A 140-bp-Long Palindromic Sequence Induces Double-Strand Breaks During Meiosis in the Yeast *Saccharomyces cerevisiae*

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ABSTRACT

Palindromic sequences have the potential to form hairpin or cruciform structures, which are putative substrates for several nucleases and mismatch repair enzymes. A genetic method was developed to detect such structures *in vivo* in the yeast *Saccharomyces cerevisiae.* Using this method we previously showed that short hairpin structures are poorly repaired by the mismatch repair system in *S. cerevisiae.* We show here that mismatches, when present in the stem of the hairpin structure, are not processed by the repair machinery, suggesting that they are treated differently than those in the interstrand base-paired duplex DNA. A 140-bp-long palindromic sequence, on the contrary, acts as a meiotic recombination hotspot by generating a site for a double-strand break, an initiator of meiotic recombination. We suggest that long palindromic sequences undergo cruciform extrusion more readily than short ones. This cruciform structure then acts as a substrate for structure-specific nucleases resulting in the formation of a double-strand break during meiosis in yeast. In addition, we show that residual repair of the short hairpin structure occurs in an *MSH2*-independent pathway.

INVERTED repeated sequences are found naturally in prokaryotic and eukaryotic genomes. Many of them are present in functionally important regions such as operator sequences, replication origins and transcription-termination sites, and play important roles with respect to the function of these regions. The sequence arrangement of inverted repeats allows the sequence to exist in one of two alternative structures: the normal interstrand base-paired DNA duplex or a cruciform structure with intrastrand base pairing. Cruciform structures are putative substrates for several structure-specific nucleases such as Holliday-junction resolvases and mismatch repair enzymes. Several key questions arise in understanding the stability of inverted repeated sequences in the genome: if cruciform structures are cleaved by cellular nucleases, how are these sequences maintained within the cell? Do these sequences form cruciform structures *in vivo*?

Several genetic experiments suggest that cruciform formation is responsible for inverted repeat-mediated genomic instability observed in various organisms. Although inverted repeats naturally occur in the DNA of many organisms, palindromic sequences longer than 150–200 bp on plasmids are difficult to maintain in *Escherichia coli* (COLLINS 1981; HAGEN and WARREN 1982; LEACH and STAHL 1983; SINDEN *et al.* 1991; LEACH 1994). Long palindromic sequences, however, can be maintained in a nuclease deficient strain of *E. coli.* A novel two component system, *sbcCD,* has been shown to regulate the stability of palindromic sequences in bacteria (LEACH 1994).

Palindromic sequences also stimulate deletion formation (GLICKMAN and RIPLEY 1984; WESTON-HAFER and BERG 1991; GORDENIN *et al.* 1993; RUSKIN and FINK 1993). Such deletion formation has been shown to occur between direct repeats that flank the inverted repeat or between directly repeated sequences in which one direct repeat occurs within and one occurs outside but near the inverted repeats. It has been suggested that palindrome-mediated deletion formation occurs when replication machinery enters the palindrome and stalls after progressing some distance. The nascent strand then dissociates and can hybridize to any complementary strand that is nearby. Such sequences are normally found downstream of the palindrome (LEACH 1994).

In vitro several different palindromic sequences have been shown to form cruciform structures in negatively supercoiled DNA (LILLEY 1980; MIZUUCHI *et al.* 1982; LEACH 1994). Initial studies involving crosslinking of DNA strands failed to detect cruciform structures *in vivo* (CECH and PARDUE 1976; SINDEN *et al.* 1983; LEACH 1994). Kinetic studies also suggested that cruciform structures are rarely or probably not formed *in vivo* (COUREY and WANG 1983; GELLERT *et al.* 1983). Recent *in vivo* experiments suggest that palindromes below the size limit that causes inviability in the wild-type host are hard to detect in the cruciform conformation. To detect significant amounts of cruciform DNA specifically designed palindromes (such as AT-rich centers and GC-

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rich stems), specialized hosts and/or conditions are necessary (McClellan *et al.* 1990; Sinden *et al.* 1991; Zheng *et al.* 1991). However, a genetic method (described below) was developed to detect hairpin structures in the heteroduplex DNA (hDNA) in the yeast *Saccharomyces cerevisiae* (Nag *et al.* 1989).

Heteroduplex DNA is formed as an intermediate of genetic recombination. In yeast, such hDNA formation and the repair of mismatches in the hDNA are usually monitored by following the segregation of heterozygous markers during meiosis (Fogel *et al.* 1981; Petes *et al.* 1991). During meiosis, two heterozygous alleles *A* and *a* usually follow the normal Mendelian segregation pattern of 4*A*:4*a* (following the nomenclature of eight-spore producing fungi). Two types of aberrant (ab.) events are also observed: gene conversion (6*A*:2*a* and 2*A*:6*a* tetrads) and postmeiotic segregation (PMS) events (5*A*:3*a*, 3*A*:5*a*, ab. 4*A*:4*a* tetrads) (Fogel *et al.* 1981; Petes *et al.* 1991). In PMS events, one or more of the spore colonies derived from a single tetrad have a sectored (*A/a*) phenotype. PMS events simply reflect the presence of hDNA in the spore DNA.

According to the current models of recombination (Meselson and Radding 1975; Szostak *et al.* 1983), hDNA is formed as an intermediate by the transfer of a DNA strand from a donor to a recipient chromatid. If the heteroduplex is formed between a wild-type gene and a mutant allele, and the hDNA covers the site of the mutation, a mismatch is generated. Gene conversion is a result of the repair of mismatches in the hDNA. A failure to repair the mismatch results in a PMS event. For most heterozygous alleles, PMS rates are low (3–19% per aberrant segregation), indicating that the mismatch repair system in yeast is very efficient (Fogel *et al.* 1981; Detloff *et al.* 1991; Alani *et al.* 1994). Base substitution mutations that lead to the generation of a C-C mismatch and palindromic-insertion mutations show a high level of PMS events (33–54% and 60–80% per aberrant segregation, respectively) (Fogel *et al.* 1981; Nag *et al.* 1989; Detloff *et al.* 1991; Alani *et al.* 1994).

In the hDNA formed between a wild-type strand and an insertion-mutant strand, a loop is generated because of the presence of extra sequences on the mutant strand. However, if the mutation is due to palindromic insertions, the inserted sequence on the mutant strand has the potential to fold back on itself to form a stem-loop (hairpin) structure. Such hairpin structures are poorly repaired by the mismatch repair system in yeast. The conclusion that the high PMS rate is due to the hairpin formation is based on the following observations: (1) disruptions of the hairpin structure caused by introducing mismatches in the stem of the hairpin decreased the PMS rate, (2) compensatory changes that restore the hairpin structure increased the PMS rate, (3) all palindromic sequences examined showed a high level of PMS, and (4) palindromic insertions at different loci similarly increased the rate of PMS events (Nag *et al.* 1989). The inefficient repair of the hairpin structure led to the proposal that hairpin structures are either ignored by the repair machinery or protected from the repair enzymes due to the binding of a structure-specific protein(s).

Mismatches in the recombination intermediates are processed in yeast by a pathway that is analogous to the *E. coli* MutHLS pathway, also known as a long-patch repair pathway (for details see Modrich 1991; Kolodner 1996; Modrich and Lahue 1996). The bacterial MutHLS pathway repairs a broad spectrum of mismatches including small insertions and deletions that are generated as a result of errors in DNA replication or in the recombination intermediates. A C-C mispair, and insertions or deletions of more than 4 bp are poorly repaired by this pathway (Modrich 1991; Parker and Marinus 1992). The MutS protein is responsible for mismatch recognition. While the functional activity of MutL is not known, it interacts with MutS bound to a mispaired base and is required for the activation of MutH, the endonuclease that initiates the excision-repair process.

Several genetic studies resulted in the identification of a number of *mutL* and *mutS* homologues of *S. cerevisiae* (Kramer *et al.* 1989; Reenan and Kolodner 1992; New *et al.* 1993; Prolla *et al.* 1994; Kolodner 1996; Modrich and Lahue 1996). Of six *mutS* homologues (*MSH1–MSH6*) that have been identified in yeast three (*MSH2, MSH3,* and *MSH6*) have been shown to participate in the mismatch repair pathway. Several genetic experiments suggested that *MSH3* and *MHS6* encode redundant functions that act in a Msh2-dependent pathway (Marsischky *et al.* 1996). Two MutL homologous activities of *S. cerevisiae,* Mlh1 and Pms1, interact with Msh2 bound to a mispaired base, suggesting that both MutL and its homologues play a similar role in mismatch repair in yeast. A mutation in any of these genes results in a mutator phenotype and an increased rate of PMS events with a simultaneous decrease in the gene conversion rate for otherwise well repaired mutant alleles (Kolodner 1996; Modrich and Lahue 1996). However, gene conversion events were not absolutely abolished in the mutant background suggesting the presence of an inefficient repair pathway that operates in an *msh* or *mlh* background. Heteroduplex DNA containing hairpin structures results in 60–80% PMS and 20–40% gene conversion events (Nag *et al.* 1989; Detloff and Petes 1992). It is not known if the residual repair occurs by the Msh2-dependent pathway.

The length of DNA processed during the repair process has been studied in detail in both bacteria and yeast. The repair tracts in different bacteria have been shown to range from 3 to 10 kb (Guild and Shoemaker 1976; Wagner and Meselson 1976; Mejean and Claverys 1984). Coconversion studies and the measurement of the length of the hDNA revealed that the repair

tracts in yeast vary from less than 1 to 3.7 kb (DICAPRIO and HASTINGS 1976; BISHOP and KOLODNER 1986; BORTS and HABER 1989; PETES *et al.* 1991; DETLOFF and PETES 1992). Bacteria and mammalian cells possess another repair pathway, a short-patch pathway, in which the repair tract is short, typically limited to 10 nucleotides or less (MODRICH 1991). The elements that control the outcome of the short-patch repair pathway are the nature of the mismatch and its immediate sequence environment. Several proteins involved in the MutHLS repair system are also required in the short-patch repair pathway (MODRICH 1991). Although the short-patch pathway has been shown to be present in bacteria and mammalian cells, such a pathway has yet to be demonstrated in yeast.

We initiated the work presented here to investigate whether the mismatches present in the stem of the hairpin structure can be recognized and repaired by the mismatch repair system in yeast and to study the effect of long palindromic sequences on meiotic recombination and mismatch repair. We were also interested to see if a short-patch repair process exists in yeast. Our results indicate that mismatches when present in the stem of the hairpin structure are not processed by either pathway and that long palindromic sequences act as meiotic recombination hotspots.

MATERIALS AND METHODS

Yeast strains: The genotypes of all yeast strains are shown in Table 1. All strains used in this study were derived from AS4 and AS13 (STAPLETON and PETES 1991). AS13 × AS4 diploids show a high rate of meiotic recombination at the *HIS4* locus (NAG *et al.* 1989). All *his4* mutant alleles (except *his4-21*) were introduced into the AS13 chromosome by replacing wild-type sequences using a two-step transplacement procedure (ROTHSTEIN 1991). The *his4-21* mutant allele was introduced into the AS4 chromosome. The His^- haploid derivatives were confirmed by Southern analysis. The His^- cells were mated with AS4 or its derivatives to make the diploids. The *rad50S* mutation was introduced by a one-step transplacement by transforming the haploid strains with *Eco*RI- and *Bam*HI-treated pNKY349 and selecting for the Ura^+ transformants. The *msh2::Tn10LUK7-7* mutant allele was introduced into the chromosome using the plasmid pII-2::Tn10LUK7-7 (REENAN and KOLODNER 1992). This plasmid was digested with *Spe*I before yeast transformation to release a 9.6-kb fragment that was separated by gel electrophoresis. Standard techniques were used for all genetic manipulations (ROSE *et al.* 1990). PD3 was kindly provided by TOM PETES. AS13 was made Leu^+ by replacing the *leu2-Bst* allele with the wild-type gene to generate DNY221.

Plasmids: Standard molecular biology procedures were used for all plasmid constructions (MANIATIS *et al.* 1992). All oligonucleotides were inserted into the unique *Sal*I site present in the plasmid pDN9 (NAG *et al.* 1989). The plasmid pDN9 was constructed by cloning the *Xho*I-*Bgl*II fragment of *HIS4* into *Bam*HI-*Sal*I-treated YIp5. The unique *Sal*I site is present in the *HIS4* sequences. The oligonucleotides have *Sal*I-compatible T-C-G-A sequence at the 5′ end (Figure 1). Oligonucleotides were made double stranded before cloning by annealing with the complementary strands. The oligonucleotide that was used to generate the *his4-9* mutant allele is perfectly palindromic and it was self annealed to make it double stranded. The *his4-140* mutant allele was constructed as follows: the plasmid pBR322-PAL114 (WARREN and GREEN 1985) was digested with *Bam*HI to release a DNA fragment containing a 114-bp palindromic sequence that was then ligated into the *Bam*HI site of pDN19. The plasmid pDN19 has a 32-nucleotide-long palindromic insertion at the *Sal*I site of pDN9. The inserted oligonucleotide has a *Bam*HI site at its center of symmetry. The resulting plasmid, pDN34, has an insertion of a 140-bp palindromic sequence at the *Sal*I site of the *HIS4* sequence. The final sequence of the inserted inverted repeat is 5′ TCGAGTACTGTATGTGGATCCGGGCAACGTTGTTG CCATTGCTGCAGGCGGAGAACTGGTAGGTATGGAAGA TCTTCCATACCTACCAGTTCTCCGCCTGCAGCAATGGC AACAACGTTGCCCGGATCCA CATACAGTAC 3′. The oligonucleotide 5′ TCGAGCGTGTCCGCTCGAGCGGACACGC 3′ was used to generate the *his4-21* mutant allele. The unique *Sna*BI site present in the *HIS4* sequence was used for targeting insertions into the chromosome.

TABLE 1

Yeast strains

Strains	Genotype
AS4	*MATα trp1-1 arg4-17 tyr7-1 ade6 ura3-52*
AS13	*MAT***a** *leu2-Bst ade6 ura3*
PD3	AS4 with *his4-Sal*
MLY15	AS4 × AS13 with *his4-7*
MLY16	AS4 × AS13 with *his4-8*
MLY20	AS4 × AS13 with *his4-9*
MLY21	AS4 × AS13 with *his4-12*
DNY64	AS4 × AS13 with *his4-140*
DNY115	AS4 × AS13 but homozygous for *rad50S*
DNY202	AS4 with *his4-21* × AS13 with *his4-12*
DNY214	DNY64 but homozygous for *rad50S*
DNY216	DNY64 but homozygous for *msh2::Tn10LUK7-7*
DNY217	MLY20 but homozygous for *msh2::Tn10LUK7-7*
DNY221	*MAT***a** *ade6 ura3*
DNY222	DNY221 with *leu2-140*
DNY224	DNY222 × PD3
DNY230	DNY221 with *rad50S* × PD3 with *rad50S*
DNY231	DNY224 but homozygous for *rad50S*

The plasmid containing the *leu2-140* allele was constructed as follows: a 33-nucleotide-long palindromic oligonucleotide (identical to that present in pDN19) containing a *Bst*EII linker at the 5′ end was inserted into the *Bst*EII site in YEp13 to generate pDN70. The *Bam*HI fragment containing the 114-bp-long palindromic sequence was then inserted into the *Bam*HI site, present at the center of symmetry of the inserted oligonucleotide within the *LEU2* gene to generate pDN71. A *Xho*I to *Sal*I fragment derived from pDN71 containing the *leu2-140* mutant allele was inserted into the *Xho*I site of pRS306. The unique *Hpa*I site present in the *LEU2* gene was used for targeting insertion into the chromosome. All plasmid constructions were confirmed by DNA sequencing.

Genetic techniques: Standard genetic methods and media were used (ROSE *et al.* 1990). All diploid strains were sporulated at 25°. The His^+ complementation tests were carried out as follows: diploid strains heterozygous for *his4* mutant alleles were sporulated at 25° on plates and tetrads were dissected on rich YPD plates (ROSE *et al.* 1990). After 3 days of growth at 30°, the spore colonies were replica plated onto a minimal plate lacking histidine. The His^- cells from the sectored colony were streaked out onto YPD plates for single colonies. Approximately five to six patches made from single

colonies were mated with F98 [*a his4-280* (c-1 missense) *trp1*] and F99 [*α his4-280* (c-1 missense) *ade2-1*] for ~20 hr. The His^+ phenotype was monitored by replica plating the mating mixture onto a minimal plate lacking histidine. For tetrad analysis of the DNY202 strain, diploids were sporulated and the tetrads were dissected as described above. After germination, the spore colonies were replica plated onto various types of omission media. The *his4-21* is a frameshift mutation that eliminates all three enzymatic activities of *HIS4*, whereas the *his4-12* mutation inactivates only His4A activity. To score these alleles, we mated spore colonies derived from DNY202 to F98 and F99 tester strains and tested for the His^+ phenotype of the resulting diploids.

Analysis of DSBs in meiotic DNA: DNA was isolated from cells (collected at different time intervals) sporulated in 1% potassium acetate as described by NAG and PETES (1993). To examine the DSBs, the meiotic DNA was digested with *Pvu*II and the resulting fragments were separated on a 0.8% agarose gel. The DNA was transferred to a nylon membrane that was then hybridized with a *Xho*I-*Bgl*II fragment of *HIS4* as a probe. The probe was obtained from pDN42 (NAG and PETES 1993) as a *Xho*I-*Xba*I fragment. The amount of DSBs was quantitated from the 24-hr time point using a Molecular Dynamics Phosphorimager.

RESULTS

Genetic assay for mismatch repair in the stem of the hairpin structure: Short palindromic-insertion mutant alleles generate hairpin structures in heterodplex DNA. Such hairpin structures are poorly repaired by the mismatch repair system in yeast. We took a genetic approach to determine if mismatches present in the stem of the hairpin structure are repaired by the cellular repair machinery. The *HIS4* gene of the yeast *S. cerevisiae* has three functional domains: *HIS4A, HIS4B* and *HIS4C* (KEESEY *et al.* 1979). Each of the three domains can be altered without affecting the others if their reading frames remain intact. A haploid strain containing a mutation in any one of the three domains is His^-. However, if such a haploid strain is mated with another haploid strain containing a mutation in a different domain, a His^+ diploid strain is generated due to intragenic complementation. We introduced several in-frame, nearly perfect, palindromic oligonucleotides into the *Sal*I restriction site (present in the *HIS4A* domain) of the *HIS4* gene. The mutant alleles were introduced into one of the two homologous chromosomes of the diploid strain by replacing the wild-type gene. The oligonucleotides were designed in such a way that when present in the hDNA, the inserted sequences on the mutant strand generate different types of mismatches in the stem of the hairpin structure (Figure 1).

The resulting mismatches, generated in the hairpin structure, can be repaired by one of two pathways. A short-patch repair pathway can repair the mismatch in the hairpin structure to make it a perfect palindrome. Alternatively, in a long-patch pathway, the repair process either eliminates all of the inserted sequence on the mutant strand and repairs the gap using the wild-type strand as a template or adds complementary sequences to the wild-type strand using the mutant strand as a template. All mutant alleles were generated by in-frame insertions and the mismatches, if repaired by the short-patch pathway, will either result in an out-of-frame insertion or generate a stop codon on the mutant strand. Since the rates of PMS and gene conversion events will not be changed in the short-patch repair process, these events can be monitored in a complementation test by mating the His^- cells of the sectored colony with a tester strain containing a *his4C* (*HIS4A HIS4B his4C*) mutation. If short-patch repair occurs, the His^- cells of the sectored colony will not generate His^+ cells when mated with a *his4C* tester strain.

In the case of a long-patch mismatch repair, the repaired product will be either the wild-type or the mutant gene. As a result, the PMS level will be reduced with a concomitant increase in the gene conversion events. In addition, there will be a strong disparity in the number of 6:2 and 2:6 events if there is a strand bias during excision repair.

Mismatch repair in the hairpin structure: To study mismatch repair in the hairpin structure we introduced *his4-7, his4-8, his4-9,* and *his4-12* mutant alleles (Figure 1) into one of the two homologous chromosomes of the diploid strain MLY15, MLY16, MLY20, and MLY21, respectively. The oligonucleotide used to make the *his4-7* allele is 33 nucleotides long and, in the hDNA, generates a single-nucleotide loop in the stem of the hairpin structure (Figure 1). If short-patch repair occurs, deletion of the base will result in a 32-mer insertion, and the addition of a base would cause a 34-mer insertion. In either situation, the end product is an out-of-frame insertion, resulting in the loss of all three (His4A His4B His4C) enzymatic activities.

The mutant allele *his4-8* generates a C-T mismatch in the hairpin structure. If repaired locally, a C-G or an A-T base pair would be formed. The C-G base pair creates a stop codon in the inserted sequence (Figure 1) causing a premature termination of the His4 protein, resulting in a polar *his4A* mutation that eliminates all three enzymatice activities. Since the A-T base pair is not detectable, only those repair events that produce G-C base pairs at the mismatched site can be detected. The *his4-12* allele generates a five-nucleotide loop in the stem of the hairpin structure. The local repair of the loop would generate an out-of-frame insertion, which can be monitored by the complementation test described above.

Diploid strains were sporulated and tetrads were dissected and analyzed. The results of the tetrad analysis are shown in Table 2. All mutant alleles that generated mismatches in the stem of the hairpin structure exhibited a high PMS rate; 16–25% of unselected tetrads had PMS events. These PMS rates are very similar to those of the control mutant allele, *his4-9* (16%), which has a perfect palindromic insertion. In addition, there

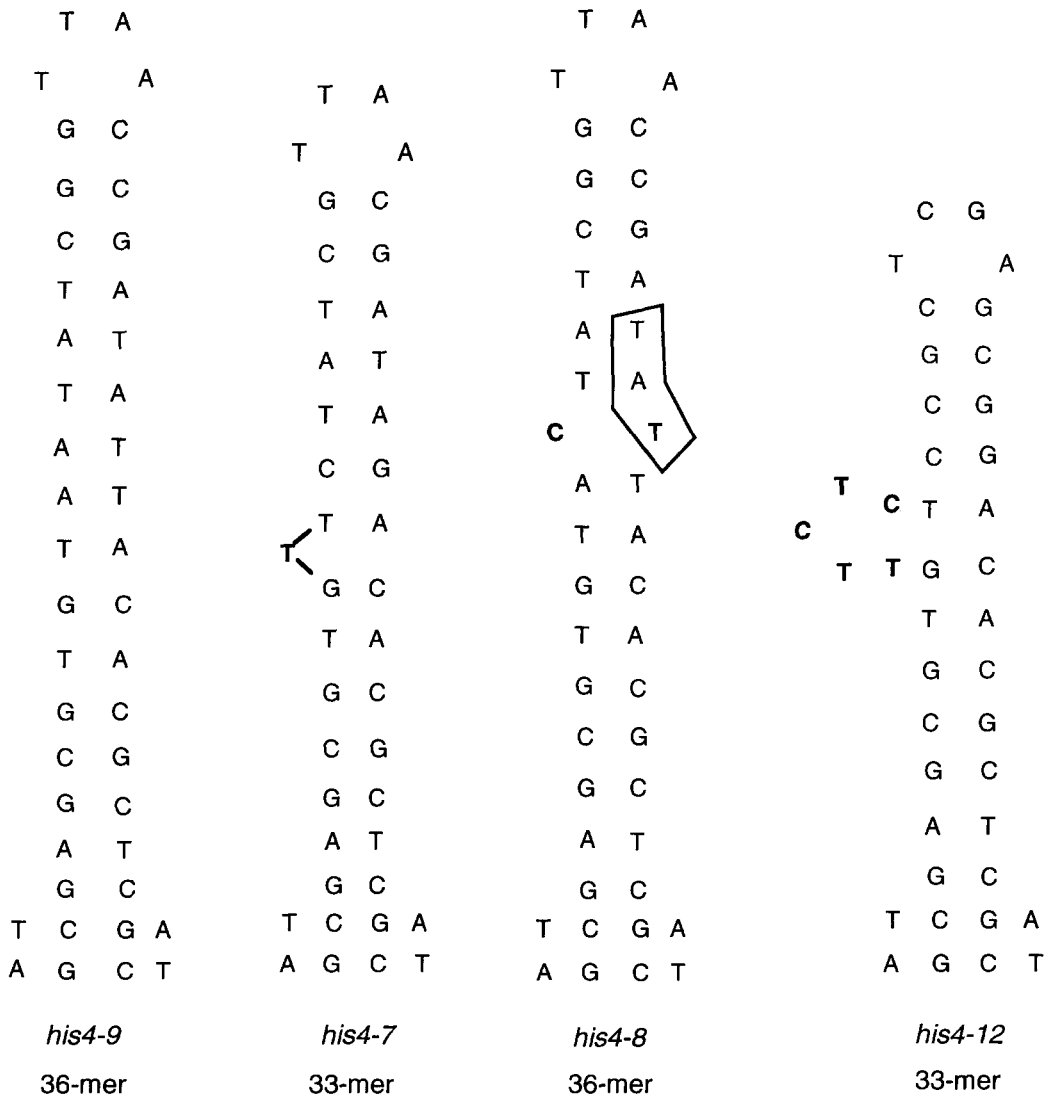

FIGURE 1.—Expected hairpin structure in the heteroduplex DNA involving one wild-type strand and one mutant strand containing a palindromic insertion. Also shown are expected mismatches in the stem of the hairpin structure. All hairpin structures are shown with a four-nucleotide-long loop, although the size of the loop can vary from two to four nucleotides (BLOMMERS *et al.* 1989; DAVISON and LEACH 1994). Since 7-bp-long inverted repeats can form stable hairpin structures *in vivo* (NAG and PETES 1991), oligonucleotides were designed to form mismatches in the stem leaving at least 7 bp on either side of the mismatch. The box in the hairpin structure derived from the *his4-8* allele indicates the formation of the TAG stop codon upon repair of the C-T mismatch to a C-G bp.

is no significant disparity between 6:2 and 2:6 events. These results suggest that mismatches in the hairpin structure were not repaired by the long-patch repair pathway. To determine if the mismatches were repaired by the short-patch repair pathway, the His^- cells of each sectored colony were mated with a tester strain containing a *his4C* mutation. We found that the resulting diploids were His^+, indicating the absence of short-patch repair events.

Since hairpin structures containing different mismatches were derived from the same basic sequence, it is possible that the failure to repair the mismatches could be due to a sequence-specific effect. To rule out this possibility, we constructed a *his4* mutant allele, *his4-21,* by inserting a palindromic oligonucleotide that is missing five nucleotides of the loop region of *his4-12.* A diploid strain (DNY202) was constructed where one homologue had the *his4-12* allele and the other homologue had the *his4-21* mutant allele. When hDNA is formed involving these two mutant alleles, a five-nucleotide loop is generated. The diploid strain was sporulated and tetrads were analyzed. If the inefficient repair is due to the primary sequence of the oligonucleotides, a high level of PMS events would be observed. As shown in Table 2, among 360 tetrads analyzed none of the events were PMS events, indicating that the inefficient repair of the mismatch-containing hairpin structure was not a sequence-specific effect. A similar conclusion was made by NAG *et al.* (1989) using different palindromic-insertion mutant alleles.

A 140-bp long palindromic-insertion mutation increases the rate of gene conversion events: Since the short hairpin structures or the mismatches in the stem of the hairpin structure are not repaired, it is possible that hairpin structures are not recognized as a mismatch by the repair system. Alternatively, the hairpin structures may be protected due to the binding of some structure-specific proteins. Such binding may take place at the loop of the stem-loop structure that could occlude the short hairpin structure. In the case of a long hairpin structure such binding of the protein might not cover the entire structure thereby exposing the stem-

TABLE 2

Segregation patterns of *his4* and *leu2* mutant alleles containing different palindromic insertions

Strain	Mutant locus	6:2	2:6	8:0	0:8	5:3	3:5	Aberrant 4:4	Other aberrants[a]	Total no. Tetrads	Aberrant segregation (% of total)	PMS (% of total)
MLY20	*his4-9*/*HIS4*	17	13	2	0	24	29	1	1	340	25.6	16.2
MLY15	*his4-7*/*HIS4*	14	13	0	0	19	22	3	5	302	25.2	16.2
MLY16	*his4-8*/*HIS4*	15	9	1	0	27	36	7	2	338	28.7	21.3
MLY21	*his4-12*/*HIS4*	16	10	1	0	33	28	9	6	302	34.1	25.2
DNY202[b]	*his4-12*/*his4-21*	51	35	1	4					360	25.2	0
DNY64	*his4-140*/*HIS4*	68	4	35	0					390	27.4	0
DNY224	*leu2-140*/*LEU2*	8	0	1	0					289	3.1	0

Diploids were sporulated at 25° for 2–4 days and the tetrads were dissected and analyzed as described in MATERIALS AND METHODS.

[a] Others include tetrads with two PMS events and two spores with the same genotype (aberrant 6:2 and 2:6), and events where each tetrad had one PMS event and three spores showing the same genotype (7:1 and 1:7). Specific classes in each strain were: MLY20 (one 7:1), MLY15 (one aberrant 6:2, one 7:1, and three 1:7), MLY16 (one aberrant 6:2 and one 7:1), MLY21 (three aberrant 6:2 and three 7:1).

[b] The first number of each class represents the number of spores with *his4-12* allele. For example, the 6:2 class represents three spore colonies with the *his4-12* allele and one spore colony with the *his4-21* allele.

loop structure to the repair enzymes for subsequent processing. In other words, the repair of the hairpin structure may depend on the stem length. To test this idea, we constructed the *his4-140* mutant allele by inserting a 140-bp-long palindromic sequence at the same *SalI* site in the *HIS4A* domain. Heteroduplex DNA formed involving the *his4-140* mutant allele and the wild-type gene is expected to contain a hairpin structure of 68-bp stem length. Tetrad analysis of the diploid strain (DNY64) containing the wild-type gene and the *his4-140* mutant allele is shown in Table 2.

About 27% of total unselected tetrads had aberrant events and none of them showed postmeiotic segregations. The absence of PMS events suggests that long palindromic insertion mutant alleles either generate mismatches that are well repaired by the mismatch repair system or the conversion events occur by a mechanism not involving heteroduplex formation. In addition, there is a strong disparity (68 *vs.* 4) between the number of 6:2 and 2:6 events (Table 2). In the case of the wild-type donor, the repair of the mismatch in favor of the invading strand results in a 6:2 event; a similar repair event results in a 2:6 event when the mutant chromosome provides the invading strand. A repair event using the recipient strand as a template results in a 4:4 restoration event (PETES *et al.* 1991). Since there was no such disparity between 6:2 and 2:6 events with short hairpin structures (TABLE 2, NAG *et al.* 1989), the disparity with the *his4-140* mutant allele indicates that the repair events favored the wild-type strand as a template. This conclusion was further substantiated by a significant increase in the number of 8:0 events.

The events showing an 8:0 segregation pattern constitute a significant proportion among the aberrant events. The lack of disparity between 5:3 plus 6:2 and 3:5 plus 2:6 events with short palindromic-insertion mutant alleles suggests that both wild-type and mutant chromatids initiate recombination with equal efficiencies. If the same is true for the *his4-140* mutant allele, most 2:6 events then could not be recovered due to the preferential repair process. Assuming that ~60 2:6 events could not be recovered, the rates of single and double events are expected to be 34% and 11%, respectively. The 8:0 events alone constitute 9% of total unselected tetrads, suggesting that the conversion events occurred by a different mechanism. The mitotic reversion rate of the *his4-140* mutant allele to wild type was 2×10^{-8} per cell per generation, indicating that the 8:0 events were not due to a high rate of mitotic reversion.

Gene conversion events by the palindromic insertion-mutant alleles are *MSH2*-independent: Since the long palindromic-insertion mutant alleles mostly generate gene conversion events, one likely possibility is that the mismatches generated in the hDNA are well recognized by the mismatch repair system. We wanted to investigate if the repair is carried out by the *MSH2*-dependent mechanism. We constructed a derivative of DNY64 by making the strain homozygous for the *msh2::Tn10LUK7-7*

TABLE 3

Segregation patterns of heterozygous mutations at several loci in the wild-type and *msh2* background

Segregation pattern	Mutant allele											
	his4-140		*his4-9*		*arg4-17*		*leu2-Bst*		*tyr7-1*		*trp1-1*	
	Wt	*msh2*	Wt	*msh2*	Wt	*msh2*	Wt	*msh2*	Wt	*msh2*	Wt	*msh2*
6:2, 2:6	68, 4	45, 9	17, 13	10, 5	100, 89	11, 9	37, 40	1, 0	34, 26	3, 1	10, 5	0, 1
5:3, 3:5	0, 0	0, 0	24, 29	11, 20	0, 0	23, 17	0, 0	5, 8	4, 3	4, 9	0	3, 1
8:0, 0:8	35, 0	9, 2	2, 0	0, 0	1, 1	0, 0	2, 0	0, 0	2, 4	0, 0	0, 0	0, 0
Ab4:4	0	0	1	2	0	3	0	2	0	1	0	1
Others[a]	0	1	1	1	0	2	0	0	0	1	0	0
Total tetrads	390	300	340	164	2032	464	2032	464	2032	464	2032	464
Aberrant events (%)	27.4	22.0	25.6	29.9	9.4	14.0	3.9	3.4	3.5	4.1	0.7	1.3
PMS (% of total aberrants)	0	1.5	63.2	69.4	0	69.2	0	93.7	9.6	79.0	0	83.3

Sporulation was carried out as described for Table 2. Wt, wild type.

[a] Others include tetrads showing aberrant 6:2 and 2:6, 7:1 and 1:7 events. Specific classes were as follows: *his4-140* (one 1:7), *his4-9* (one 7:1 in the Wt strain and one 1:7 in the *msh2* strain), *arg4-17* (one ab. 2:6 and one 1:7), *tyr7-1* (one ab. 2:6).

mutant allele (REENAN and KOLODNER 1992). The tetrad analysis of the resulting strain (DNY216) is shown in Table 3. The rates of gene conversion of *arg4-17, leu2-Bst, tyr7-1,* and *trp1-1* were decreased with a concomitant increase in the rate of PMS events. Conversely, 22% of unselected tetrads showed gene conversion events at the *his4-140* mutant allele without a significant increase in the number of PMS events. In addition, the disparity between 6:2 and 2:6 events was retained among tetrads showing conversions and 82% of the aberrant events were 6:2 and 8:0 events. These results suggest that repair of the long hairpin structure is carried out by a Msh2-independent mechanism or these events were generated by a mechanism not involving heteroduplex formation.

Heteroduplex DNAs containing short hairpin structures are repaired with an efficiency of 20–30% (NAG *et al.* 1989; DETLOFF and PETES 1992). This residual repair might occur by the Msh2-dependent mechanism or by a different, inefficient repair mechanism. We constructed a *msh2* derivative of MLY20 (DNY217) to test if the residual repair of the short hairpin structure is mediated by a Msh2-dependent mechanism. The PMS rate (for the *his4-9* allele) among aberrants remained unchanged in both *MSH2* and *msh2* strains, 63% and 69%, respectively (Table 3). These results suggest that the residual repair of the hairpin structure is carried out by a different mechanism that is Msh2 independent.

The *his4-140* mutant allele generates double-strand breaks during meiosis: A high rate of gene conversion events at the *his4-140* allele in the *msh2* background indicates that either there is a different mechanism to repair the long hairpin structure or long palindromic insertion-mutant alleles generate double-strand breaks (DSB) during meiosis. If a double-strand break occurs in the palindromic sequence on the mutant chromatid, the inserted sequence must be degraded by exonucleases before the strand-exchange reaction occurs, so that the invading strand can pair with the complementary strand on the intact wild-type chromatid. As a consequence, the repair of the double-strand break will result in a 6:2 event. Similarly, if the break occurs on both mutant chromatids, an 8:0 event would result. We examined the meiotic DNA from a *HIS4/HIS4* homozygous diploid (DNY115) and a *HIS4/his4-140* diploid (DNY214) to determine if the long palindromic insertion causes a DSB formation. Both diploid strains had the *rad50S* mutation in the background, which prevents processing of the broken chromosomes (ALANI *et al.* 1990).

Meiotic DNA was isolated from DNY115 and DNY214 and analyzed. A *Pvu*II digestion of the DNY115 DNA generates a 2.4-kb fragment that contains most of the *HIS4* gene and a portion of the *BIK1* gene (Figure 2A). A similar digestion of the DNY214 DNA would yield a 2.4-kb fragment (resuting from the wild-type chromosome) and a 2.6-kb fragment (resulting from the *his4-140* mutant chromosome). As shown in Figure 2B and observed previously (NAG and PETES 1993), in addition to the 2.4-kb band, we found an additional band (1.9 kb) in DNY115 DNA, which resulted from the DSB at the promoter region of *HIS4* (site I). In the DNY214 DNA, in addition to the DSB at the *HIS4* promoter, we found two additional bands indicating the presence of a new DSB site (site II). The sizes of those bands (1.4 and 1.2 kb) correspond to a break site within the inserted sequence. The smaller band (1.2 kb) showed a weak signal due to a shorter region of sequence homology with the probe. The amount of DSB at site I (*i.e.,* the *HIS4* promoter region) varies from 2 to 5% of the total DNA (FAN *et al.* 1995) and sporulation efficiency in our experiments varies from 35 to 40% (NAG *et al.*

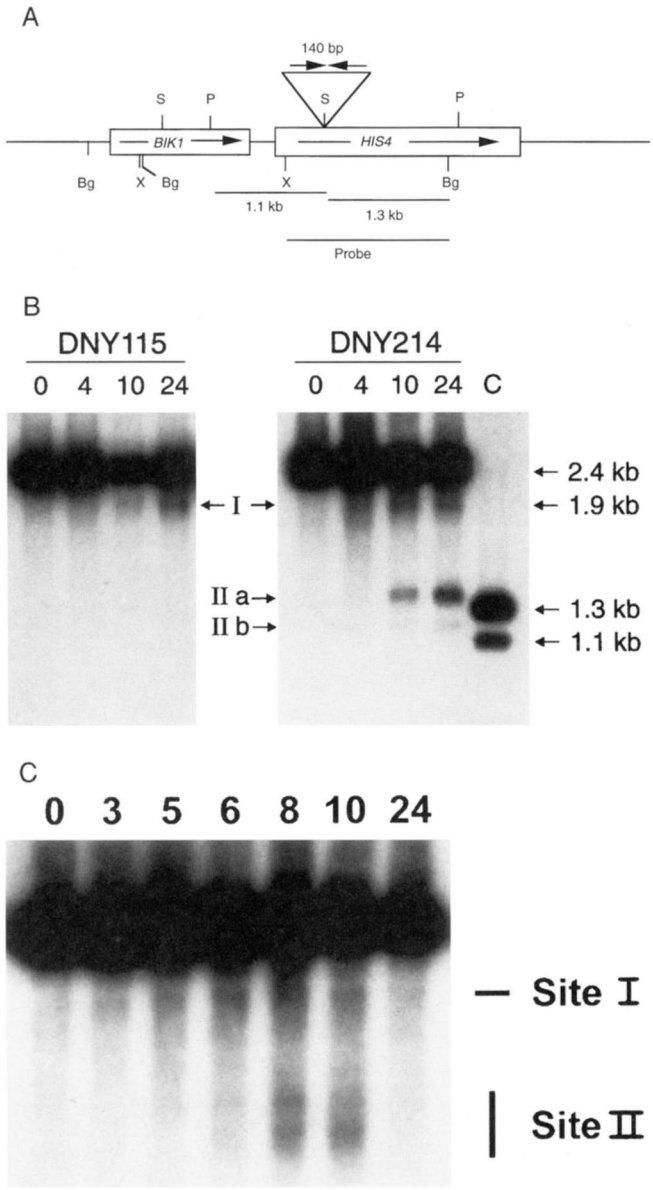

FIGURE 2.—Double-strand break formation in wild-type strain (DNY115) and in a strain containing a heterozygous *his4-140* allele (DNY214). (A) Partial restriction map of the *HIS4-BIK1* region. Only relevant restriction sites are shown. The boxes indicate the coding region of the genes and the arrows indicate the direction of transcription. Expanded region over the linear map represents the 140-bp palindromic insertion. Abbreviations: Bg, *Bgl*II; P, *Pvu*II; S, *Sal*I; X, *Xho*I. (B) Analysis of DSB formation in the *HIS4-BIK1* region. DNA was isolated at various time intervals after induction of sporulation and digested with *Pvu*II. The fragments were separated on a gel, transferred to nylon membrane and analyzed by Southern hybridization. The *Xho*I-*Bgl*II fragment containing most of the *HIS4* gene and a portion of *BIK1* was used as a probe. The number above each lane indicates the time (in hr) of sample collection during sporulation. Lane C had DNY115 DNA from the 0-hr time point digested with *Sal*I and *Pvu*II. A *Pvu*II digestion of the meiotic DNA generates a 2428-bp fragment for DNY115, and 2428- and 2568-bp fragments for DNY214. The top band in DNY214 is a doublet. Arrow I indicates the DNA fragment generated due to a DSB at the normal *HIS4* initiation site. Arrows IIa and IIb indicate the fragments generated due to the DSB at the palindromic insertion site. Approximate sizes of the fragments are shown at the right side. (C) DSB formation in DNY64. Meiotic DNA was digested with *Pvu*II and analyzed by Southern hybridization. The *Xho*I-*Bgl*II fragment was used as a probe. Numbers above the lanes indicate the time of sample collection. DSBs at site I and at site II are indicated.

1995). In our experiments, the amounts of DSB at the normal site in DNY115 and DNY214 were 1.3% and 1.5%, respectively, and the amount of DSB at site II was 3.4%. The occurrence of the new DSB explains the observed disparity between 6:2 and 2:6 events and a significant increase in the 8:0 events, since the breaks occurred only on the mutant chromatid and were repaired by the information present on the wild-type chromatid.

Physical analyses of DSB formation described above were carried out in strains that are homozygous for the *rad50S* mutation. To analyze the nature of DSBs in the wild-type background, meiotic DNA was isolated from DNY64 and digested with *Pvu*II. Results are shown in Figure 2C. DSBs at both site I and site II were diffused as the broken ends are processed to become engaged in the recombination process. These results suggest that DSBs at site II, as in site I, are protected by the *rad50S* mutation. The kinetics of DSB formation indicate that DSBs at both sites appear nearly at the same time.

These results also indicate that DSBs at site II are not a result of processing of the hDNAs that are formed due to the recombination initiation at site I. If the DSB at site II was repair induced, it would not be formed in the *rad50S* background since the break at site I would not be processed to generate hDNA. The results shown in Figure 2 indicate that the DSB at site II is independent of the recombination initiation at site I.

DSB formation by the 140-bp-long palindromic sequence is not specific for the *HIS4* locus: The *HIS4* locus in the AS4/AS13 background has a high rate of meiotic recombination. It is possible that the 140-bp-long palindromic sequence fortuitously created a DSB-initiation site at a region that is already primed for DSB initiation. To eliminate this possibility, we inserted the same 140-bp-long palindromic oligonucleotide into the *Bst*EII site, present in the coding region of the *LEU2* gene, to generate the *leu2-140* mutant allele. Tetrad analysis of the diploid strain DNY224, containing the wild-type *LEU2* gene and the *leu2-140* mutant allele, is shown in Table 2.

When compared with the recombination rate of the *leu2-Bst* allele (generated by filling in the *Bst*EII site in the *LEU2* gene, Table 3), our results indicate that the presence of the long palindromic sequence did not increase the recombination rate at the *LEU2* locus. However, the segregation pattern in the case of the *leu2-140* allele is different from that of the *leu2-Bst* allele. All events were either 6:0 or 8:0 events, which is consistent with the hypothesis that the 140-bp-long palindromic sequence generates a site for a DSB during mei-

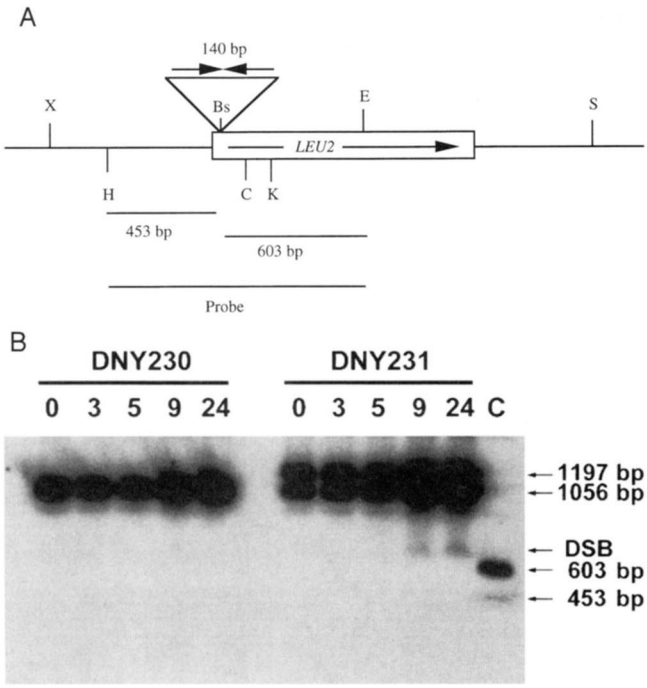

FIGURE 3.—Double-strand break formation in DNY230 (*LEU2/LEU2*) and DNY231 (*LEU2/leu2-140*). (A) Partial restriction map of the *LEU2* region. Only relevant restriction sites are shown. Abbreviations: Bs, *Bst*EII; C, *Cla*I; E, *Eco*RI; H, *Hpa*I; K, *Kpn*I; S, *Sal*I; X, *Xho*I. (B) Analysis of DSB formation at the *LEU2* locus. Meiotic DNA was digested with *Hpa*I and *Eco*RI, separated on a gel and analyzed by Southern hybridization. The *Hpa*I-*Eco*RI fragment was used as a probe. The amount of DSBs was quantitated from the 24-hr time point. The number above each lane indicates the time of sample collection. Lane C had DNY230 DNA from the 0-hr time point digested with *Hpa*I, *Eco*RI and *Bst*EII.

osis in yeast. To provide a physical evidence for the DSB formation, we constructed two diploid strains, DNY230 (*LEU2/LEU2*) and DNY231 (*LEU2/leu2-140*). Both are homozygous for the *rad50S* mutation. DNA samples were isolated from cells undergoing meiosis and analyzed for DSB formation.

A *Hpa*I and *Eco*RI digestion of DNY230 DNA generates a 1056-bp fragment that contains a portion of the *LEU2* gene and a portion of the upstream region (Figure 3A). A similar digestion of DNY231 DNA would yield a 1056-bp fragment (resulting from the wild-type chromosome) and a 1197-bp fragment (resulting from the chromosome containing the *leu2-140* mutant allele). As shown in Figure 3B, in addition to the parental fragments, an additional band of ~750 bp was observed in the late DNA sample of DNY231 indicating the presence of a DSB site. The site for the normal DSB at the *LEU2* locus is not known. The second band could not be observed due to short homology with the probe. The amount of DSB formed by the *leu2-140* mutant allele was 0.42% of total meiotic DNA.

DISCUSSION

From our genetic and physical analyses of *his4* mutant alleles containing different palindromic insertions, we conclude that (1) mismatches in the stem of the short hairpin structure are not repaired in yeast, (2) a 140-bp-long palindromic insertion creates a meiotic recombination hotspot by generating a site for a DSB, and (3) the residual repair of the hairpin structure occurs in an Msh2-independent pathway. Each of these conclusions is discussed below.

We followed the repair of different types of mismatches in the DNA hairpin structure. Our results indicate that neither short-patch nor long-patch repair pathways operate to eliminate the mismatches present in the stem of DNA hairpin structures. These results suggest that mismatches in the hairpin structure are treated differently than those present in the interstrand base-paired duplex DNA. Although the short-patch repair pathway was not found to be operating on the substrates examined here, it could work on other substrates in a different sequence context. As observed in bacteria, there could be different short-patch repair pathways operating on different substrates (MODRICH 1991).

One possible explanation for inefficient repair of mismatches in the hairpin structure is that proteins may exist that specifically bind at the short hairpin structure in the DNA molecule. The binding of such proteins at the stem-loop structure would also protect the protruding mismatches in the hairpin structure. One line of evidence in favor of this hypothesis is that the level of PMS of a poorly repaired allele is reduced by the presence of a nearby well repaired allele. Although the PMS level of the well repaired allele is usually not influenced by the poorly repaired allele (PETES *et al.* 1991), a palindromic-insertion mutation, *his4-IR9,* does significantly increase the PMS level of *his4-519,* a well-repaired allele located 26 bp downstream of *his4-IR9* (DETLOFF and PETES 1992). It is suggested that proteins that protect the hairpin structure from recognition or repair by the mismatch repair system also protect the closely spaced well repaired mismatch. A 38-bp deletion-mutant allele of the *ADE8* locus, *ade8-18,* also shows a high level of postmeiotic segregation (WHITE *et al.* 1988). Although the loops generated by most deletion- or insertion-mutant alleles are efficiently repaired, WHITE *et al.* (1988) proposed that the deletion in the *ade8-18* allele fortuitously created a protein binding site that protects the loop structure in the hDNA from repair by the mismatch repair system. An alternative possibility is that while mismatches in the hairpin structure can be recognized, the duplex DNA on either side of the mismatch is not long enough for the mismatch repair enzymes to bind and repair it.

We showed that insertion of a 140-bp-long palindromic sequence generates a site for a DSB that initiates meiotic recombination (PETES *et al.* 1991; PADMORE *et al.* 1992). The lesion that is produced on the mutant chromosome is repaired by the information on the intact homologous chromosome thereby causing a strong

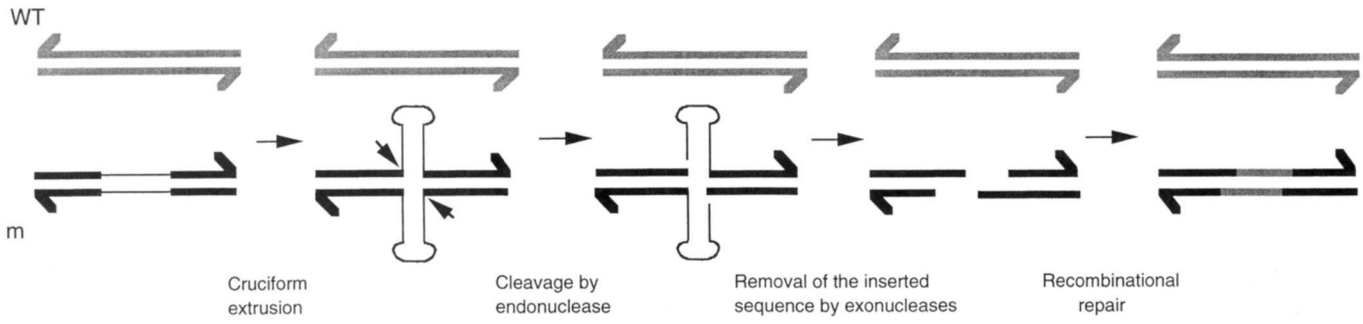

FIGURE 4.—A model for palindrome-induced meiotic recombination. Thick lines indicate the wild-type chromosomal DNA and the thin lines indicate the inserted palindromic sequences. Homologues are indicated by different shading. Only two non-sister chromatids are shown. A certain fraction of DNA containing the palindromic sequence undergoes cruciform extrusion that is cleaved by structure-specific endonucleases during meiosis. For simplicity, only one cleavage site at the base of the cruciform is shown. The cleavage may also occur at the stem of the cruciform structure. After cleavage, the inserted DNA must be degraded by exonucleases so that the resulting single-stranded region can pair with the homologous chromatid during strand invasion. The gap thus formed on the mutant chromatid is sealed by recombinational repair. WT indicates the wild-type chromosome and m indicates the mutant chromosome.

disparity between 6:2 and 2:6 events. Meiotic recombination occurs after DNA replication (PETES *et al.* 1991; PADMORE *et al.* 1992). If both sisters containing the insertion are broken and the breaks are repaired by the information present on the wild-type chromosome, the result is an 8:0 event, with a strong disparity between 8:0 and 0:8 events.

One obvious issue is: why does a long palindromic sequence act as a meiotic recombination hotspot? One possibility is that the palindromic sequence may act as an entry point for the recombination enzymes. This possibility is less likely since the short inverted repeats do not act as recombination hotspots. An alternative and more likely possibility is that palindrome inviability in bacteria and DSB formation in yeast occur by the same mechanism, *i.e.*, by extrusion into a cruciform conformation. Long palindromic sequences may adopt a cruciform structure at a frequency higher than those for the short ones, thus attracting endonucleases such as Holliday-junction resolvases to make a double-strand break (Figure 4). We do not know the minimum length of the inverted repeat that is necessary to make a recombination hotspot, however, the required length of the inverted repeat should be between 36 and 140 bp, as the 36-bp insertion does not create a hotspot and the 140-bp insertion does (Table 2 and Figure 2). Since only one long palindromic sequence has been tested, we cannot rule out the effect of the primary sequence present in the inserted sequence on the generation of the double-strand break. We, however, believe this possibility to be unlikely, since all short hairpins tested, so far, behave identically and the long palindromic sequences are difficult to maintain in other organisms (LEACH 1994). In *E. coli,* long palindromic sequences are unstable, however, they can be maintained in a nuclease deficient *sbcCD* strain (LEACH 1994). The inviability of long palindromes is believed to be due to the formation of a cruciform structure and subsequent processing by the structure-specific nucleases.

Our results show that 9% of total spores of DNY64 had one conversion event assuming that 8:0 events were due to DSBs on both sister chromatids (Table 2). In DNY214, 70% of total DSBs were at the hotspot and 30% were at the *HIS4* promoter. Based on 40% sporulation efficiency and one break per event, we calculate that 1.1% and 2.5% of total DNA should have DSBs at the *HIS4* promoter (site I) and at site II, respectively. These values are close to the observed levels of DSBs (1.5% and 3.4%, respectively). However, there still remains a strong disparity between 6:2 and 2:6 events, if 30% of the aberrant events have originated at the *HIS4* promoter. This disparity can be explained by a preferential deletion of the stem-loop structure in the hDNA that is formed due to the recombination initiation at the *HIS4* promoter. BISHOP *et al.* (1987) demonstrated the existence of a *PMS1*-independent pathway that operates to repair the insertion-loop hDNA in favor of the deletion. These insertion loops are similar to *his4-140* as they are palindromic in nature but much shorter in length. A similar system may operate to delete the stem-loop structure from the hDNA containing a long stem-loop structure.

The amounts of DSB at the *HIS4* promoter were similar in both DNY115 and DNY214. It has been shown that the presence of a strong recombination-initiation site suppresses the weak site nearby due to local depletion of limiting factors (WU and LICHTEN 1995; XU and KLECKNER 1995). This result can be explained as follows: either the competition between the recombination-initiation sites cannot be monitored at the observed low level or DSBs at site I and site II originate by two different enzymes (Figure 4). If the DSBs at site II are due to the resolution of the cruciform structure, mismatches in the hDNA that are formed due to initiation at site I are repaired in favor of deletion as described above resulting a disparity between 6:2 and 2:6 events.

It has been shown previously that short cruciform

structures when formed in the hDNA are not processed in yeast (NAG and PETES 1993). The first 16 bp of the *his4-140* allele are identical to the *his4-IR16* allele, used to study cruciform formation for physical detection of hDNA. One possible explanation for this discrepancy is the hairpin-protection hypothesis. Short hairpin structures are protected by binding of some structure-specific proteins. Such binding may occur through the loop of the stem-loop structure that also masks the short hairpin structure. In the case of a long hairpin structure, the binding of the protein at the loop of the hairpin does not cover all of the extruded stem-loop structure, thereby exposing the structure to the processing endonucleases and resulting in the formation of a DSB. An alternative explanation is that processing of the cruciform structure by endonucleases depends on the length of the arm of the stem-loop structure. The endonuclease in yeast may require a longer arm. Dependence on the arm length has been observed with bacteriophage T4 endonuclease VII that has been shown to act on Holliday junctions (MUELLER *et al.* 1990). Similarly, the endonuclease in yeast may require a longer arm for resolution of Holliday junctions.

The position of the DSB appears to be within or near the inserted sequence. If the break occurs at or near the base of the cruciform structure, each fragment would contain ~140 nucleotides overhang (Figure 4). The results shown in Figure 2B indicate that both fragments generated by the DSB had the expected size. Since Southern analysis cannot indicate the position of the break at the nucleotide level, our analysis of the location of the DSBs does not allow us to determine exactly where they fall with respect to the cruciform structure. It has been shown that insertion of a telomeric sequence upstream of the *HIS4* gene creates a recombination hotspot (WHITE *et al.* 1993). However, DSBs were found to occur upstream of the inserted sequence (XU and PETES 1996). One likely explanation of these results is that the endonuclease (that makes the DSB) binds to a particular sequence and then cleaves at a distance. If the DSB generated by the *his4-140* mutant allele occurred on one side of the insertion, at least one of the two fragments would be equal to or less than the sizes of the control fragments (1.1 and 1.3 kb; lane C, Figure 2B) that were generated by digesting DNY115 DNA (0-hr time point) with *Sal*I and *Pvu*II. Our results indicate that both fragments were larger than the control fragments suggesting that the DSB did not occur on one side of the insertion. A similar result was also obtained at the *LEU2* locus.

If the cruciform formation is responsible for generation of the DSB, it occurs only during meiosis but not in mitosis. Several groups have reported a correlation between the nuclease hypersensitive sites in chromatin and the DSB sites (WU and LICHTEN 1994; FAN and PETES 1996). Although the patterns of hypersensitive sites were similar in both meiotic and mitotic nuclear preparations at most loci examined, OHTA *et al.* (1994) reported that the sites associated with double-strand break formation at the *ARG4* and *CYS3* loci are several-fold hypersensitive during meiosis compared to mitosis. Such alteration in the chromatin structure might alter the local superhelicity of the DNA resulting in cruciform extrusion. It is also likely that cruciform structure is formed both during meiosis and mitosis; however, the enzyme that makes the DSB is induced during meiosis. We favor this later explanation since palindromic sequences stimulate deletion formation. However, chromatin structure is likely to play a significant role in cruciform extrusion since the recombination rate was not increased when the 140-bp-long palindrome was inserted into the *LEU2* gene.

The occurrence of gene conversion events with both short and long palindromic insertion-mutant alleles suggests that there is an inefficient repair mechanism that repairs the hairpin structure. Tetrad analysis showed that the spectrum of events remained unchanged in both wild-type and *msh2* strains suggesting that the residual repair occurs by a *MSH2*-independent mechanism.

An important implication of these findings is that short palindromic sequences are highly stable *in vivo,* since the hairpin structures are not processed even though there are mismatches within the stem of the hairpin structure. Both perfect and quasi-palindromic sequences are present at functionally important regions in the chromosome, and it is important that such short palindromic sequences be maintained in the genome. Mechanisms have been developed to maintain such sequences even when fortuitously extruded into the cruciform conformation. Long palindromic sequences on the other hand, make the genome unstable and such sequences are removed from the genome by recombinational repair.

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