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Identification of Double Holliday Junctions as Intermediates in Meiotic Recombination

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Summary

During meiosis, branched DNA molecules containing information from both parental chromosomes occur in vivo at loci where meiosis-specific double-stranded breaks occur. We demonstrate here that these joint molecules are recombination intermediates: they contain single strands that have undergone exchange of information. Moreover, these joint molecules are resolved into both parental and recombinant duplexes when treated in vitro with Holliday junction-resolving endonucleases RuvC or T4 endo VII. Taken together with previous observations, these results strongly suggest that joint molecules are double Holliday junctions.

Introduction

During meiosis, reciprocal exchange between homologous nonsister chromatids (crossing over) is required for faithful disjunction of maternal and paternal homologs at the first meiotic division (Hawley, 1988; Carpenter, 1994). Meiotic recombination can also result in the local exchange of genetic information without accompanying exchange of chromosome arms; the function of such noncrossover recombination is unknown (e.g., Storlazzi et al., 1995). Both crossovers and noncrossovers occur during meiosis at frequencies several orders of magnitude higher than in mitotic cells (e.g., Malone et al., 1980) and appear to arise via a common pathway (Malone et al., 1980; Storlazzi et al., 1995).

Detailed mechanistic information about meiotic recombination has emerged from analysis of recombination in vivo by physical methods in studies of the budding yeast Saccharomyces cerevisiae (Haber and Sugawara, 1995). For most or all recombination events, the first chemical change in DNA is a double-stranded break (DSB). This feature was originally proposed on theoretical grounds from parallels between meiotic recombination and recombinational repair of DSBs (Resnick, 1976; Szostak et al., 1983). DSBs occur at genomic loci that are hotspots for meiotic recombination and are a general feature of recombination at all loci (Game et al., 1989; Sun et al., 1989; Cao et al., 1990; Zenvirth et al., 1992; Goldway et al., 1993; Wu and Lichten, 1994; Fan et al., 1995).

DSBs occur via nonspecific nuclease cleavage in regions of chromatin where DNA is accessible, i.e., sites that are nuclease hypersensitive in both mitotic and meiotic chromatin (Wu and Lichten, 1994; Ohta et al., 1994; Liu et al., 1995; Xu and Kleckner, 1995; de Massy et al., 1995). Cleavage is accompanied by covalent attachment of a protein, presumably the DSB nuclease, to the 5′ termini of the DSB (Liu et al., 1995; de Massy et al., 1995; Keeney and Kleckner, 1995). Cleavage is followed rapidly by exonucleolytic degradation of the two 5′ strand termini, a step that yields molecules having 3′ terminal single-stranded regions of about 600 nt; there is no indication of resection at 3′ termini (Alani et al., 1990; Sun et al., 1991; Bishop et al., 1992).

The current work addresses the nature of later stages of recombination. Some models of DSB repair postulate that a double Holliday junction is a key intermediate (Strathern et al., 1982; Szostak et al., 1983). In pioneering work, Bell and Byers isolated branched molecules from total meiotic DNA whose features suggested that they were recombination intermediates, and many of these molecules had an eye-form structure likely to represent double Holliday junctions; by contrast, structures expected for single Holliday junctions were absent (Bell and Byers, 1983; Byers and Hollingsworth, 1994).

More recent studies have identified analogous branched joint molecules (JMs) at specific chromosomal loci (Schwacha and Kleckner, 1994; Collins and Newlon, 1994). These JMs have the properties expected for a recombination intermediate that arises from a DSB: they form preferentially between homologous nonsister chromosomes; the relative levels of JMs, DSBs, and recombinants vary coordinately at the loci examined; JM formation requires meiotic recombination functions including those needed for formation of DSBs; and, finally, JMs occur with appropriate kinetics during meiosis, subsequent to DSBs but prior to mature crossover and noncrossover products (Schwacha and Kleckner, 1994; Collins and Newlon, 1994; Padmore et al., 1991; Storlazzi et al., 1995).

JMs from one meiotic recombination/DSB hotspot, *HIS4–LEU2*, have been examined in more detail. Using a procedure in which the molecules are dissociated and their component single strands are analyzed, Schwacha and Kleckner (1994) demonstrated that all of the single strands within JMs are uninterrupted. Thus, if JMs arise from DSBs, they represent a stage at which all physical traces of the initiating lesion have been eliminated. Furthermore, when JMs are isolated from a strain in which the two parental chromosomes have flanking restriction sites at different positions, all component single strands have a parental configuration of flanking markers, with the two parental types represented equally. This latter feature implies that JMs do not comprise single (or odd-numbered) Holliday junctions: in those forms, two of the four strands have a recombinant configuration of flanking markers (see below). Several alternative structural possibilities for these species could not be eliminated, however.

Despite considerable circumstantial evidence suggesting that JMs should be recombination intermediates, previous work did not critically address the possibility that these molecules might represent "side-by-side" DNA–DNA interactions, stabilized by psoralen treatment, that serve exclusively to mediate the pairing of homologous

chromosomes without ever leading to genetically or physically recombinant products. Indeed, stable side-by-side interactions are likely to occur (Xu and Kleckner, 1995; V. Rocco and A. Nicolas, personal communication); moreover, two prominent features of JMs, the absence of interrupted strands and the absence of recombinant strands, would be predicted features of such pairing interactions.

In the work presented, component single strands have been analyzed with respect to physical markers located exactly at the major DSB site of this locus, as well as with respect to flanking markers. In addition, the susceptibility of purified JMs to Holliday junction-specific nucleases has been analyzed in vitro. These findings, in combination with previous work (Schwacha and Kleckner, 1994), provide strong physical evidence that JMs are recombination intermediates and, more specifically, that they are double Holliday junctions. These findings confirm the inference of Bell and Byers (1983) and one important prediction of the meiotic recombination model of Szostak, Rothstein, Orr-Weaver, and Stahl (1983). In addition, further consideration of these findings supports the proposition that the meiotic recombination involves two separate rounds of mismatch correction.

Results

Identification and Purification of Interhomolog JMs at *HIS4-LEU2*

The *HIS4-LEU2* locus contains two meiotic DSB sites, I and II (Figure 1). During meiosis, 10%–15% of DNA duplexes undergo a DSB at site I, the junction between *LEU2* and distal sequences; 3%–5% of duplexes undergo a DSB at site II, located at the proximal end of the *LEU2* segment (Cao et al., 1990; Xu and Kleckner, 1995).

JMs that form at this locus are analyzed below using a diploid strain homozygous for the *HIS4-LEU2* construct but heterozygous for XhoI restriction site differences flanking the locus and for a pair of restriction site alleles, *MluI* versus *BamHI*, that are tightly linked to DSB site I (Figure 1). The central marker site lies within the "zone of resection" for DSBs at site I (Bishop et al., 1992; Figure 1, legend). Thus, for any chromosome that undergoes a DSB at site I, the corresponding allele will always be present within one of the 3′ single-stranded regions (assuming every molecule that undergoes a DSB will also undergo resection; Figure 1). DNA species derived from each parental chromosome, "Mom" or "Dad," may be identified individually using homolog-specific sequences inextricably linked to the corresponding *HIS4*-distal XhoI sites.

Interhomolog JMs are harvested from yeast cultures undergoing synchronous meiosis (Schwacha and Kleckner, 1994). At the desired time, cells are treated with psoralen and ultraviolet light, a procedure that cross-links the Watson and Crick strands of DNA duplexes and increases the yield of JMs about 20-fold. Meiotic DNA is then extracted and analyzed by an appropriate gel electrophoresis protocol; species of interest are visualized by Southern blot analysis (Schwacha and Kleckner, 1994; see below).

In a native two-dimensional gel analysis in which DNAs are first separated according to mass and then according to shape, branched molecules migrate more slowly than linear molecules, and interhomolog JMs form a characteristic comet-shaped signal to one side of the arc of unbranched linear molecules (Figure 2A).

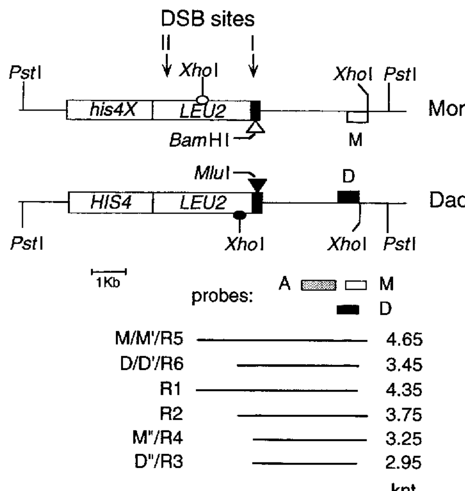


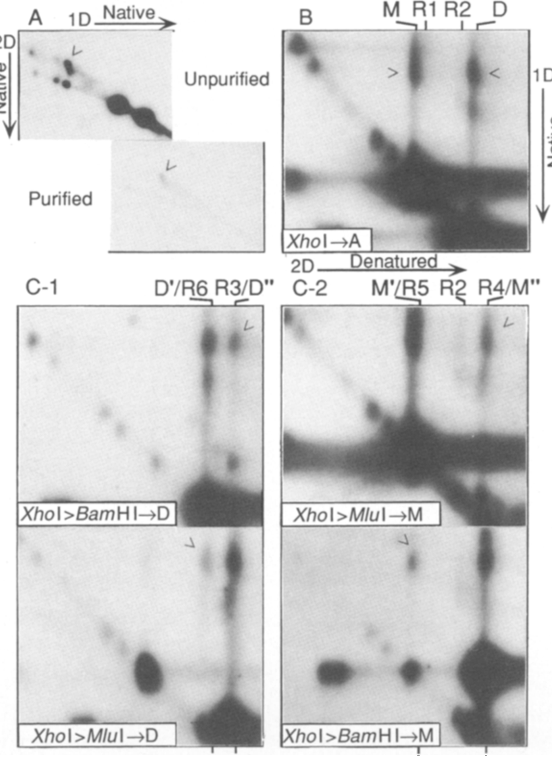
Figure 1. Genetic Constructs at *HIS4-LEU2*

The *HIS4-LEU2* locus is a hotspot for meiotic recombination, comprising a segment encoding *LEU2* inserted distal to the *HIS4* locus on chromosome III plus a 77 bp segment of bacterial DNA at the *LEU2*-distal junction (Xu and Kleckner, 1995). A strong site for meiotic DSBs coincides with this latter segment; DSBs at this site occur over a region of 150 bp (Xu and Kleckner, 1995) and are resected relatively uniformly for ~600 nt at both 5′ termini (Bishop et al., 1992). A weaker DSB site (II) occurs about 2 kb upstream within the *LEU2* segment. In the diploid strain analyzed, NKY2598, the *HIS4-LEU2* regions on the two homologs are marked as indicated (see text). Most of the JMs analyzed here probably arose from DSBs at site I (Schwacha and Kleckner, 1994). The *BamHI* allele (open triangle) was constructed by insertion of a BamHI linker into the MluI site of the *MluI* allele (closed triangle); DSBs at site I occur with equal frequency on the two homologs in this strain (A. S., unpublished data). The extents and sizes of single-stranded species observed in this work are indicated. Species were detected with homolog-specific probes Mom (M) and Dad (D) or with a probe that recognizes both homologs (A). knt, kilonucleotides.

Interhomolog JMs Are Recombination Intermediates

To search for direct physical evidence that JMs are recombination intermediates, we analyzed their composition with respect to the DSB-linked *MluI/BamHI* alleles. These alleles are directly involved in a high fraction of all recombination events promoted by *HIS4-LEU2*: in a strain closely related to that analyzed here, 40% of tetrads exhibit aberrant segregation of these alleles, with or without associated crossing over of flanking markers (A. Storlazzi, L. Cao, and N. K., unpublished data). We expected therefore that a significant fraction of the component single strands within JMs might have undergone a change in information at the DSB-linked marker with respect to the flanking marker(s), which is always in nonrecombinant configuration (Schwacha and Kleckner, 1994).

The procedure used to evaluate the status of the *BamHI* and *MluI* alleles is as follows: JMs are released from bulk cross-linked meiotic DNA by primary digestion with XhoI



D Single stranded species observed

Probe	*XhoI* only	*XhoI* > *BamHI*	*XhoI* > *MluI*	Average
Mom	M R2 (~5%)	M" (*) R5 (17±1%)	(•) M' R4 (28±4%)	23%
Dad	D R1 (<2%)	(*) D' R3 (36±3%)	D" (•) R6 (17±6%)	26%

Corresponding duplex not cleaved with *BamHI*(*) or *MluI*(•)

Figure 2. Purification and Analysis of Interhomolog JMs

(A) Identification of JMs by two-dimensional gel electrophoresis. Branched species migrate off the arc of linear molecules; JMs are indicated by carets. In some experiments, JMs were first purified away from species that would normally comigrate in the first dimension; the purity of the resulting preparation is documented by inset.

(B and C) In each panel, meiotic DNA was digested with XhoI, and JMs were separated from other species by gel electrophoresis in one dimension. The corresponding gel slice was then digested with BamHI, MluI, or no enzyme as indicated, subjected to strand dissociation and denaturing gel electrophoresis in a second dimension, and then blotted and probed as indicated. Carets in (B) denote the parental species. In (C), each recombinant species of interest (R3–R6) is indicated with a caret; the other important species is parallel with the recombinant species in the first dimension and at the second dimension position indicated at the top of the panel. (B) and (C) analyze a single preparation of total meiotic DNA; the top of C-1 and the bottom of C-2 are the same blots, analyzed sequentially with each homolog-specific probe following removal of the previous probe as needed; the same is true for the top of C-2 and the bottom of C-1. (C) is one of four identical experiments, which all gave the same results.

(D) summarizes the average of results from the four experiments; values represent the fraction of total strands detected by the indicated probe. A technical concern with these experiments is the presence of cross-hybridizing species that migrate with the same mobility as JMs in the first dimension and thus might contribute to signals observed in the second dimension. One of the four experiments therefore utilized a preparation of JMs that were specifically purified in such a way as to eliminate such comigrating species (A). Restriction site markers are denoted as in Figure 1.

and then separated from other species by one-dimensional gel electrophoresis. The corresponding gel slice is excised, digested secondarily in situ with either BamHI or MluI to cleave susceptible JMs at the central marker position, treated in situ with alkali to remove cross-links and denature DNA into single strands, and then subjected to electrophoresis in a second dimension under denaturing conditions (Schwacha and Kleckner, 1994). XhoI-digested JMs, which all migrate at the same specific position in the first dimension, separate into component (cleaved or uncleaved) single-stranded species in the second dimension. Following Southern blotting, DNA species of interest were identified with appropriate radiolabeled probes.

In previous work, JMs were analyzed analogously, except that secondary digestion, with MluI or BamHI, was not performed. In such an experiment, virtually all component single strands are found to be identical to those present in the two types of parental duplexes (Mom [M] and Dad [D]); the two "recombinant" strand types, R1 and R2, are rare or absent (e.g., Figures 2B and 2D).

To assess the status of the central marker, we analyzed duplicate aliquots from a single meiotic DNA sample in parallel, using either BamHI or MluI for the secondary restriction digestion. Then, for each of the two cases, strands containing the two homolog-specific terminal markers were examined individually by successive probings of the same blot (Figures 2C and 2D).

The combination of two restriction digests and two probes provides four different analyses. In considering the results of these analyses it is convenient to divide them into two categories.

In two of the four analyses, the secondary enzyme and the homolog-specific probe recognize different parental chromosomes. In each of these cases, a single-stranded species is detected which, unlike the corresponding parental chromosome, has been cleaved at the central marker position. These strands are by definition recombinant (R3 and R4; Figure 2D). Within the JMs that gave rise to such strands, the relevant segment of site I–encoding DNA duplex must have contained "cleavable" information on both strands. R3 and R4 comprise 36% and 28% of total detected strands in their respective analyses. Clearly, therefore, JMs contain substantial levels of single strands that have undergone genetic alteration at the central marker position.

In each of these two analyses, one other single-stranded species is detected. Each of these second species extends from the left-most to the right-most XhoI marker of the corresponding (detected) parental chromosome, and they are therefore designated D′ and M′, respectively (Figure 2D). The majority of these strands are presumably truly parental in genetic composition and arose from JMs in which the corresponding duplex segment contained uncleaved information on both of its two complementary single strands. A minority of these strands, however, could have arisen from a JM in which the corresponding duplex segment contained heteroduplex DNA at the central marker position, as such heteroduplex DNA would also be

refractory to cleavage. For such JMs, the released single strand might be either genetically parental or genetically recombinant.

In the other two analyses, the secondary enzyme and the homolog-specific probe recognize the same parental chromosome. In each of these cases, a single-stranded species is detected that is uncleaved at the central position (R5 and R6; Figure 2D). These species necessarily arose from JMs in which some change in genetic content had occurred at the central marker position within the relevant duplex segment. R5 and R6 comprise 17% of total detected strands in their respective cases, again suggesting that JMs contain substantial levels of genetically altered single strands. Within such JMs, the relevant duplex segment either could have the same (uncleaved) information on both strands at the central marker position or could be (uncleaved) heteroduplex at that position. In each of these two analyses, one additional single-stranded species is detected, which is both physically and genetically parental (M″ and D″; Figure 2D).

There is a discrepancy between the levels of recombinant strands detected in the two types of analyses, 36% versus 17% for strands detected with the Dad probe and 28% versus 17% for strands detected with the Mom probe. This discrepancy is not expected. It is likely attributable to some peculiarity of the analysis rather than to the nature of JMs, because it is observed irrespective of the nature of the secondary restriction digest or the particular central marker involved.

This discrepancy appears to reflect an excess of the shorter species as compared with the longer species in each of the four analyses, i.e., R3 versus D′, M″ versus R5, R4 versus M′, and D″ versus R6. Two possible explanations for this discrepancy are, first, preferential failure to recover longer strands owing to nonspecific nicking or inefficient cross-link removal (or both) or second, double-stranded cleavage at molecules heteroduplex at the central marker site. According to either of these explanations, the true percentage of recombinant strands visualized with each probe would be approximately the average of the two disparate values, 23% of Mom-containing strands and 26% of Dad-containing strands, or ~25% of all strands.

The observed discrepancy cannot be explained by the presence of large numbers of JMs containing uncleaved heteroduplex sites, as such sites would normally be refractory to cleavage, and their presence would thus result in an excess of the longer species.

JMs Are Double Holliday Junctions

Previous analysis has placed limits on the possible structures of interhomolog JMs (Schwacha and Kleckner, 1994; Byers and Hollingsworth, 1994). Since XhoI-digested JMs contain only parental-length single strands, JMs cannot contain an unrepaired DSB. Furthermore, a JM cannot comprise a single Holliday junction, because that structure contains two parental and two recombinant strands (Figure 3A). Several structures that are compatible with experimental data have been suggested previously (Schwacha and Kleckner, 1994). The two component duplexes might lie side by side, connected only by psoralen cross-links

Possible JM structures		Recomb. *XhoI* single strands ?	Resolved by RuvC/EndoVII ?
A	single HJ	+	+
B	side-by-side duplexes	-	-
C	hemicatenane	-	-
D	double HJ (2 chain)	-	+
E	double HJ (4 chain)	+	+
F	antiparallel double HJ	n.a.	+
	JMs (observed)	-	+

Figure 3. Evaluation of Possible Interhomolog JM Structures

Structures A–F represent conceivable forms for branched molecules containing strands corresponding to two homologous nonsister duplexes. The observation that XhoI-digested JMs contain single strands only of parental length excludes structures A, E, and F; the observation that XhoI-digested JMs are cleaved into sensible products by RuvC excludes structures B and C; structure D is compatible with all observations. The Holliday junctions (HJs) in structures D and E are parallel; that in structure F is one of several types of anti-parallel Holliday junctions (for discussion see Fu and Seeman, 1993). n.a., not applicable.

(Figure 3B). A JM could be a hemicatenane in which the two duplexes are topologically intertwined at a single point (Figure 3C). Or, finally, JMs could contain two (or any even number of) Holliday junctions involving the same two crossing strands (Figure 3D).

To distinguish among these possibilities, we analyzed JMs for their susceptibility to RuvC and T4 endonuclease (endo) VII. RuvC cleaves the noncrossover strands of Holliday junctions and *recA*-promoted three-stranded junctions, but does not cleave Y junctions, base pair mismatches, heteroduplex loops, or single-stranded extensions (Iwasaki et al., 1991; Dunderdale et al., 1991; Benson and West, 1994; Bennett and West, 1995). T4 endo VII cleaves Holliday junctions efficiently, but also recognizes other related structures not cleaved by RuvC (Kemper et al., 1984; Jensch and Kemper, 1986; Kleff and Kemper, 1988). Both enzymes would resolve a multiple Holliday junction structure into two separate duplexes that could have either a parental or a recombinant configuration of flanking markers, depending upon the isomeric state of the junction. Neither enzyme has been tested explicitly on side-by-side duplexes or hemicatenanes; RuvC, however, exhibits a sufficiently narrow substrate specificity that reactivity with these types of structures is not expected.

For this analysis, a preparation of JMs was purified from XhoI-digested psoralen-treated meiotic DNA (see Figure 2, legend; Experimental Procedures). One aliquot of the preparation was examined in the appropriate two-dimensional analysis to confirm the absence of recombinant single strands (Figure 4A). Two other aliquots were treated separately with each of the two resolvases, and the products were analyzed by one-dimensional gel electrophoresis without prior removal of psoralen cross-links. Treatment of purified JMs with RuvC converts more than 90% of JMs into separated duplexes, of which approximately half are parental and half are recombinant for flanking

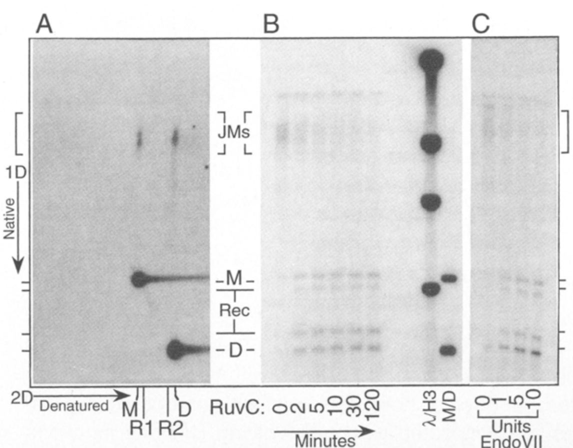


Figure 4. Susceptibility of Purified JMs to Enzymes That Cleave Holliday Junctions

(A) Approximately 4 pg of JMs was analyzed to confirm the absence of component single strands recombinant for XhoI markers (as in Figure 2B; control fragments are a mixture of the two parental XhoI species).

In (B) and (C), about 1 pg of JMs per lane was treated with RuvC and endo VII, respectively. Unbranched duplex products are either parental (Mom [M] or Dad [D]) or recombinant (Rec) with respect to flanking XhoI sites (see Figure 1). Size markers are λH3 (21–4.4 kb) and a mixture of the two parental XhoI duplex fragments (M/D). First dimension separation, hybridization, and detection with probe A (see Figure 1) was carried out identically in (A) and (B) to permit direct comparisons among corresponding species. (A) and (B) analyze the same preparation of purified JMs shown in Figure 2A; (C) analyzes an analogous preparation.

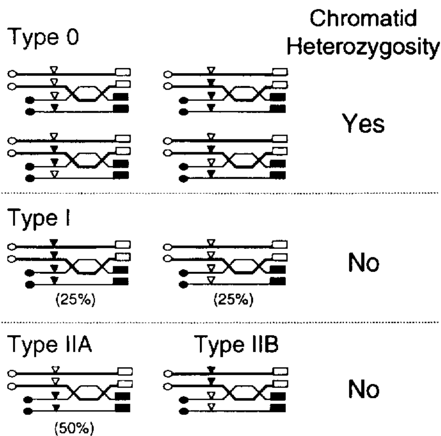


Figure 5. Chromatid Heterozygosity within Double Holliday Junctions

Three types of *HIS4–LEU2* JMs. Closed and open triangles indicate the *MluI* and *BamHI* alleles, as in Figure 1. Type 0 JMs exhibit chromatid heterozygosity, while type I and type II JMs do not (see text). Distribution inferred for JMs at *HIS4–LEU2* is 50% type I plus 50% type IIA. The two types of type I JMs are those expected from DSBs on the two parents. Type IIA JMs are symmetrical with respect to marker information and would thus arise irrespective of which parent had the DSB. Type IIB JMs are unlikely to occur at any appreciable frequency; resolution of these forms would yield two-strand double crossovers, which are infrequent relative to simple gene conversions in yeast in general (Fogel et al., 1979) and this locus in particular (A. Storlazzi, L. Cao, and N. K., unpublished data).

markers (Figure 4B). Treatment with T4 endo VII produces an identical result (Figure 4C).

We conclude from these results that JMs comprise an even number of Holliday junctions, presumably two.

Physical Properties of JMs

The physical properties of JMs can be defined further in two respects. First, if the structure is drawn in planar projection with the two arms of each parental duplex in *cis* to one another, the same pair of DNA single strands is involved in the "crossing" at both junctions (see Figure 3D). Either both crossed strands are Watson and both noncrossed strands are Crick, or vice versa. If the two junctions involved two different pairs of strands, all four strands within the molecule would be recombinant with respect to flanking markers (see Figure 3E) rather than none, as is observed.

Second, the arms emanating from each of the two component Holliday junctions must globally be in a parallel configuration rather than an anti-parallel configuration (for discussion see Fu et al., 1994). Anti-parallel junctions would contain strands that are neither parental nor recombinant with respect to terminal XhoI sites (see Figure 3F; Fu and Seeman, 1993). This feature corresponds to the fact that genetically sensible products arise only if the double junctions are parallel.

Any single Holliday junction might exist in space as either of two geometric isomers depending upon the relative positions of flanking chromosome arms (Sigal and Alberts, 1972; Sobell, 1974; Meselson and Radding, 1975), and JMs might thus exist as any of four possible geometric isomers. The only information provided by this analysis regarding isomerization status is that the JM population must comprise more than one geometric isomer before and/or during the course of the RuvC cleavage reaction. Since RuvC cleaves only the noncrossover strands of a Holliday junction (Bennett and West, 1995), cleavage of a homogeneous JM population would yield only parental or only recombinant duplexes.

Constrained Genetic Composition of Double Holliday Junctions

Within any particular JM, one pair of complementary strands is a continuation of the Mom chromatid and the other is a continuation of the Dad chromatid. With respect to any particular genetic marker, then, the two complementary strands of any given pair may contain either the same or different information. We define the latter condition as chromatid heterozygosity. Examples of JMs that do and do not exhibit chromatid heterozygosity for a DSB-associated marker (e.g., *MluI/BamHI*) are shown in Figure 5.

Existing observations suggest that double Holliday junction recombination intermediates, as a general feature, do not exhibit chromatid heterozygosity.

Any JM that exhibits chromatid heterozygosity should contain heteroduplex DNA. In fact, however, cross-linked and noncross-linked DNA both have been examined for the presence of heteroduplex in three independent studies involving poorly corrected DSB-associated markers (Goyon and Lichten, 1993; Nag and Petes, 1993; Schwacha and Kleckner, 1994). In all three cases, DNA

restriction fragments containing heteroduplex DNA were not observed at times when JMs would have been present in vivo. Instead, such fragments are detected only at the the very end of the recombination process, concomitant with the appearance of mature crossover and noncrossover products and, in the one case examined, concomitant with the disappearance of JMs. The failure to detect heteroduplex DNA early is unlikely to reflect a failure to recover heteroduplex-containing fragments from branched intermediates: one study specifically examined very small fragments that should have been recoverable from a JM (Goyon and Lichten, 1993).

Furthermore, if meiotic DNA is prepared without psoralen cross-linking treatment, the yield of JMs is reduced by ≥80% (Bell and Byers, 1983; Schwacha and Kleckner, 1994), and no other branched molecules appear in their stead (Schwacha and Kleckner, 1994). Since we now know that JMs are double Holliday junctions, we can infer that when cross-linking treatment is eliminated each JM is converted to a pair of simple linear duplexes, almost certainly by branch migration of the two junctions that move in tandem toward and then off one end of the molecule (Fu et al., 1994; see Experimental Procedures).

Given that type 0 JMs are absent, the experimental data presented here specify the composition of the current JM population in more detail. JMs that lack chromatid heterozygosity are of two general types. In type I JMs, all four component strands have identical central marker information; in type II JMs, one pair of complementary strands has MluI information and the other has BamHI information (Figure 5). In type I JMs, 50% of single strands are recombinant by the experimental criteria defined in the current analysis; in type IIA JMs, 0% of single strands are recombinant; type IIB JMs are not plausible intermediates in DSB-promoted recombination (Figure 5, legend). To obtain the observed level of 25% recombinant strands, 50% of JMs must be of type I and 50% must be of type IIA. It should be noted that this conclusion does not require any assumption regarding the extent to which *MluI/BamHI* heteroduplexes are, or are not, readily mismatch corrected.

Discussion

Temporal Progression of Meiotic Recombination

The current observations, together with previous physical and temporal analysis, provide the following picture of the progression of meiotic recombination in yeast. Meiosis-specific DSBs appear in early prophase, at about 2.5 hr after initiation of meiosis in the current situation and prior to initiation of synaptonemal complex (SC) formation. DSBs are resected very rapidly and then persist in resected form for about 15–30 min. DSBs then disappear, and, concomitantly, double Holliday junctions appear; these events are also concomitant with formation of the SC. Double Holliday junctions persist through much of the 60–90 min when SC is full length (pachytene) and then disappear concomitant with the appearance of both crossover and noncrossover recombination products. SC disassembles shortly thereafter (Cao et al., 1990; Sun et al., 1989, 1991; Schwacha and Kleckner, 1994; Padmore et al., 1991; L. Xu and N. K., unpublished data).

These temporal studies and all other available information are consistent with conversion of one intermediate into the next via a single pathway that branches into crossover and noncrossover components at the chemical level at the very end. The evidence that crossover and noncrossover products both arise from DSBs and that double Holliday junctions arise from DSBs is now very good (Storlazzi et al., 1995; this work). It is likely a priori that double Holliday junctions ultimately yield crossover products, and all existing data are consistent with the possibility that they yield noncrossover products as well. Viable models have been suggested, however, in which noncrossover products are generated without involving Holliday junctions (Resnick, 1976; Hastings, 1988).

Physical analysis has also shown that experimentally detectable heteroduplex DNA appears just before, or concomitant with, the appearance of mature recombination products. This heteroduplex DNA almost certainly corresponds to that detected genetically as postmeiotic segregation: in each of three analyses involving poorly corrected allelic pairs, the final level of heteroduplex DNA observed by physical analysis corresponds closely to that observed genetically in the same strain: 0.7% and 0.5%, respectively, at *ARG4*, 6% and 5% at *HIS4*, and 2% and 3% at *HIS4–LEU2* (Lichten et al., 1990; Nag and Petes, 1993; Schwacha and Kleckner, 1994; Storlazzi et al., 1995). Thus, assuming that a double Holliday junction is an obligatory intermediate for all observed products of meiotic recombination (see above), the appearance of experimentally detectable heteroduplex DNA very likely reflects a late and relatively short-lived transitional stage in the recombination process that is concomitant with Holliday junction resolution.

These considerations lead to the specific notion that double Holliday junctions form in a way that precludes the occurrence of type 0 JMs and are then resolved in such a way as to yield both the late-arising heteroduplex DNA detected in physical experiments and the patterns of genetic marker segregation known to characterize recombination in yeast. A model for meiotic recombination that has these features is presented in Figure 6. One important feature of this model, which is required by the constrained composition of JMs, is that correction of mismatched base pairs occurs twice, during both formation and resolution of double Holliday junctions. In both cases mismatch correction is biased by interruptions in the DNA: DSBs at the first stage and nicks resulting from junction resolution in the second stage. Resolution-directed mismatch correction has been suggested by Alani et al. (1994) as one possible explanation for the patterns of aberrant segregation observed in wild type and in mutants deficient in mismatch correction.

Topological Resolution of Double Holliday Junctions into Noncrossover Products

A double Holliday junction potentially can be converted into a pair of mature noncrossover recombination products without the action of a junction-specific nuclease via the

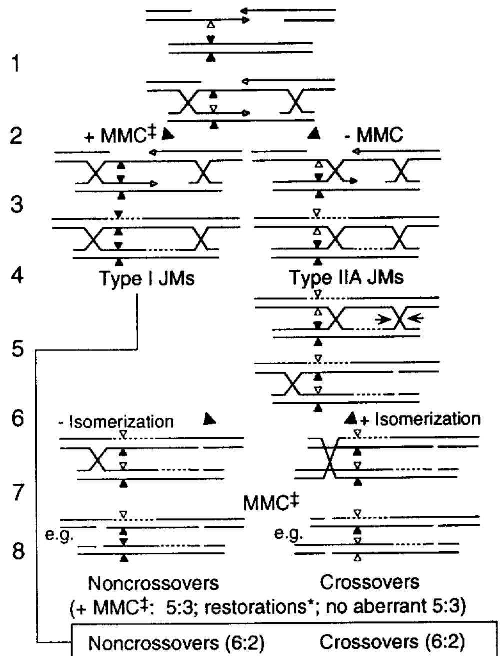


Figure 6. Model for DSB-Promoted Meiotic Recombination

(1–3) Formation of double Holliday junctions. The 3′ single-stranded tails of the DSB invade an intact duplex (1). Heteroduplex DNA formed at the resection-associated marker may be eliminated by mismatch correction (2, left). In accord with biochemical data (Holmes et al., 1990; Thomas et al., 1991), correction is biased such that the intact strand is used as template (MMC‡, biased mismatch correction). Double Holliday junctions formed after such a process are type I exclusively (3, left). Alternatively, or possibly always, the Holliday junctions migrate to an asymmetric position such that both junctions are located to one side of the mismatch (2, right). As long as such migration precedes filling in of the gap opposite the marker in question, filling in will yield a type IIA JM (3, right). By these two scenarios, then, chromatid heterozygosity within JMs is avoided, and the two necessary JM forms are produced. Reverse branch migration of a single strand exchange junction has been suggested previously as one possible mechanism for eliminating the formation of symmetric heteroduplex DNA at this stage (Radman, 1989; Alani et al., 1994).

(4–8) Resolution of double Holliday junctions. Type IIA JMs must be resolved in a way that yields physically detectable heteroduplex DNA and the patterns of marker segregation observed genetically. One of the two Holliday junctions is resolved by cleavage specifically of crossing strands (4). Then, at least in events that ultimately yield heteroduplex DNA and postmeiotic marker segregation, the remaining junction migrates backward across the marker site, thus yielding (transient) symmetrical heteroduplex DNA (5). Next, in all events, the remaining junction either isomerizes or does not (6) and is then resolved, again by cleavage of the crossing strands (7). A second round of mismatch correction then occurs prior to ligation of the cleaved strands, again biased such that the intact strand is used as a template and at an appropriate efficiency that is less than 100% (8). Correction of one mismatch at this stage will yield tetrads exhibiting 5:3 marker segregation; correction of both mismatches at this stage will yield normal 4:4 marker segregation (restorations*). Type I JMs that undergo this same sequence of resolutions will ultimately yield tetrads that exhibit 6:2 segregation.

This model explains several constraining genetic observations. For example, in noncrossover tetrads, 5:3 segregations are potentially of two types, normal and "aberrant" (see Figure 3 in Szostak et al., 1983); in yeast, only the former are observed. More generally, aberrant 4:4 and 6:2 segregations, as well as aberrant 5:3 segregations, are expected to arise from random mismatch correction of symmetrical heteroduplex DNA, and all of these are rare in yeast. The current model involves formation of symmetric heteroduplex DNA (5). Recent observations have been interpreted as evidence that symmetric heteroduplex DNA occurs in yeast in the absence of mismatch correction (Alani et al., 1994); in the model shown, symmetrical heteroduplex DNA is a regular feature of meiotic recombination in a wild-type cell. In the current model, all three prohibited segregation patterns are excluded as a consequence of the proposed nick-biased mismatch correction, a possibility also suggested by Alani et al. (1994). This explanation will hold for any model in which noncrossovers arise by nuclease cleavage of the two junctions: proper mismatch correction bias is maintained independently of whether resolution occurs by cleavage of crossing or noncrossing strands (or both). This explanation will not hold for models in which noncrossovers arise exclusively via topoisomerase-mediated resolution. Genetic data also indicate that for a marker in a coding region, with DSBs upstream, the sense strand is always the donor of information in 5:3 tetrads (e.g., Lichten et al., 1990; Nag and Petes, 1990). The current model is consistent with these data. For previous considerations of these issues, see Figure 9 in Szostak et al. (1983), Lichten et al. (1990), Porter et al. (1993), and Alani et al. (1994).

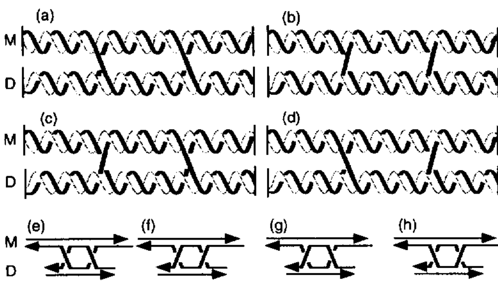


Figure 7. Four Types of Parallel Double Holliday Junctions

(a) Standard structure in which both junctions are unbraided.

(b–d) Structures in which both junctions are braided (b) or the left or right junction only is braided (c and d). In an unbraided junction, one crossing strand passes across the front face of both helices and in front of the other crossing strand; in a braided junction, one crossing strand passes across the back of one helix, on top of the other crossing strand, and then across the back of the other helix (Fu et al., 1994). Braided junctions are energetically less favorable than unbraided junctions (Sobell, 1974; Fu et al., 1994).

(e)–(h) show representations of the molecules in (a)–(d), respectively, in which interlinks between complementary strands have been omitted. In structures (e) and (f), participating chromatids are connected by a single interlink between same-polarity strands; in structures (g) and (h), no such interlink is present.

action of a topoisomerase (e.g., Thaler et al., 1987). This type of reaction could be relevant during meiosis in either of two situations. First, during normal meiotic recombination, noncrossovers might arise via a topoisomerase route, while crossovers arise via a junction-specific nuclease (contrary to the model proposed above). Second, topoisomerase action could provide a backup mechanism for resolving double Holliday junctions that persist aberrantly beyond pachytene and into anaphase of meiosis I.

Topoisomerase-mediated processing of a double Holliday junction would have to take into account several features not previously appreciated in this context. A parallel double Holliday junction is usually represented as a molecule in which the pair of same-polarity crossing strands at each junction is in an unbraided form (Fu et al., 1994; Figures 7a and 7e). In vivo, the two chromatids participating in such a structure would be topologically linked via two different types of connections: intertwinings between complementary strands in the region between the two junctions (Figure 7a), plus an additional single interlink

between noncomplementary same-polarity strands (Figure 7e).

The intertwinings between complementary strands can, in principle, be eliminated directly by a type I topoisomerase or indirectly by conversion of interstrand "twist" to duplex backbone "writhe," which is in turn eliminated by a type II topoisomerase. Elimination of these intertwinings would convert the double Holliday junction structure into a hemicatenane structure (Figure 3C). The remaining single same-polarity intertwining could then be resolved via either another topoisomerase reaction, for which only a type I activity will suffice, or a nick ligation process.

Additional complexities are possible if braided crossing strands are allowable within parallel double Holliday junctions (Figures 7b–7d and 7f–7h, legend).

Experimental Procedures

Strains, Growth Conditions, and DNA Extraction

NKY2598 is identical to NKY1962 (Schwacha and Kleckner, 1994) except for the addition of a BamHI linker at the MluI site within the Mom construction (Figure 2). The resulting *BamHI* allele is analogous to a better-characterized version (Xu and Kleckner, 1995), but was constructed independently; the number of BamHI linkers is unknown. Synchronous meiosis, DNA extraction, and psoralen cross-linking were performed as described previously (Schwacha and Kleckner, 1994), with DNA samples taken 4 hr after initiation of meiosis.

JM Purification

JMs were purified away from cross-hybridizing material that comigrates with JMs under gel electrophoresis conditions in which molecules migrate according to mass (e.g., Figure 2A). Meiotic DNA was separated by one-dimensional electrophoresis under conditions in which migration of branched DNA is retarded (1.3% Seakem Gold agarose [FMC BioProducts] in 1 × TBE without ethidium bromide at 1.6 V/cm for 14 hr). JMs were eluted from an appropriate gel fragment using a Qiaex purification kit (Qiagen). Recovery was estimated to be 10% of total JMs. The final preparation also contains a 100-fold excess of nonhybridizing DNA.

Two Dimensional Electrophoresis

Analyses were performed as described previously (Schwacha and Kleckner, 1994), with the addition that, for in situ digestion, each gel slice was equilibrated with appropriate digestion buffer and then incubated with 1000 U of the appropriate enzyme for 18 hr at 37°C. The resulting gel slices were then rinsed exhaustively in 10 mM EDTA and 0.1% SDS and subjected to cross-link removal as described previously (Schwacha and Kleckner, 1994). Digestion was ≥96% efficient in every case. In experiments using purified JMs, internal standards of purified Mom and Dad duplex fragments were mixed with JMs at the beginning of the experiment. Hybridization probes were generated by random priming or symmetric PCR (GIBCO BRL kit 10199-016) and thus detected both strands in the region of interest. DNA species were quantitated using a Fuji BAS2000 phosphoimager (Schwacha and Kleckner, 1994).

Resolution of JMs with RuvC and Endo VII

Each reaction contained ~1 pg of JMs plus a 100-fold excess of copurifying, nonhybridizing DNA. RuvC reactions contained in addition 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 2.5 mM DTT, 250 µg/ml acetylated BSA, and 25 ng of RuvC protein in a 20 µl volume; reaction mixtures were incubated at 55°C for the indicated times and stopped by addition of EDTA to 10 mM and SDS to 0.1% final concentrations, followed by transfer to ice. Endo VII reactions were identical, except that digestion was carried out at 37°C for 30 min with the indicated units of protein. Gel electrophoresis was in 0.6% agarose for 24 hr at 1.6 V/cm. RuvC was provided by H. Dunderdale and S. West; endo VII was a gift from B. Kemper.

Decay of Noncross-Linked JMs

JMs likely decay into two linear duplexes by tandem branch migration of the two junctions out of the region or off the ends of restriction-digested molecules during the isolation or digestion procedure (Fu et al., 1994). The decay of double Holliday junction intermediates is specifically counteracted by treatment with psoralen (Schwacha and Kleckner, 1994), an agent that prevents branch migration but should not stabilize intermediates against decay by nicking of strand exchange junctions. Also, all of the DNA extraction procedures used for heteroduplex DNA analysis involve conditions that favor Holliday junction branch migration, i.e., high temperatures in the absence of divalent metal ions (Panyutin and Hsieh, 1994; Panyutin et al., 1995). Furthermore, branch migration likely occurs to a limited extent even in psoralen-cross-linked molecules. Efficient resolution of Holliday junctions by RuvC requires branch migration, and JM resolution by RuvC is enhanced by the same reaction conditions (elevated temperatures and alkaline pH) that stimulate both the resolution and branch migration of synthetic single Holliday junctions (data not shown; Shah et al., 1994). We note, however, that the conclusions drawn above would apply even if JMs decay by nonspecific nicking at the Holliday junctions, rather than by branch migration.

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