints do not coincide with recombination hotspots and therefore are unlikely to be the result of meiotic programmed DSBs, although MRE11 is likely to be involved. Previous analysis indicated that the DSBs appear to be repaired by a mechanism similar to nonhomologous end joining (NHEJ), although NHEJ is normally suppressed during meiosis. Taken together, these studies support the hypothesis that physical proximity between 11q23 and 22q11—but not typical meiotic recombinational activity in meiotic prophase—plays an important role in the generation of the constitutional t(11;22) rearrangement.

The t(11;22)(q23;q11) is the only known recurrent, non-Robertsonian constitutional translocation in humans.^{1,2} Factors that predispose to recurrence of this rearrangement have been difficult to identify. In general, 11;22-translocation carriers are phenotypically normal and are often identified only after the birth of abnormal offspring with an unbalanced form of the translocation. The supernumerary-der(22)t(11;22) syndrome (Emanuel syndrome [MIM #609029]) occurs as the result of 3:1 meiotic mal-segregation of der(22).³ Individuals with Emanuel syndrome have a karyotype of 47,XX or XY,+der(22)t(11;22), and they present at birth with multiple congenital anomalies.^{1,4} The 11;22 translocation is rare, and its exact prevalence has not been determined. Clinical data indicate that there is a paucity of de novo translocation carriers in the population, and only a limited number of such occurrences have been identified in previous studies.^{5,6} Only one example was amenable to parent-of-origin determinations, and it was paternally derived. Translocation-specific der(11) and der(22) PCR products have been observed in DNA from sperm but not in somatic tissues isolated from karyotypically normal individuals, indicating that de

novo translocations occur during male meiosis with a measurable frequency.^{7,8} Although the translocation has been identified in the sperm of normal males, it is not known whether it arises in female meiosis as well.

The genomic configuration of the breakpoint regions is likely to contribute to their propensity to rearrange. The 11q23- and 22q11-translocation breakpoint regions contain palindromic AT-rich repeats (PATRRs)^{9,10} consisting of a long AT-rich DNA sequence followed by its inverse complement. Palindromic sequences have the ability to self-pair, forming intrastrand hairpin or cruciform structures,¹¹ and cloned DNA sequences derived from the 11q23 palindromic breakpoint form such structures in vitro.^{8,12} Further, polymorphisms of the PATRR11 sequence at the breakpoint alter the frequency of de novo translocations in gametes of normal males.⁸

Palindromic sequences represent a source of genetic instability in the genomes of many organisms through their potential to adopt these secondary structures, which can perturb a variety of biological processes. Hairpin structures can halt the progress of the replication fork and are also known to be intermediates in specialized mammalian re-

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combination reactions such as V(D)J recombination.¹³ In mice, rearrangements of a palindromic transgene are consistent with a center-break mechanism where double-strand breaks (DSBs), created by hairpin nicking of an extruded cruciform, are imperfectly rejoined, creating microdeletions of a few nucleotides.¹⁴ In *Escherichia coli*, long palindromes are highly unstable and cannot be directly PCR amplified or sequenced because of their propensity to form intrastrand hairpins. The *E. coli* SbcCD-enzyme complex cuts near the tip of the hairpin, allowing homologous recombination to restore DNA replication.¹⁵ Thus, it has been suggested that cruciform extrusions of the palindromes at the 11q23 and 22q11 breakpoints might be similarly sensitive to enzymes that induce meiotic DSBs.^{7,9} In fact, when the 11;22 translocation forms, it sustains small symmetric central deletions at the site of the breakpoints, which supports the hypothesis that hairpin nicking of an extruded cruciform is a likely prelude to the repair that generates the translocation.^{7,9}

Meiosis must provide the enzymatic machinery for translocation initiation and resolution, since the t(11;22) does not occur in somatic cells. Programmed DSBs, followed by DNA repair and recombination, are required for proper segregation of homologous chromosomes during meiosis. A break at the chromosome 11 or 22 PATRR hairpin extrusion might represent either (1) one of these programmed meiotic DSBs or (2) a break arising solely from the secondary structure of the palindrome. If the 11;22-translocation breakpoints are located at programmed DSBs, they should coincide with cytologically mapped hotspots of recombination. If they are rare palindrome-associated events and not programmed DSBs, the translocation breakpoints would be less likely to correspond to recombination hotspots. Whereas genetic analysis of chromosome 22 has shown that a peak of meiotic recombination coincides with the breakpoint region at 22q11,¹⁶ cytological examination of sites of crossing over has shown that distal events are twice as frequent as pericentromeric events in the vicinity of the breakpoint in males.^{17,18} In contrast, chromosome 11 has not yet been the subject of detailed cytological analysis, although this type of study has been performed for numerous other human chromosomes.^{19,20} Thus, the role of normal meiotic recombination in the genesis of the 11;22 translocation is unknown.

Programmed DSBs occur throughout the genome during meiotic prophase. This prompted us to seek a physical explanation for why the t(11;22) constitutional translocation recurs, whereas other balanced translocations, which occur in ~0.1% of newborns,²¹ do not. There is evidence that individual chromosomes occupy compartmentalized domains and unique nuclear positions in the interphase nucleus, rather than being distributed in a random fashion.^{22–25} Studies of somatic cells have demonstrated that regions that preferentially rearrange with one another to produce nonrandom, tumor-associated rearrangements are more likely to be in closer proximity to one another in the interphase nucleus.^{26,27} Although analysis of sperm

demonstrates the prevalence of chromosome 11 and 22 rearrangement in meiosis,^{7,8} little has been done to examine meiotic prophase chromosomal domains and recombinational behavior as a prelude to this rearrangement.

Molecular cytogenetics (i.e., FISH) and immunocytochemistry (i.e., fluorescent antibody localization [FAL]) provide powerful tools to address the question of meiotic chromosomal position and recombinational activity as a prelude to interchromosomal rearrangement. MLH1, a mismatch-repair protein, is one of several required for reciprocal recombination (crossing over).^{28,29} Antibodies to MLH1 can be used to identify sites of crossing over during meiosis.^{28,30,31} These combined FISH and FAL techniques have yet to be used to address the question of meiotic chromosomal position and recombinational activity. To our knowledge, the present study is the first to use FISH and FAL together to examine the location of chromosomes 11 and 22 during both male and female meiosis and the position of recombination events relative to the translocation breakpoint positions as determined by MLH1 foci. From these studies, it can be concluded that the position and secondary DNA structure—but not the programmed recombinational activity of 11q23 and 22q11 in the meiotic prophase nucleus—play an important role in the generation of the recurrent constitutional t(11;22) rearrangement.

Material and Methods

Ovarian and Testicular Samples

All samples were obtained with the appropriate Institutional Review Board approval. Fetal ovaries from first-trimester abortuses were obtained, by M.M.C., from Greater Baltimore Medical Center's Cytogenetic Laboratory, and adult testicular samples were obtained either from the Cooperative Human Tissue Network or from biopsies performed by A.S. at University Hospitals of Cleveland or Cleveland VA Medical Center.

Tissue Preparation

Two different protocols were used: "classic" 3:1 methanol:acetic acid fixation and "microspread" paraformaldehyde fixation. For the classic preparations, the tissue was minced in Hanks balanced salt solution. Following hypotonic treatment (0.075 M KCl solution), the meiotic cells were fixed in a 3:1 solution as described elsewhere,³² with minor modifications. The microspreads of spermatocytes were prepared as described elsewhere.¹⁷

FAL

Antibodies for the following proteins were used: SCP3, a component of the axial/lateral elements of the synaptonemal complex³³; MLH1 (Pharmingen), a mismatch-repair protein that is required for meiotic recombination²⁸; and human CREST, an autoimmune serum that detects kinetochores (centromeres) derived from individuals with CREST (calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia). The antibodies were: goat anti-SCP3 diluted 1:1,000,³⁴ mouse monoclonal anti-MLH1 (Pharmingen) diluted 1:200, and CREST diluted 1:2,000. Secondary antibodies included: rhodamine (TRITC)-labeled anti-goat, FITC-labeled anti-mouse, and Cy5-labeled anti-human (all from Jackson ImmunoResearch). Application of the antibody

ies and their detection was as described elsewhere.³⁵ After detection, the spermatocytes were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

DNA Probes

Prelabeled chromosome-specific whole-chromosome paint (WCP) probes for chromosomes 6, 11, and 22 were purchased from Vysis. The locus-specific FISH probe for chromosome 21 was the pre-labeled LSI 21 probe purchased from Vysis. The probe covers ~200 kb in 21q22.13-22.2 and encompasses markers *D21S342*, *D21S341*, and *D21S529*. It is located at a distance ~38 Mb from the centromere of chromosome 21 (UCSC Genome Browser). The chromosome 22 locus-specific FISH probe, c87f9, was a cosmid isolated from the LL22NCO3 cosmid library. The chromosome 11 locus-specific probe (BAC 442e11 [GenBank accession number AC007707]) and the 6q26 control locus-specific probe (BAC 849d12) are from the RPC11 human BAC library (Roswell Park Cancer Institute). The BAC and cosmid DNA were isolated with Qiagen Maxi Prep kits (Qiagen) and were labeled by nick translation with tetramethyl-rhodamine-5-dUTP or fluorescein-12-dUTP (Amersham).

FISH

FISH of the 3:1 fixed material was performed as described elsewhere,³⁶ with minor modifications. After image acquisition of the FAL signals, the microspread spermatocyte preparations were denatured, and the probes were hybridized as described elsewhere,³¹ with the following exceptions: the probes were denatured at 85°C for 6 min and were preannealed for 15 min, and the hybridization step was extended to 72 h.

Image Analysis

All images were digitally captured using IP Lab Software (Applied Imaging). For the classic preparations, tools within IP Software were used to measure (in pixels) the distance between the FISH signals of the locus-specific probes. For chromosomes 11 and 21, the distance between the 11q BAC and the short arm of chromosome 21 was measured by specifying 21p as the end of the bivalent farthest from the LSI 21 signal. Measurements in pixels were converted to micrometers (1 μm = 7.5 pixels) with use of a slide micrometer.

Collection of the FISH and FAL images from the microspread spermatocytes was a two-step procedure. The FAL data were first imaged on a Nikon Eclipse E800 fluorescence microscope equipped with a Photometrics-cooled charge-coupled device camera CH350, and the location of each cell was recorded electronically with IPLab software (version 3.6.3) (Scanalytics). Following completion of the FISH procedure, each nucleus previously imaged for the FAL experiment was electronically relocated and the FISH images captured. Although much of the antibody staining was lost during the denaturation step for FISH, enough remained to superimpose the images.

Chromosomes 11 and 22 were identified by the BAC and cosmid signals. MLH1 distribution and probe-separation distances along these synaptonemal complexes (SCs) were measured using MicroMeasure, a 32-bit Windows application,³⁷ and the locations of each MLH1 focus and probe on the respective SCs were recorded as a relative position, with use of distance (fraction of SC arm length) from the centromere (identified by CREST).

Sequence Analysis

Ensembl (Build 35) was used to ascertain the available sequence for chromosome 21. Database searches were performed on the sequence from chromosome 21, to identify palindromes. The sequence for chromosome 21 was divided into two large contigs, 9,719,768–10,210,000 and 13,260,001–46,944,323. The sequence was examined using the EMBOSS palindrome-recognition program (PALINDROME). The following parameters were applied to the sequence contigs: arm size >60 bp, mismatch between arms <10 bp, and spacer <20 bp. Only AT-rich palindromes thus identified were subjected to further analysis with use of mfold,³⁸ to calculate the free energy for each. This was determined by entering the obtained sequences into the mfold server.

Results

FISH Analysis of Chromosomes 11 and 22 in Oocytes (3:1 Preparations)

To determine whether regions that preferentially rearrange are located in proximity to one another in the meiotic prophase nucleus, the distance between 11q23 and 22q11 in oocytes and spermatocytes was assessed. Oocyte nuclei were first classified as to meiotic stage of meiotic prophase on the basis of microscopically observed cytologic characteristics: degree of chromosomal pairing and condensation. Of the 200 oocyte nuclei analyzed, 13.5% were in leptotenema, 24% in zygonema, 49% in pachynema, and 13.5% in diplonema. Since recombination occurs during pachynema, only oocytes in this stage were selected for further analysis after FISH analysis with DNA probes.

Dual-color FISH was performed on the fetal pachytene oocytes with use of four or six probes simultaneously. WCP probes labeled with either FITC or rhodamine were used to identify individual bivalents (11 with 22; 6 with 22; 11 with 21 and 22), and single-copy, locus-specific probes were used to identify the breakpoint regions on the test bivalents (11 and 22) or the selected regions on the control bivalents (6 or 21). The single-copy probes were labeled with the alternative color fluor to that of the WCP. The WCP- and probe-labeling combinations for the individual experiments are shown in table 1.

To determine the distance between 11q23 and 22q11 in oocytes, two experiments were performed (experiments 1 and 2). The labeling scheme is described in table 1, and representative images are shown in figure 1A and 1B. See table 2 for data.

As a control, the distance between 6q26 and 22q11 was compared with the distance between 11q23 and 22q11. Chromosome 6 was selected as a control for three reasons. Chromosome 6 is similar in size to chromosome 11, 6q26 is approximately the same fractional distance from the long arm telomere as is 11q23, and chromosome 6 has not been involved in recurrent rearrangements with 22q11. A BAC that maps to 6q26 (849d12) was selected as the control probe. The labeling scheme is described in table 1 (experiments 3 and 4). Representative images are shown in figure 1C and 1D). See table 2 for data.

In these experiments, the distances between the rele-

Table 1. Probes and Labeling Scheme for FISH Experiments

Experiment	WCP for Chromosome				Probe (Chromosome)				No. of Cells	Sample Type
	11	22	6	21	c87f9 (22)	B442e11 (11)	B849d12 (6)	LSI 21 (21)		
1	Rhodamine	FITC	Rhodamine	FITC	50	Oocytes (3:1)
2	Rhodamine	FITC	Rhodamine	FITC	50	Oocytes (3:1)
3	...	Rhodamine	FITC	...	FITC	...	Rhodamine	...	50	Oocytes (3:1)
4	...	Rhodamine	FITC	...	FITC	...	Rhodamine	...	48	Oocytes (3:1)
5	FITC	Rhodamine	...	FITC	FITC	Rhodamine	...	Rhodamine	50	Oocytes (3:1)
6	FITC	Rhodamine	FITC	Rhodamine	50	Spermatocytes (3:1)
7	...	Rhodamine	FITC	...	FITC	...	Rhodamine	...	47	Spermatocytes (3:1)
8	FITC	Rhodamine	...	FITC	FITC	Rhodamine	...	Rhodamine	50	Spermatocytes (3:1)
9 ^a	Rhodamine	FITC	144	Spermatocytes (microspread)

^a In experiment 9, the marker for chromosome 21 was the CREST antibody.

vant 11q23 and 22q11 or 6q26 and 22q11 single-copy probes were measured, and the two experiments for each bivalent pair were combined. The 98 oocytes were ranked by increasing probe-to-probe distance, and the data were plotted. Figure 2 compares the distance between chromosome 22 and either (1) chromosome 6 (open squares) or (2) chromosome 11 (filled circles) from the combined experiments (experiments 1–4). The Y-axis indicates the distance between probes from individual oocytes, which are enumerated on the X-axis to indicate the trend. Overall, it appears that 11q23 is closer to 22q11 than is 6q26, with average separation distances of 11–14.5 μm and 18–35 μm , respectively (table 2). These differences were statistically significant ($P \leq .02$).

The distance of chromosome 21 (short arm) to 11q23 was selected as another control, to compare with the distance between the breakpoints on 22 and 11. Chromosome 21 was selected as a control for chromosome 22, because it is similar in size and morphology to 22, 21p is adjacent to the centromere of a small acrocentric chromosome, and chromosome 21 has not been involved in recurrent rearrangements with 11q23. For these experiments, all three bivalents (11, 21, and 22) were painted with WCPs, and the locus-specific probes were labeled in the fluor color alternative to that of the WCP (fig. 1E and 1F). Although there were several instances where chromosome 21 was closer to 11q23 than was 22q11, 22q11 was usually closer to 11q23 than was chromosome 21 in the 50 oocytes evaluated. The average distance (\pm SD) between 22q11 and 11q23 was $14.5 \pm 9.4 \mu\text{m}$, whereas that between 21 and 11q23 was $26.2 \pm 12.1 \mu\text{m}$. This difference is significant ($P < .001$), although less striking than that observed for the distances between 11 and 22 versus between 6 and 22.

FISH Analysis of Chromosomes 11 and 22 in Spermatocytes (3:1 Preparations)

Similar experiments were performed on spermatocytes. As can be seen from the representative images in figure 3A and 3B, 11q23 and 22q11 were often in very close proximity to one another, even occasionally overlapping in the vicinity of the breakpoint probe. The distance between 11q23 and 22q11 or 6q26 and 22q11 (fig. 3C and 3D) was

measured in 50 (11q23) or 47 (6q26) spermatocytes (table 2 [experiments 6 and 7]). The average distance between 6q26 and 22q11 was more than four times greater than between 11q23 and 22q11 (table 2), a value that was highly significant ($P < .001$).

In experiments corresponding to those for oocytes, the distance between 21p11 and 11q23 was compared with the distance between the breakpoints on 22 and 11 in the spermatocytes. For these experiments, see table 1 for the labeling scheme, table 2 (experiment 8) for the data, and figure 3E and 3F for examples. On average, 22q11 is closer to 11q23 ($20.5 \pm 11.7 \mu\text{m}$) than is chromosome 21 ($24.4 \pm 10.8 \mu\text{m}$) in the 50 spermatocytes evaluated, but this difference was not statistically significant. This is in contrast to the oocyte experiments, in which the average separation distances between these two probes were significantly different. Whereas there is less separation between the 22q11 breakpoint region and the 11q23 breakpoint region, as compared with the same measurement for the 21p11 control region, the difference between the measurements was less striking than it was in the experiments comparing 6q26 and 22q11 versus 11q23 and 22q11. This suggests that proximity is not the only factor that plays a role in generating the translocation.

FISH Analysis of Chromosomes 11 and 22 in Spermatocytes (Microspread Preparations)

To determine whether preparative technique might affect proximity results, the distances between 11q23 and 22q11 versus 11q23 and 21p11 were evaluated in microspread spermatocytes. For these experiments, locus-specific probes were used to identify chromosomes 11 and 22. No probe was necessary to identify chromosome 21, since it is the smallest autosomal bivalent. The CREST antibody signal, rather than a locus-specific probe, was used for chromosome 21, and measurements were made from the distal border of the CREST signal. The average distances between either the 11q23 and 22q11 probes or 11q23 and 21q11 probes were not significantly different from those observed using 3:1 fixation (table 2 [experiment 9]). Unlike the 3:1 fixation in spermatocytes, the distances between chromosomes 11 and 22 and between chromosomes 11 and 21 were different for the microspread spermatocytes

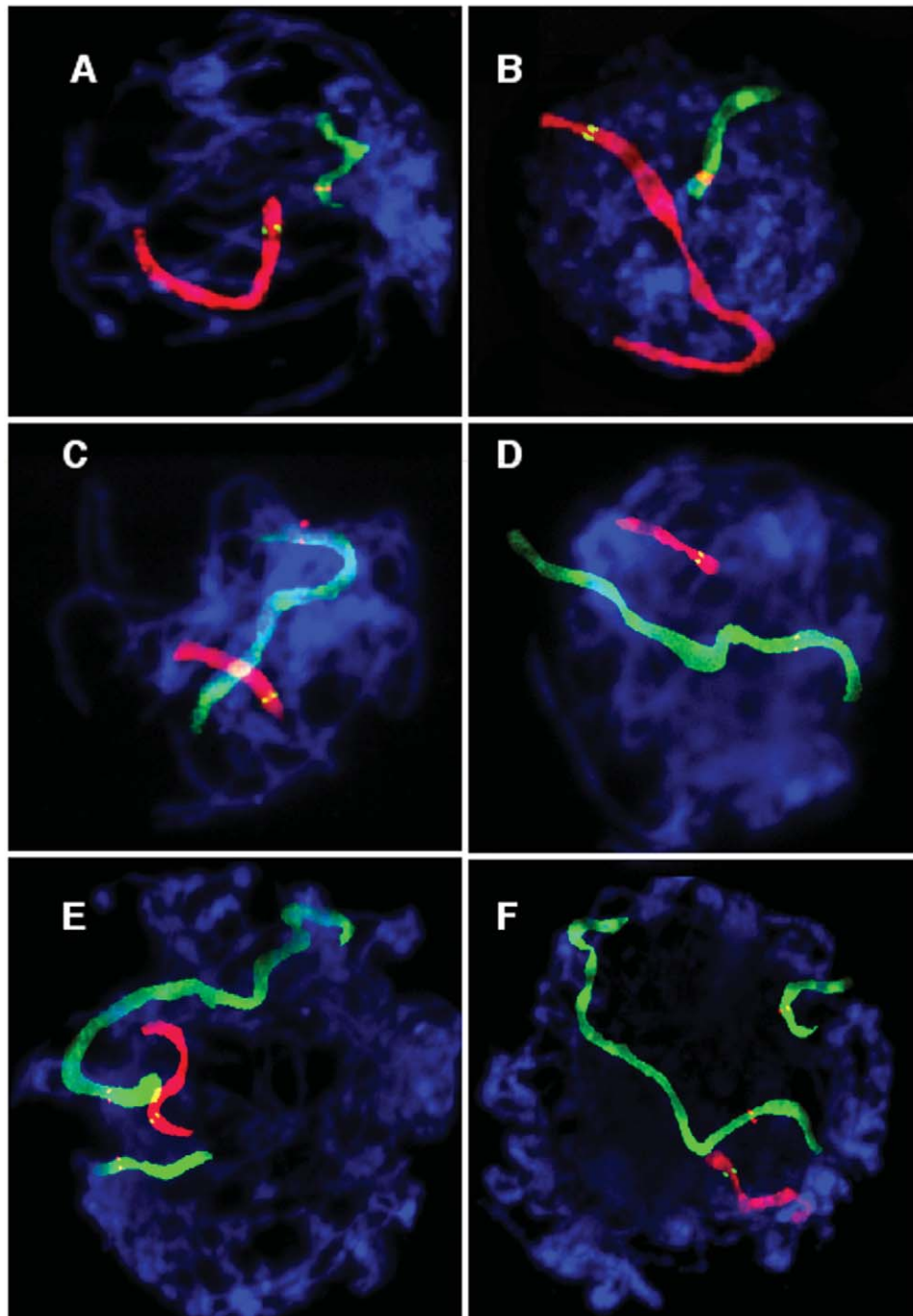


Figure 1. FISH-labeled human pachytene oocytes (3:1 preparation). Chromosomes were cohybridized with WCPs and locus-specific probes. For details of the probes and labeling scheme, see table 1. *A* and *B*, WCP11 is shown in red, with the 11q23 locus-specific probe shown in green; WCP22 is shown in green, with the 22q11 locus-specific probe in red (table 1 [experiments 1 and 2]). *C* and *D*, WCP6 is shown in green, with the 6q26 locus-specific probe shown in red; WCP22 is shown in red, with the 22q11 locus-specific probe shown in green (table 1 [experiments 3 and 4]). *E* and *F*, WCP11 is shown in green, with the 11q23 probe in red; WCP22 is shown in red, with the 22q11 probe shown in green; WCP21 is shown in green, with the 21q11 probe in red (table 1 [experiment 5]).

($P = .04$). However, the level of significance is not high, and the large number of observations ($n = 145$ and $n = 144$) presumably contributed to the ability to detect the slight difference between the two probe combinations.

Comparison between Oocyte and Spermatocyte Preparations

To begin to address the question of whether there are differences in chromosomal position between oocytes and

Table 2. Distances between Probes for Three Females and Three Males

Experiment and Sample	Probe Combinations	No. of Cells Observed	Distance between Probes (μm)		P^a
			Mean (SD)	Range	
1:					
Female 1	11q23+22q11	50	14.5 (8.5)	0–33.2	
3:					
Female 1	6q26+22q11	50	18.1 (6.7)	5.2–32.1	.02
2:					
Female 2	11q23+22q11	50	11.0 (8.8)	0–39.6	
4:					
Female 2	6q26+22q11	48	35.2 (11.3)	4.1–65.0	<.001
6:					
Male 1	11q23+22q11	50	8.5 (7.2)	0–28.0	
7:					
M1	6q26+22q11	47	35.6 (10.0)	14.6–55.5	<.001
5:					
Female 3	11q23+22q11	50	14.5 (9.4)	0–35.2	
Female 3	11q23+21p11	50	26.2 (12.1)	7.1–52.2	<.001
8:					
Male 2	11q23+22q11	50	20.5 (11.7)	0–47.8	
Male 2	11q23+21p11	50	24.4 (10.8)	3.9–52.2	>.05 ^b
9:					
Male 3	11q23+22q11	145	21.1 (9.3)	1.1–44.9	
Male 3	11q23+21q11	144	23.4 (9.8)	3.8–43.0	.04

NOTE.—All measurements were taken from 3:1 fixed cells, except male 3, which was from microspread preparations.

^a Between probes in same individual (*t* tests).

^b Not significant.

spermatocytes, the data sets for the two sexes were compared. For both oocyte and spermatocyte 3:1 preparations, there was at least one instance of zero distance between the 11 and 22 single-copy probes, whereas control probes (6–22 or 11–21) always had at least some distance between them (table 2). The distance between 6 and 22 is similar in oocytes and spermatocytes for experiments 4 and 7. The two data sets indicate that there is little effect of possible size differences between the two cell types. This is not the case when experiment 3 is compared with experiment 7. The distances between 6 and 22 are greater than the distances between 11 and 22 in both oocytes and spermatocytes. In addition, there appears to be a trend for 11 and 22 to lie closer to one another in oocyte 3:1 preparations than in spermatocytes prepared the same way (except for male 1). The data suggest a potential interindividual difference, as well as differences between oocytes and spermatocytes.

PATRRs and Chromosome 21

Since chromosomes 21 and 22 seem to lie at similar distances from chromosome 11q23 in meiotic prophase nuclei, most notably in spermatocytes, nuclear position alone is insufficient to explain the recurrent t(11;22). The presence of palindromic sequences on any given chromosome might be predicted to influence translocation permissiveness.³⁹ To determine whether there exists a PATRR on chro-

mosome 21 that is as conducive to translocation with 11q23 as is the PATRR on chromosome 22, a sequence search with the DNA sequence for chromosome 21 was performed with the PALINDROME software. The minimum length of a palindrome was set at 60 nt, the gap length was set at 20 nt, and mismatch was set at 10 nt. The search identified 160 palindromic sequences within the two large sequence contigs that exist for chromosomes 21. Formation of hairpin secondary structures requires denaturation of double-stranded chromosomal DNA containing an inverse complement such that the single-stranded DNA can self-anneal into a stem loop. The stability of a particular strand of DNA in its double-stranded configuration or in a secondary structure formed within a single strand can be described by separate Gibbs free energy (*G*) values. Because a maximum amount of base pairing occurs in double-stranded DNA, it is inherently more stable and always has a free energy value (G_{DS}) that is more negative than that of a single strand folding within itself (G_{STRUC}). The free energy of a single strand of sequence forming a stem loop depends on the position and number of complementary base pairs, as well as the GC content of these nucleotides.

The palindromic sequences were submitted to the mfold server that provided free energy values (G_{STRUC}) as part of the output for the PATRRs. Of the 160 palindromes studied, 25 sequences were identified with a G_{STRUC} lower than –50. For these 25 sequences, the G_{DS} was calculated (table 3). This was accomplished by “annealing” the sequence to its reverse complement *in silico*, reanalyzing the DS sequence, and halving the output. Free energy for the formation of a secondary structure (ΔG) is the $G_{\text{DS}} - G_{\text{STRUC}}$ difference. Small ΔG s are predicted to be more susceptible to hairpin extrusion and breakage. When the ΔG s of the 25 chromosome 21 PATRR-like sequences were compared with those for PATRR17, -11, and -22, it was determined that all of the chromosome 21 PATRR ΔG values were greater than those calculated for PATRR17, -11, and -22. These data indicate that there is no PATRR on chromosome 21 that is likely to engage in translocation with 11q23 at a frequency similar to what has been determined for the translocation-permissive chromosome 17 and the chromosome 22 PATRRs. This suggests that proximity and presence of PATRRs alone do not predispose to translocation; rather, palindromes with specific characteristics are required.

FAL Analysis to Determine Crossover Distribution on Chromosomes 11 and 22

Reciprocal exchange between chromosomes 11 and 22 requires DSBs and DNA repair. To determine whether the position of the breakpoints coincides with normal hotspots of meiotic recombination during pachynema, MLH1 was used to identify crossover sites. In addition, an antibody to SCP3, a component of the axial/lateral elements of the SC, was used to visualize the SCs, and CREST, a

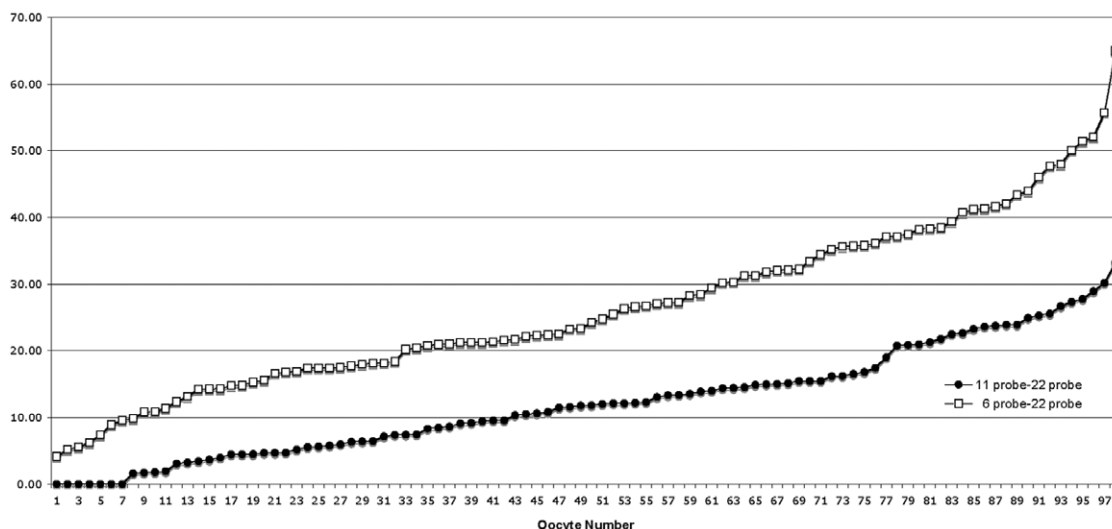


Figure 2. Comparison of distances between 11q23 and 22q11 and between 6q26 and 22q11. Probe-to-probe distance is shown in micrometers on the Y-axis. Distances between 11q23 and 22q11 locus-specific probes are shown as filled circles and between 6q26 and 22q11 as open squares.

human autoimmune serum that localizes to kinetochores, was used to identify the centromere regions and distinguish between the long and short arms (fig. 4).

For chromosome 11, FISH with the BAC 442e11 probe provided both a positive identification of the bivalent and a marker for the breakpoint region. Several previous human MLH1 mapping studies have divided the chromosomal arms into fourths and have assigned each focus to quarter segments.^{17,19} To better compare the distribution patterns for chromosomes 11 and 22 at a higher resolution, we used the procedure of Froenicke et al.³¹ to map MLH1 foci in 0.2- μm segments for the relevant chromosomal bivalents. The distribution graphs for MLH1 are shown in figures 5 and 6.

The number of MLH1 foci on the chromosome 11 bivalent varied between one and four, with a mean of 2.17 (SD 0.70) ($n = 101$). Of these pachytene nuclei, 16% had a single MLH1 focus, 52% had two foci, and 31% had three foci. In addition, one chromosome 11 bivalent had four MLH1 foci. The distribution of MLH1 foci along the SC bivalent in each category is presented in figure 5. No MLH1 foci localized near the centromere (located at $\sim 4.8 \mu\text{m}$ on the histograms), consistent with previous observations on binned chromosomes in human spermatocytes.^{17,19} Consistent with reported high rates of recombination in human males, the largest MLH1 peak on chromosomal bivalent 11 was near but not at 11qter. However, there were also a few MLH1 foci in the terminal 0.2 μm of 11q.

It has long been recognized that when two crossovers occur on the same chromosome, they do not occur close together, a phenomenon called “positive crossover interference.”⁴⁰ Therefore, it is not surprising that a clear bi-

modal distribution pattern exists in those chromosome 11 bivalents with two foci (figs. 5 and 7). Within this group, one MLH1 focus was generally located on each arm, which suggests that the crossover interference signal can be transmitted across the centromere. In the middle of each of these “peaks,” there was a distinct trough—a 0.2- μm region that had no MLH1 foci.

Given the inhibitory effect of a second crossover occurring in close proximity to a first, one would predict the widest distribution spread in those bivalents with the highest number of crossovers—that is, very proximal and very distal crossovers on these bivalents. Consistent with this expectation, both the two-focus group and the three-focus group have MLH1 foci nearer the telomeres than any observed in the one-focus group. Since there was only one chromosome 11 bivalent with four foci, the sample size was too small to draw any further conclusions.

Chromosome 22 is the second-shortest in the human complement, and all chromosome 22 bivalents had either one or two foci, with the majority (73% [$n = 92$]) having only one (mean 1.26; SD 0.44 [$n = 90$]). The distribution of MLH1 foci along the SC of chromosomes 22 is presented in figure 6. Although only three MLH1 foci mapped to the terminal 0.2 μm of 22q, there was a major MLH1 peak near the end of 22q. Only one MLH1 focus mapped to the short arm of chromosome 22. When the fact that 22p consists of highly repetitive ribosomal sequences—in which recombination is known to be repressed—is considered, the paucity of MLH1 foci in this region is hardly surprising. However, unlike chromosome 11, there were a few crossovers very close to the centromere on chromosome 22.

When there were two MLH1 foci on the 22 bivalent,

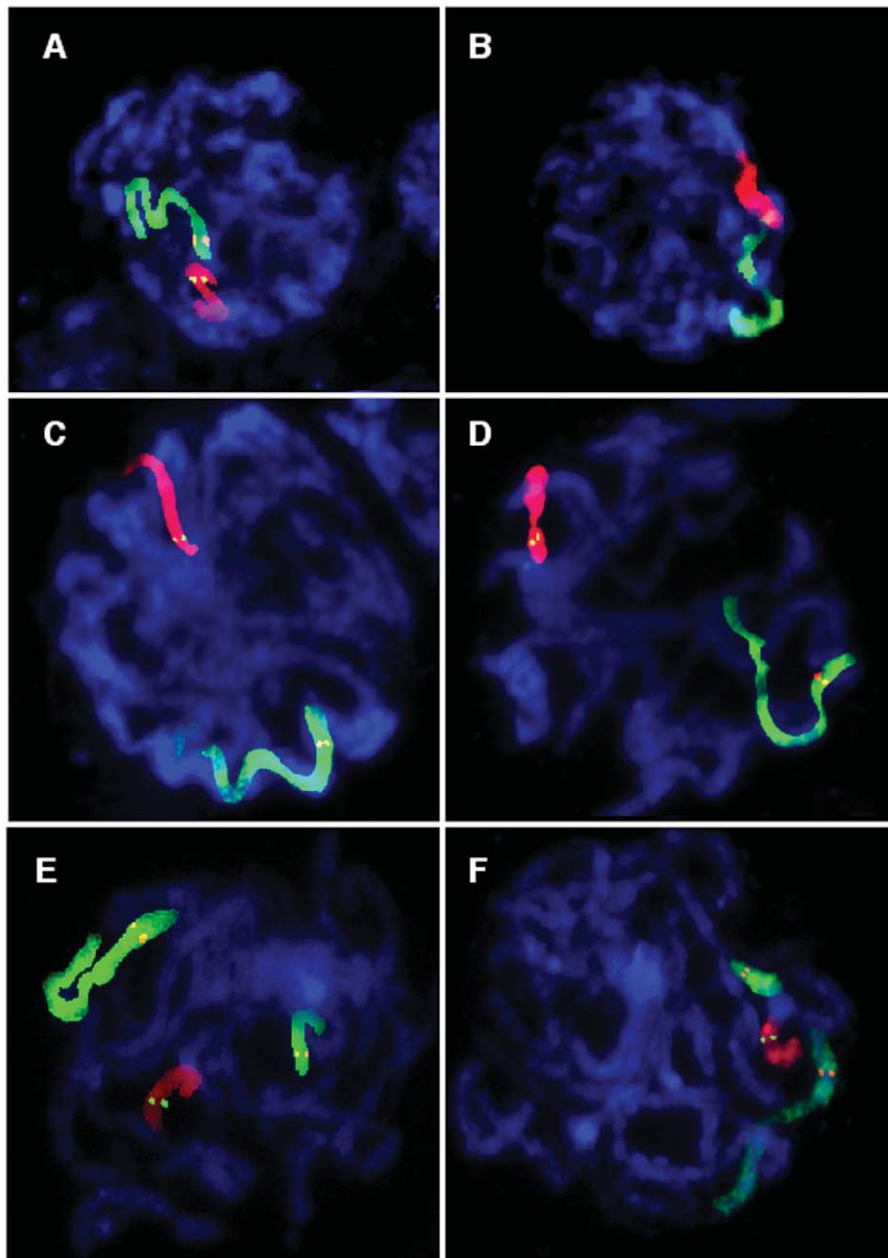


Figure 3. FISH-labeled human pachytene spermatocytes (3:1 preparation). Chromosomes were cohybridized with WCPs and locus-specific probes. For details of the probes and labeling scheme, see table 1. *A* and *B*, WCP11 is shown in green, with the 11q23 locus-specific probe shown in red; WCP22 is shown in red, with the 22q11 probe shown in green (table 1 [experiment 6]). *C* and *D*, WCP6 is shown in green, with the 6q26 probe shown in red; WCP22 is shown in red, with the 22q11 probe shown in green (table 1 [experiment 7]). *E* and *F*, WCP11 is shown in green, with the 11q23 probe shown in red; WCP22 is shown in red, with the 22q11 probe shown in green; WCP21 is shown in green, with the 21q11 probe shown in red (table 1 [experiment 8]).

the distribution was bimodal. The distal peak on 22q in the group with two foci coincides with the main peak in the single-focus group. Rather than a second sharp peak of MLH1 foci on proximal 22q, there was a broad distribution of foci in the two-focus group. Contrary to the expectation of suppression of crossover near the centromere, in both the one- and two-focus groups, there were a few foci in the intervals adjacent to the centromere.

Location of the Breakpoints Relative to the Peaks of Recombination

The 11;22 translocation could arise as a result of an error in a normal meiotic recombination event (repair of a meiotically programmed DSB) or as an attempt to repair an unprogrammed DSB that arose as a result of the secondary structure of the PATRRs. To discriminate between these two

Table 3. Palindromic Sequences Identified on Human Chromosome 21

Palindrome ^a	Location in Chromosome 21		Length (nt)	G _{STRUC} (kcal/mol)	G _{DS} (kcal/mol)	ΔG (kcal/mol)	ΔG/nt (kcal/mol)
	Start	End					
5	16108261	16108407	147	-62.69	-94.97	32.28	.2196
10	17041640	17041792	153	-55.27	-71.21	15.94	.1042
22	20574998	20575241	244	-119.23	-135.13	15.90	.0651
27	20951787	20951942	156	-65.74	-87.45	21.71	.1391
35	24454851	24455058	208	-81.69	-129.21	47.52	.2285
36	24615664	24615817	154	-5.40	-82.27	31.87	.2069
38	24927548	24927717	170	-51.76	-81.01	29.25	.1720
39	25121832	25122002	171	-61.11	-102.27	41.16	.2407
48	28030093	28030220	128	-54.32	-73.10	18.78	.1467
55	29263048	29263183	136	-54.58	-98.61	44.03	.3238
83	33617249	33617425	177	-56.89	-89.28	32.39	.1830
84	33617282	33617448	167	-61.12	-82.33	21.21	.1270
86	33617284	33617461	178	-65.93	-88.45	22.52	.1265
89	33617296	33617477	182	-67.52	-9.31	22.79	.1252
91	33617305	33617496	192	-77.01	-97.06	2.05	.1044
93	33617312	33617489	178	-74.11	-87.74	13.63	.0765
94	33617314	33617515	202	-8.63	-10.71	2.08	.0994
96	33617342	33617515	174	-68.09	-86.28	18.19	.1045
99	33758611	33758766	156	-72.62	-113.39	4.77	.2613
104	37508486	37508642	157	-7.80	-11.29	39.49	.2515
147	42468805	42468949	145	-63.73	-96.84	33.11	.2283
149	43404786	43404925	140	-67.39	-96.42	29.03	.2073
152	44600367	44600499	133	-7.47	-99.51	29.04	.2183
153	45475447	45475586	140	-6.17	-104.79	44.62	.3187
154	46446323	46446567	245	-142.41	-18.51	38.10	.1555
PATRR11 ^b			445	-178.40	-196.25	17.85	.0401
PATRR17 ^c			187	-89.47	-92.11	2.64	.0141
PATRR22 ^{d,e}			582	-313.60	-324.75	11.15	.0192

^a Palindromes with G_{STRUC} less than -50 kcal/mol were analyzed.

^b GenBank accession number AF391129.

^c GenBank accession number AB195814.

^d GenBank accession number AC087065 (location 38923-39213) and AC074203 (location 11820-11530).

^e PATRR22 was inferred from the two junction-fragment sequences on der(11) and der(22).

possibilities, probes from the breakpoint regions on chromosomes 11 and 22 were hybridized and FISH mapped to microspread spermatocytes. The microspread preparative technique preserves the chromatin structure better than does the 3:1 fixation. Consequently, both the chromosome 11 BAC and the chromosome 22 cosmid produce a FISH signal that is more like that seen in fiber-FISH. Consistent with the differences in size, the chromosome 11 BAC (180 kb) produces a more elongated signal than does the cosmid on 22 (fig. 4). These signals represent the chromatin loops extending from the SC. The position where the signals from the two homologues crossed the SC was measured and mapped to a region 11.2 μm from the distal end of the chromosome 11 bivalent. As seen in figure 7, the location of the BAC 442e11 does not coincide with a cytological region of high recombination activity but with a trough of MLH1 on 11q. On the basis of the combined MLH1 and probe map, the exchange event that leads to the t(11;22) cannot be attributed to a hotspot of normal meiotic recombinational activity.

The 87f9 cosmid from the breakpoint region of chromosome 22 mapped ~1.8 μm from 22pter and ~0.8 μm

distal to the centromere (fig. 7). Again, on the basis of the MLH1 map, this region is one of low recombinational activity, which argues against typical meiotic recombination as the mechanism of DSB repair leading to the t(11;22).

Discussion

The remarkable similarity between numerous constitutional t(11;22) breakpoint junctions derived from unrelated individuals suggested that, in addition to the presence of PATRRs at both breakpoints, proximity between these two chromosomal regions in the meiotic prophase nucleus is likely to facilitate the rearrangement. In recent years, studies on the spatial organization of chromosomes within the mitotic interphase nucleus have indicated that the arrangement of chromosomes and genes is nonrandom (for reviews, see Misteli⁴¹ and Parada et al.⁴²). Gene-dense, euchromatic chromosomes are located more toward the center of the nucleus, and gene-poor, G-band-positive chromosomes are more peripherally positioned.²⁴ Nonrandom proximity between nonhomologous chromosomes has been observed and proposed as playing a role

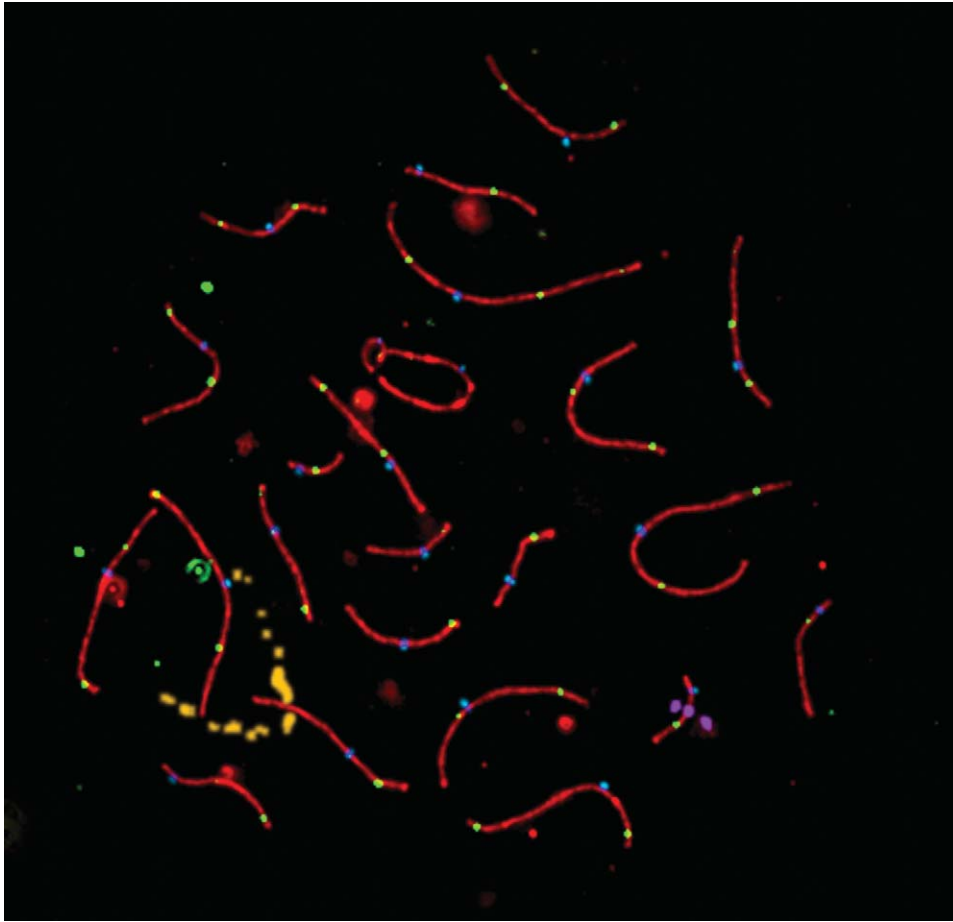


Figure 4. Immunostained and FISH-labeled human pachytene spermatocyte (microspread preparation). SCP3 is shown in red, MLH1 in green, and CREST in blue. In addition, the 11q23 probe is shown in gold, and the 22q11 probe is shown in magenta. The entire nucleus is stained with DAPI (cyan). MLH1 foci mark crossover sites.

in recurrent chromosomal rearrangements.⁴³ Moreover, there seem to be changes in the position of specific loci and regions in different cell types.⁴³ However, few such studies have attempted to examine chromosomal position during mammalian meiosis, and most of these have focused on alignment of homologues prior to synapsis.⁴⁴ Only a few have looked at the spatial disposition of specific chromosomes during meiotic prophase.^{45,46} Thus, the current studies offer a first glimpse at comparative chromosomal organization of pachytene nuclei and indicate that chromosomal domains may not be randomly distributed and that relative positions may differ between the sexes.

These studies demonstrate that both chromosomes 21 and 22 reside close to 11q23 in the meiotic prophase nucleus, especially in males. It is not surprising that 21 and 22 reside close to one another, since they are both small chromosomes and previous ultrastructural studies found that the nucleolar organizer regions of human acrocentric bivalents associate with the nucleolus during meiotic prophase.⁴⁷ However, despite the fact that they are close to one another and are both in proximity to 11q23, chro-

mosome 21 does not engage in recurrent translocation with 11q23. The results reiterate the idea that proximity alone is insufficient to promote chromosomal rearrangement.

Specific DNA-sequence content and genomic configuration undoubtedly play significant roles in translocation permissiveness. For example, even though Robertsonian translocations take place between acrocentric chromosomes with homologous DNA sequence on their short arms, the prevalence of specific translocations among the potential chromosomal combinations differs. Thus, the more common recurrent Robertsonian translocations— $t(13;14)$ and $t(14;21)$ —have translocation breakpoints in the same chromosomal region with respect to specific satellite DNA subfamilies that are shared between them. These translocations arise primarily during oogenesis, when the chromosomes are in close proximity, perhaps through a common sequence-based mechanism.⁴⁸ Similarly, the common breakpoints for the $t(11;22)$ suggest that the recurrence of the translocation is based not only on the proximity of the two chromosomes but also on the presence of the rearrangement-permissive PATRRs.

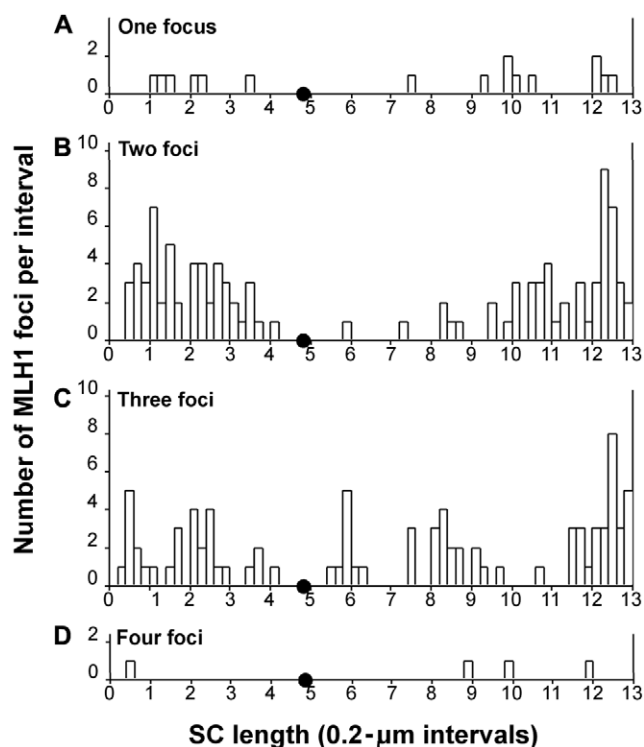


Figure 5. Histograms of the distribution of MLH1 on chromosomal bivalent 11. The X-axis indicates the position (in μm) of MLH1 foci along the length of the bivalent. The position of the centromere is indicated by a small blackened circle; the p arm lies to the left, and the q arm lies to the right. The Y-axis indicates the number of MLH1 foci mapped to each interval. *A*, Distribution of MLH1 on bivalents with only one MLH1 focus. *B*, Distribution of MLH1 on bivalents with two foci. *C*, Distribution of MLH1 on bivalents with three foci. *D*, Distribution of MLH1 on the single bivalent with four foci.

The role of polymorphic variation in PATRR11 DNA sequence in generating the t(11;22) has recently been demonstrated.⁸ Polymorphisms of the PATRR11 in normal males results in a greater than threefold variation in susceptibility for generating the recurrent translocation in male gametes. Although DNA sequence variation influences translocation frequency, it is important to note that the chromosome 11 and 22 PATRRs appear to have limited cell-type susceptibility to interchromosomal rearrangement. Previous studies of DNA samples from normal cell lines from a variety of tissues—as well as those derived from individuals with Bloom syndrome or ataxia-telangiectasia, which are DNA-instability syndromes—did not identify translocation-specific PCR products.⁹ This indicates that production of the recurrent t(11;22) requires the conditions that exist during meiosis.

Since the t(11;22) rearrangement occurs during meiosis and obviously requires DNA breakage and repair, it is important to consider the likely enzymes and their availability during meiotic prophase. The MRE11 complex (MRE11/RAD50/NBS1 in mammals) is probably a key

player. This complex has been implicated in both major DNA-repair pathways: nonhomologous end joining (NHEJ) and homologous recombination (see Assenmacher and Hopfner⁴⁹ for review). The Mre11 complex recognizes and cleaves hairpin structures such as those adopted by the PATRRs on chromosomes 11 and 22⁵⁰; thus, it is logical to predict binding under the current circumstances. Intriguingly, the Mre11 complex is also required for meiotic synapsis and recombination.⁵¹ Another prerequisite for meiotic recombination is programmed DSBs executed by SPO11, a topoisomerase.⁵²

The Mre11 complex is not only a key component of DSB repair, but it is often prepositioned at sites vulnerable to a variety of DSBs. In addition to hairpin binding,⁵³ the mammalian Mre11 complex binds to transcription factors at replication origins⁵⁴ and continues to colocalize with replication forks as DNA synthesis proceeds.⁵⁵ In meiosis in *Saccharomyces cerevisiae*, the Mre11 complex is part of a large prerecombination complex that includes SPO11 and is required for programmed meiotic DSBs.⁵¹ A similar role for the Mre11 complex has been proposed in mammals.⁵⁶

Therefore, in humans, association of the Mre11 complex with the PATRRs during early stages of meiosis might theoretically assure their inclusion in a prerecombination complex. Alternatively, the Mre11 complex might associate with the PATRR hairpins and cleave them independent of SPO11. If binding of the Mre11 complex to the PATRR hairpins on chromosomes 11 and 22 were to assure

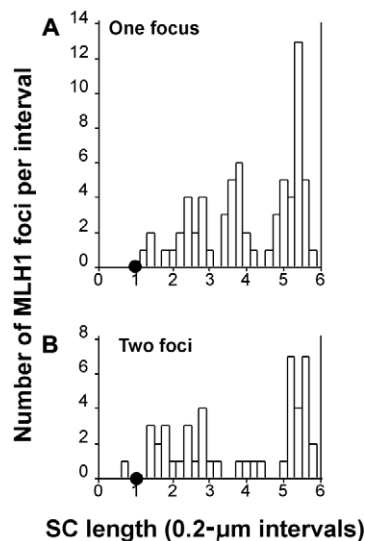


Figure 6. Histograms of the distribution of MLH1 on chromosomal bivalent 22. The X-axis indicates the position (in μm) of MLH1 foci along the length of the bivalent. The position of the centromere is indicated by a small blackened circle; the p arm lies to the left, and the q arm lies to the right. The Y-axis indicates the number of MLH1 foci mapped to each interval. *A*, Distribution of MLH1 on bivalents with only one focus. *B*, Distribution of MLH1 on bivalents with two foci.

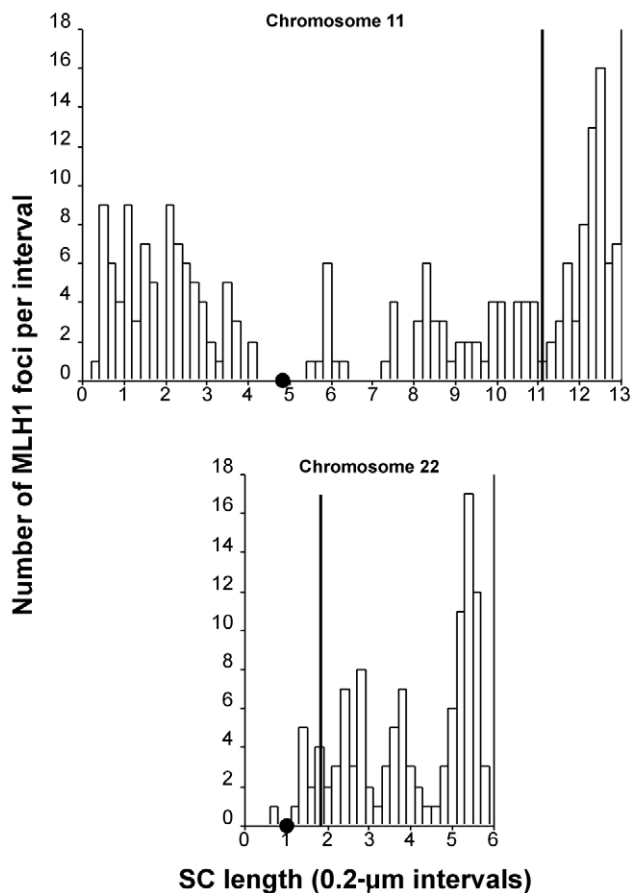


Figure 7. Histograms of the distribution of all MLH1 foci relative to the position of the probe for chromosomal bivalents 11 and 22. The X-axis indicates the position (in μm) of MLH1 foci (*unblackened bars*) and the probe (*solid line*) along the length of the bivalent. The chromosomes are positioned relative to one another on the X-axis on the basis of centromere position, which is indicated by a small blackened circle; the p arm lies to the left, and the q arm lies to the right. The Y-axis indicates the number of MLH1 foci mapped to each interval, as well as the location of the probe.

inclusion of these sequences in a prerecombination complex, it would be logical to expect that most DSBs would be repaired via homologous recombination between homologues. If this scenario were correct, the breakpoints on chromosomes 11 and 22 should coincide with hotspots of meiotic recombination. As shown in the current study, this is not the case. The lack of such a correlation suggests that the DSB(s) giving rise to the translocation are not programmed meiotic DSBs but occur independently, as a result of the hairpin structure itself. Although programmed meiotic DSBs are thought to occur during leptotema or early zygotema, the time of breakage as a result of extruded hairpins remains unresolved.

Once the breaks occur, they must be repaired, a process that can leave “footprints” that offer clues related to the repair pathway used. Reconstruction of the original genomic DNA configuration that is based on end products

of the translocation event for both derivative chromosomes indicates that the rearrangement resembles a process seen in mice and other eukaryotes called “center break palindrome modification.” The palindromic DNA sustains small central deletions that create junction fragments, a hallmark of NHEJ.⁵⁷ Despite their AT-richness, no substantial homology has been observed between the PATRR11 and the PATRR22. The breakpoints on the two chromosomes possess only a small number of identical nucleotides. Since the DNA sequences that reside on 22 and 11 are not homologous to one another, it is unlikely that a typical homologous recombination pathway is responsible. In contrast, little homology is required for NHEJ. Thus, it appears that the mechanism for this recurrent chromosomal translocation involves DSBs at the two PATRRs, followed by their repair through a pathway that resembles NHEJ.

However, the meiotic process has evolved to assure homologous recombination. The rarity of ectopic recombination or exchange between homologous or homeologous sequences on nonhomologous chromosomes in mammals is testimony to the success of this strategy. One component of this meiotic tactic is the suppression of NHEJ during meiotic prophase. In somatic cells, NHEJ repairs hairpin lesions with the ku70/ku80/DNA-PK(cs) complex.⁵⁸ The ku proteins are not present during early meiotic prophase in spermatocytes when programmed meiotic DSBs occur, even though DNA-PK is expressed throughout meiotic prophase. In fact, ku70 and ku80 do not reappear until midpachynema,^{56,59} approximately three to four *days* after meiotic programmed DSBs are presumed to occur. There are at least two explanations for this conundrum: (1) the palindromic breaks occur long after meiotic recombination events have been initiated and after the ku proteins are again available or (2) repair is accomplished without ku70/ku80 via a mechanism currently indistinguishable from ku-assisted NHEJ.

Variation in the regional rates of recombination has led to the identification of recombination hot- and coldspots on individual human chromosomes.⁶⁰ In a detailed analysis of the genomic sequence of chromosome 22, a significant correlation between long-tandem GT repeats and recombination hotspots on human chromosome 22 was observed.¹⁶ One of these hotspots appears to reside in the general vicinity of the PATRR in 22q11. However, since the 22q11 PATRR represents one of the unclonable gaps in the sequence of chromosome 22, it is likely to have eluded this analysis. In addition, although the known PATRR sequence does not contain the GT repeat-motif characteristic of such a meiotic hotspot, the PATRR itself likely leads to a DNA structure susceptible to DSBs. This would occur as a result of cruciform extrusion promoting the DSBs that initiate stabilizing rearrangements or recombination events.⁶¹

Disease-related recombination on proximal 22 seems to contradict expectations of typical meiotic behavior. Haplotype reconstruction of 22q11-deletion cases demon-

strates proximal interchromosomal exchanges between homologs giving rise to *de novo* deletions in 95% of those studied.¹⁸ By contrast, the normal chromosome 22 in the same deleted probands showed interchromosomal exchanges in <14% of informative meioses, a rate more in keeping with the genetic distance and cytological observations. Recombination, visualized as MLH1 foci, localizes to the distal long arm of chromosome 22 in the majority of human spermatocytes examined, also reflecting the genetic map.^{17,18} The MLH1 data presented here support the previous cytological studies indicating that more crossover events take place on the distal long arm than in 22q11.2. Thus, it is interesting that this chromosome is extremely susceptible to rearrangements of the proximal long arm but that these rearrangements do not appear to have been repaired via homologous recombination.

In fact, proximal chromosome 22q demonstrates a propensity to undergo aberrant meiotic homologous or non-homologous repair events that result in translocations, interstitial deletions, and small marker chromosomes. The 11;22 translocation breakpoint at 22q11 has been localized within one of the low-copy repeats (LCRs) on 22q that produce other human chromosomal disorders, most notably the 22q11 deletion syndrome.^{3,10,62,63} Each of the LCRs on 22q11 extends over several hundred kilobases, and they share >95% sequence homology over short stretches. Synapsis of homologous chromosomes requires a molecular check for homology that involves single-strand DNA (ssDNA).^{64,65} If pairing problems resulted in sequence similarities but not identities between the LCRs, extensive unwinding of the DNA can be predicted to follow.^{64,65} This would permit LCR-B and the PATRR22 contained within it to persist as ssDNA, rendering it susceptible to cruciform extrusion and creating sites for Mre11 binding. In fact, the potential for unwinding longer stretches of DNA than would normally occur during replication in somatic cells might provide an explanation for the preferential occurrence of the 11;22 translocation during meiosis.

In addition to the t(11;22), other 22q11 translocation breakpoints cluster within the chromosome-specific LCR region that encompasses the PATRR22.^{12,39,66-71} The PATRR22 itself has been implicated in the etiology of these rare 22q11-related translocations. The data presented herein suggest that physical proximity between 11q23 and 22q11, in addition to the genomic instability introduced by the PATRRs, plays a role in facilitating the t(11;22). Greater physical separation in the prophase nucleus may account for the infrequent occurrence of these other PATRR22 single-translocation events. Verification of this hypothesis awaits further investigation. Thus, additional analysis of chromosomal domains and proximities in meiotic prophase may be warranted. The recurrence of the t(11;22) translocation reminds us of the potential for non-homologous exchange during meiosis. Given the minimal estimate of ~150 DSBs during meiotic prophase in human spermatocytes⁷² and the fact that a significant proportion of the human genome is repetitive DNA, such detailed anal-

ysis might provide us with an understanding of why there are so few constitutional chromosomal rearrangements.

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Web Resources

Accession numbers and URLs for data presented herein are as follows:

GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for BAC 442e11 [accession number AC007707], PATRR11 [accession number AF391129], PATRR17 [accession number AB195814], and PATRR22 [accession numbers AC087065 and AC074203])
 mfold, <http://www.bioinfo.rpi.edu/applications/mfold/dna/form1.cgi>

MicroMeasure, <http://www.colostate.edu/Depts/Biology/MicroMeasure>

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for Emanuel syndrome)

PALINDROME, <http://bioweb.pasteur.fr/seqanal/interfaces/palindrome.html> (for the EMBOSS palindrome recognition program)

UCSC Genome Browser, <http://genome.ucsc.edu/>

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