Physical Detection of Heteroduplexes during Meiotic Recombination in the Yeast *Saccharomyces cerevisiae*

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We describe a general physical method for detecting the heteroduplex DNA that is formed as an intermediate in meiotic recombination in the yeast *Saccharomyces cerevisiae*. We use this method to study the kinetic relationship between the formation of heteroduplex DNA and other meiotic events. We show that strains with the *rad50*, but not the *rad52*, mutation are defective in heteroduplex formation. We also demonstrate that, although cruciform structures can be formed in vivo as a consequence of heteroduplex formation between DNA strands that contain different palindromic insertions, small palindromic sequences in homoduplex DNA are rarely extruded into the cruciform conformation.

The exchange of DNA strands between interacting chromosomes, the formation of a heteroduplex, is a central feature of homologous recombination. In the yeast *Saccharomyces cerevisiae*, the first clear evidence that heteroduplexes were formed during meiotic recombination was based on classical genetic analysis (8). In a diploid strain heterozygous at a single locus (alleles A and a), although most tetrads segregate 2A:2a spores, two types of aberrant segregants are detected: (i) gene conversion events (3A:1a or 1A:3a segregation), found at a median frequency of about 5% (9), and (ii) postmeiotic segregation (PMS) events, found at a median frequency (for most alleles) of less than 1% (9, 20). PMS events are signalled by a spore colony in which the two heterozygous alleles segregate (8). In general, in *S. cerevisiae*, the most common classes of PMS are those with a single PMS event (9, 20), either 2A:1a:1A/a spore colonies (called a 5A:3a segregation) or 1A:2a:1A/a spore colonies (3A:5a). To retain consistent nomenclature, we will describe gene conversion events as 6:2 or 2:6 events, and normal Mendelian segregation as 4:4.

Since a PMS event is the segregation of two markers from a spore containing one haploid genome, these events are most simply interpreted as reflecting heteroduplex formation between the two alleles, with failure to repair the resulting mismatch (12). Most meiotic gene conversion events in yeast cells are most simply interpreted as reflecting heteroduplex formation between the two alleles, followed by mismatch repair (9, 20). For most mutations, gene conversion events are much more frequent than PMS events, indicating that most mismatches are efficiently repaired, although mutations caused by the insertion of small palindromic oligonucleotides result in poorly repaired mismatches (19). Since PMS and gene conversion events are often associated with crossing over of flanking markers, in most models of recombination, the heteroduplex is an intermediate in the production of a crossover (20).

The genetic assay for heteroduplex formation in yeast cells has two limitations. First, since the assay involves sectoring of spore colonies, the kinetics of heteroduplex formation during meiosis cannot be examined. Thus, one cannot determine the timing of heteroduplex formation rel-

ative to other types of meiotic events. Second, since viable spores are required to detect a PMS event, one cannot examine heteroduplex formation in meiotic mutants that fail to sporulate or that fail to yield viable spores. The obvious solution to these limitations is to use a physical assay for heteroduplex formation. Lichten et al. (16) developed a denaturing gel procedure to detect heteroduplexes formed at the *ARG4* locus during meiotic recombination. In this report, we describe a simple general method of detecting heteroduplexes. We use this method to examine the timing of heteroduplex formation relative to other meiotic events and the properties of several mutants that affect meiotic recombination.

For our studies, we analyzed the *HIS4* locus, which has an unusually high level of meiotic recombination in our genetic background (5, 6, 19, 31). At two other recombination hotspots in yeast cells, *ARG4* (28) and an ectopically located *LEU2* locus (4), meiosis-specific double-strand breaks have been observed. In this study, in addition to examining the formation of heteroduplexes at *HIS4*, we also found evidence for a double-strand break.

MATERIALS AND METHODS

Construction of plasmids. The plasmid pDN9 was constructed by cloning a *XhoI*-*BglII* fragment of the *HIS4* gene in *BamHI*-*SalI*-treated YIp5 (19). The plasmid pDN11 was constructed by allowing the palindromic oligonucleotide DNO14 to self-anneal and then inserting the annealed product into the *SalI* site of pDN9; this *SalI* site is within the *HIS4* coding sequence (Fig. 1A). The plasmid pDN35 was constructed comparably with the oligonucleotide DNO16. The oligonucleotide DNO14 is 32 bases long (5' TCGAG TACTGTATGTGGATCCACATACAGTAC) and results in a frameshift mutation in *HIS4* (*his4-IR16*). The oligonucleotide DNO16 is 30 bases long (5' TCGAGCGTGTCTATC TGCAGATAGACACGC) and results in the in-frame mutation *his4-IR15*. The plasmid pSP3, a gift from S. Porter (University of North Carolina), has the DNO14 oligonucleotide inserted within the *SalI* site of the *BIK1* gene which is located on a *XhoI* fragment inserted at the *SalI* site of the YIp5 derivative B142. The plasmid pNKY349, provided by N. Kleckner (Harvard University), has a *rad50S* mutation marked with *URA3* inserted in a pSP65-derived vector (1).

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FIG. 1. Restriction sites relevant to the detection of crossovers and heteroduplexes. (A) Partial restriction map of the *HIS4-BIK1* region. The boxes indicate the coding regions of the genes, and arrows indicate the direction of transcription. The line under the restriction map represents the region used as a probe for Southern analysis as described in Materials and Methods. Abbreviations: Bg, *BglII*; P, *PvuII*; S, *SalI*; X, *XhoI*; SP, *SpeI*. (B) Expected cruciform conformation resulting from strand exchange involving the *his4-IR16* and *his4-IR15* alleles. The DNA strands derived from the two alleles are indicated by lines of different thickness. Thick arrows in inverted orientation represent the palindromic insertions of the two alleles. Lines at the bottom represent the lengths of the DNA fragments generated after digestion of the sample with the three enzymes *PvuII*, *PstI*, and *BamHI*. Distances representing the sizes of the fragments are not drawn to scale. Bars within the arrowhead indicate the insensitivity of the site to that enzyme.

The plasmids pSM22 and pNKY83 contain null alleles of the *rad52* and *rad50* genes, respectively, in which the coding sequences are replaced by *URA3* (2, 24). The plasmid pMW33 (constructed by M. White, University of North Carolina) contains the *URA3* gene inserted into a *SpeI* site located about 4 kb downstream of *HIS4*. This region was cloned as a *BglII* fragment into the *BamHI* site of YIp5. The plasmid pDN42 contains a *XhoI-BglII* fragment containing most of *HIS4* inserted into the *BamHI-XhoI*-treated Bluescript vector pBSII SK(-).

Construction of yeast strains. Most of the yeast strains were derived from the haploid strain AS4 (α *trp1 arg4 tyr7 ura3 ade6*) or AS13 (α *leu2 ura3 ade6*) (27, 29) by transformation. The haploid strains were constructed by either two-step (abbreviated TST; 23) or one-step (abbreviated OST; 22) transplacement procedures as follows: DNY72 (TST of AS4 with pDN35), DNY75 (TST of AS13 with pDN35), DNY45 (TST of AS13 with pDN11), DNY84 (OST of DNY72 with pMW33), DNY85 (TST of DNY45 with pSP3), DNY88 (OST of DNY45 with pNKY83), DNY89 (OST of DNY72 with pNKY83), DNY91 (OST of DNY45 with pNKY349), DNY92 (OST of DNY72 with pNKY349), DNY100 (OST of DNY45 with pSM22), DNY101 (OST of DNY72 with pSM22), DNY107 (OST of AS4 with pNKY349), and DNY106 (OST of AS13 with pNKY349). All strains were confirmed by Southern analysis. The diploid strains were constructed as follows ($\times$ signifying a mating): DNY78 (DNY72 $\times$ DNY75), DNY86 (DNY84 $\times$ DNY85), DNY90 (DNY88 $\times$ DNY89), DNY93 (DNY91 $\times$ DNY92), DNY102 (DNY100 $\times$ DNY101), DNY112 (AS4 $\times$ AS13), and DNY115 (DNY106 $\times$ DNY107). The diploid MS1 was previously constructed by mating an AS13 derivative (MW1) with the *his4-Sal* mutation to an AS4 derivative (PD23) with the *his4-713* mutation (30). Haploid strains used for *his4*

allele testing were F98 (α *his4-280 trp1*) and F99 (α *his4-280 ade2*).

Media and genetic techniques. The media utilized were standard (25). The presporulation medium had 0.5% yeast extract, 1% peptone, 0.17% yeast nitrogen base, 1% potassium acetate, 0.5% ammonium sulfate, and 0.05 M potassium biphthalate (pH was adjusted to 5.5).

For tetrad analysis, the diploid strain DNY86 was sporulated at 18°C and the tetrads were dissected onto rich medium. After 3 days of growth at 30°C, the spore colonies were replica plated onto various types of omission media. The *HIS4* gene has three functional domains (*HIS4A*, *HIS4B*, *HIS4C*), each responsible for a different enzymatic activity (13). The diploid strain DNY86 is heterozygous for *his4-IR16* and *his4-IR15*, both mutant alleles representing insertions at the same restriction site in *HIS4A*. The *his4-IR16* allele is a frameshift mutation that eliminates all three enzymatic activities, whereas the *his4-IR15* mutation eliminates only *HIS4A* activity. To score these alleles, we mated spore colonies derived from DNY86 to tester strains (F98 and F99) that contained *his4-280* (*his4C* mutation).

Physical analysis of DNA from meiotic cells. Most of the methods for preparation of meiotic DNA were based on protocols provided by C. Goyon and M. Lichten (11). To prepare meiotic cells, we inoculated a single colony into 2 ml of yeast extract-peptone-dextrose (YPD) liquid medium (32). After overnight growth at 30°C, a 0.15-ml inoculum of this culture was used to inoculate 250 ml of presporulation medium. This culture was incubated at 30°C until the cell concentration was 2×10^7 to 3×10^7 /ml. The cells were then washed with 50 ml of 1% potassium acetate and suspended in a total volume of 250 ml of 1% potassium acetate (supplemented with one-fifth the normal concentration of the nutritional requirements) at a cell concentration of 2×10^7 /ml.

The culture was incubated at 25°C. Fifteen-milliliter aliquots were taken at different time intervals, fixed by the addition of 15 ml of cold (−20°C) 95% ethanol and 0.75 ml of 0.5 M EDTA, and stored at −20°C.

For DNA isolation, the cells were pelleted by centrifugation, washed once with 0.5 ml of 10 mM Tris (pH 8.0)–1 mM EDTA (TE), and then suspended in 0.5 ml of lysis buffer (10 mM Na₂HPO₄, 10 mM EDTA, 1 M sorbitol). 2-Mercaptoethanol (3 µl) and 5 µl of 25-mg/ml Zymolyase (20,000T) were added to this suspension, and the mixture was incubated at 37°C for 30 min. The resulting spheroplasts were harvested by centrifugation and resuspended in 0.5 ml of 50 mM EDTA. The spheroplasts were lysed by the sequential addition of 7.5 µl of 20% sodium dodecyl sulfate and 10 µl of 10-mg/ml proteinase K; this mixture was incubated at 65°C for 30 min. Two hundred microliters of 5 M potassium acetate was added to each tube, and the mixture was kept on ice for 45 min. The mixture was then centrifuged in the cold for 15 min, and the supernatant was collected. The DNA in the supernatant was precipitated with isopropanol, and the resulting pellet was resuspended in 200 µl of TE containing 50 µg of RNase per ml. After incubation at 65°C for 15 min, the DNA was reprecipitated and the pellet was washed once with 70% ethanol. After being washed, the pellet was dried and dissolved in 50 µl of TE.

For each meiotic time point, the isolated DNA was treated with a 25-fold excess of the desired restriction enzymes, and the resulting fragments were separated on a 0.8% agarose gel. Standard Southern blot procedures were performed (17). A *Xba*I-*Xho*I fragment derived from pDN42 containing a *Bgl*II-*Xho*I fragment of *HIS4* was used as a hybridization probe; the *Xba*I site is derived from the polylinker in pDN42. The relative amount of DNA present as heteroduplex was determined by densitometric analysis of the autoradiographs. After very long exposures, a small amount of undigested DNA is detected in all lanes. For the physical analysis of crossovers, since the 21-kb parental DNA fragment transferred poorly relative to the 11-kb parental DNA fragment, we calculated the total amount of hybridization to the parental bands by doubling the amount of hybridization to the 11-kb fragment.

Commitment to meiotic recombination; return-to-growth experiment. Cells become committed to meiotic levels of recombination before they become committed to meiotic division (26). Commitment to meiotic recombination can be determined by transferring a diploid (containing heteroalleles for auxotrophic mutations) to sporulation medium and, at various time intervals, returning the cells to medium allowing vegetative growth of prototrophs. The diploid MS1 is heteroallelic for mutations in *HIS4*. We sporulated a culture of MS1 using the same conditions described for the physical analysis of heteroduplexes. Aliquots were collected at various times during meiosis, diluted, and analyzed on plates containing synthetic dextrose (SD) complete medium (to monitor cell viability) and SD medium lacking histidine (to detect meiotic recombination).

Assay for nuclear division (32). Aliquots (1 ml) of the sporulating culture were collected at different time points. Cells were harvested by centrifugation and fixed in 0.5 ml of 4% formaldehyde at room temperature for 20 h. The fixed cells were washed with 1 ml of water and stained with 0.5 ml of 1-µg/ml 4',6-diamidino-2-phenylindole (DAPI). After incubation at room temperature for 5 min, the cells were washed with water and resuspended in 1 ml of water. The nuclei were visualized with a Nikon fluorescence microscope.

RESULTS

System for detection of heteroduplex DNA. If a heteroduplex is formed between two genes, one of which contains an insertion, the inserted sequences would be expected to be in a single-stranded conformation. With few exceptions, heterozygous insertion or deletion mutations in yeast cells show very little PMS, suggesting that these single-stranded regions are usually efficiently recognized by cellular mismatch repair systems (9, 20). We have previously shown that a mismatch resulting from a heteroduplex formed between a wild-type gene and a gene with a palindromic insertion leads to high frequencies of PMS (19). We interpret this result as indicating that the stem-loop or hairpin configuration expected for a heterozygous palindromic insertion in a heteroduplex is inefficiently recognized by cellular mismatch repair systems.

We constructed a diploid strain (DNY86) that had one mutation caused by insertion of a 30-bp palindrome into the *Sal*I site of *HIS4* (*his4-IR15*) and a second mutation (on the opposite homolog) with a 32-bp palindromic insertion of unrelated sequence into the same restriction site in *HIS4* (Fig. 1). A heteroduplex formed between strands derived from these two genes would be expected to form a cruciform (Fig. 1B). Another feature of the palindromic insertions is that each contained a different restriction site in its center of symmetry: *his4-IR15* had a *Pst*I site and *his4-IR16* had a *Bam*HI site. Since bases at the tip of a palindrome are unpaired, the restriction sites of the insertions in the cruciform configuration should not be substrates for either *Pst*I or *Bam*HI. Thus, if the cruciform structure shown in Fig. 1B is not well-recognized by the mismatch repair systems, one should be able to detect heteroduplex DNA as DNA fragments of the appropriate size that are resistant to cleavage by *Pst*I and *Bam*HI.

Genetic evidence that heteroduplex-induced cruciforms are poorly recognized by mismatch correction mechanisms. As described above, heterozygous palindromic mutations have high levels of PMS relative to gene conversion, indicating that hairpin structures are poorly repaired by mismatch correction systems. To determine whether the cruciform structure shown in Fig. 1B is also poorly repaired, we examined the patterns of aberrant segregation at the *HIS4* locus in DNY86.

The *HIS4* protein has three enzymatic activities encoded by complementing regions within the gene called *HIS4A*, *HIS4B*, and *HIS4C* (13). Although both *HIS4* genes in DNY86 contain mutations, since the *his4-IR15* mutation eliminates only *HIS4A* activity, whereas *his4-IR16* eliminates all three enzymatic activities, by complementation testing (described in Materials and Methods), one can examine the segregation of the two mutations separately. To describe the tetrad analysis, we use the nomenclature established for eight-spored fungi (since many of the aberrant segregants are PMS events), and the first number of each class represents the number of spores with the *his4-IR15* allele. For example, the 6:2 class represents tetrads with three *his4-IR15* spore colonies and one *his4-IR16* spore colony. Of 316 tetrads examined, we found 18 6:2, 12 2:6, 32 5:3, 32 3:5, 7 aberrant 4:4, 3 7:1, 2 1:7, 2 aberrant 6:2, and one deviant 5:3. Approximately 34% of the tetrads had an aberrant segregation at *HIS4*, and 72% of the aberrant segregants were PMS events. These numbers are very similar to those observed for strains that were heterozygous for single palindromic insertions at the *Sal*I site. For example, we previously observed 35% aberrant segregation (72% of the aberrant segregation events were PMS) in a strain

FIG. 2. Physical detection of heteroduplex DNA during meiosis. DNA samples isolated from meiotic cells (derived from the strain DNY86) were digested with *PvuII*, *PstI*, and *BamHI*. The resulting fragments were separated by gel electrophoresis and examined by Southern analysis with the region shown in Fig. 1A as a hybridization probe. The sizes of the fragments are indicated by arrows on the right, and the numbers on the top indicate the time (in hours) after the induction of meiosis. The 2.4-kb fragment represents the heteroduplex DNA (hDNA).

heterozygous for the *his4-lop* mutation (19). We conclude, therefore, that the cruciform structure shown in Fig. 1B, like the hairpin structure, is poorly recognized by the mismatch repair system.

Physical detection of heteroduplexes. As shown in Fig. 1A, most of the *HIS4* gene and a portion of *BIK1* are located on a 2.4-kb *PvuII* fragment. If the parental chromosomes of DNY86 are treated with *PvuII*, *BamHI*, and *PstI*, two fragments of 1.1 and 1.3 kb would be observed, since one insertion has a *BamHI* site and the other has a *PstI* site. If a heteroduplex involving the palindromic insertions is formed, then a band of 2.4 kb will also be observed. As described above, we have genetic evidence for heteroduplexes involving the palindromic insertions. To obtain supporting physical evidence, we transferred DNY86 to sporulation medium and collected cells at various time intervals. DNA was isolated from these cells and treated with *PstI*, *BamHI*, and *PvuII*. The resulting fragments were separated by gel electrophoresis, transferred to membranes, and hybridized to a probe containing sequences from the 2.4-kb *PvuII* fragment (Fig. 1A). A representative example of the time course of appearance of the heteroduplex DNA during meiosis is shown in Fig. 2. Heteroduplex DNA begins to appear at about 6 h after induction of meiosis and reaches a maximum level of about 6% of the total *HIS4*-specific DNA.

Since it is apparent from Fig. 2 that the 2.4-kb band is meiosis specific, as expected for heteroduplex DNA, it is unlikely that this band represents partially digested DNA. However, the 2.4-kb band might reflect a cruciform extruded from a single chromatid (as shown in Fig. 3A) rather than a cruciform reflecting heteroduplex formation between two nonsister chromatids. To check this possibility, we constructed a diploid strain, DNY78, that was homozygous for the *his4-IR15* mutant allele. If meiosis-specific intrachromatid cruciforms could form, one should see a 2.4-kb band. Since no such band was observed (Fig. 3B), we conclude that a 30-bp palindrome is rarely extruded into a cruciform *in vivo*; a similar conclusion was also reached from *in vitro* studies on the kinetics of cruciform formation (10). In addition, this result indicates that the 2.4-kb band observed in DNY86 represents heteroduplexes formed at the *HIS4* locus. Additional data supporting this interpretation based on the analysis of *Rec⁻* mutants will be discussed below.

Kinetic relationship of heteroduplex formation with other meiotic events. The level of heteroduplex formation as a

FIG. 3. Cruciform formation in homoduplexes. (A) Restriction fragments expected as a consequence of intramolecular cruciform formation in DNY78, a strain homozygous for the *his4-IR15* mutation. If cruciforms were extruded from a homoduplex, one would expect a 2.4-kb fragment. (B) Southern analysis of DNA isolated from meiotic cells of DNY78. The DNA samples were treated with *PstI* and *PvuII*. No fragment with the size expected for a cruciform extruded from a homoduplex (2.4 kb) was observed. Numbers on left show size in kilobases.

function of time in sporulation medium is shown in Fig. 4. Heteroduplex formation increases to a maximum of 6% of the total DNA at 16 h after induction of meiosis and then declines as spores are formed. As described in the Discussion, this decline is likely to reflect the difficulty of isolating meiotic DNA from spores. In addition to examining the timing of heteroduplex formation, we monitored several other landmark events in meiosis including commitment to meiotic recombination, formation of mature crossover products, meiotic nuclear divisions, and spore formation. These results are summarized in Fig. 4 and discussed in detail below.

(i) **Commitment to meiotic recombination.** Meiotic levels of recombination can be induced in diploid cells without commitment to meiotic divisions, if the cells are withdrawn from the sporulation medium and transferred to nutrient medium (26). Meiotic recombination is generally monitored with heteroallelic markers. In our experiments, we used the diploid MS1, which was heteroallelic at the *HIS4* locus, and monitored meiotic recombination by plating the cells on medium lacking histidine. In general, heteroallelic recombination reflects gene conversion (20). As shown in Fig. 4, commitment to meiotic recombination begins at about 4.5 h after the induction of meiosis, and the maximum level of recombination is achieved at about 12 h. The timing of commitment to meiotic levels of recombination appears to precede slightly heteroduplex formation, suggesting that some recombination events initiated during meiosis can result in heteroduplex formation in vegetative medium.

(ii) **Timing of conversion and crossovers.** As stated in the Introduction, in most models of recombination, the heteroduplex is an intermediate in the production of a reciprocal exchange. Thus, one would expect that formation of the heteroduplex should precede formation of the mature crossover. Crossovers can be monitored physically with strains containing heterozygous restriction sites (3). We constructed

FIG. 4. Appearance of heteroduplex DNA relative to commitment to meiotic recombination, mature crossovers, binucleate cells (meiosis I), and spore formation. Commitment to meiotic recombination was measured by scoring His⁺ prototrophs in return-to-growth experiments with the heteroallelic diploid MS1. All other measurements were done in single cultures of DNY86. Heteroduplex DNA and crossovers were assayed as described in the legends to Fig. 2 and 5, respectively. Spores in formaldehyde-fixed cells were counted under the microscope; two-, three-, and four-spored asci were counted. A total average of 200 cells were counted for each point. Binucleate cells were scored with the fluorescent stain DAPI, as described in Materials and Methods. Two graphs are used for clarity of presentation. (A) Heteroduplex formation relative to commitment to meiosis and crossovers. The levels of heteroduplexes and crossovers are shown as a fraction of the maximum observed level (indicated by the number 1 on the ordinate). The commitment data are shown on an exponential scale. The maximum observed values were 6% of total meiotic DNA for the heteroduplex DNA, 6 to 7% of total for the crossover fragment, and a frequency of 1.4×10^{-2} (His⁺ spores per total cells) for the commitment analysis. (B) Heteroduplex formation relative to formation of binucleate cells and spore formation. All data are shown as a fraction of the maximum observed levels (6% of total meiotic DNA for the heteroduplexes, 8% for the frequency of binucleate cells, and 53% for the frequency of sporulated cells).

DNY86 with two heterozygous restriction site markers flanking the *HIS4* region (Fig. 5A). One marker was an insertion of *URA3* (disrupting a *SpeI* site) located about 4 kb downstream of *HIS4*. The other marker was a palindromic insertion (containing a *BamHI* restriction site) disrupting a *SalI* site in *BIK1*, located about 1 kb upstream of *HIS4*. As shown in Fig. 5A, one of the expected crossover products is a *HIS4*-specific *SalI*-*SpeI* fragment of 7 kb, a size smaller than either parental *SalI*-*SpeI* fragment. This product was

FIG. 5. Physical analysis of crossovers. (A) Restriction fragments expected as a consequence of crossing over in DNY86. The heterozygous sites and relevant homozygous sites on the two homologs are indicated. Any crossover between the heterozygous *BIK1* *SalI* site (wild-type *BIK1* gene with *SalI* site; mutant *bik1* gene with an insertion of a palindromic oligonucleotide [with a *BamHI* site] that deletes the *SalI* site) and the heterozygous *SpeI* site (wild-type chromosome with *SpeI* site; other chromosome with insertion of *URA3* that deletes this site) will produce crossover *SalI*-*SpeI* fragments of 7 and 25 kb. The expected sizes of the parental fragments are 21 and 11 kb. Abbreviations: Sp, *SpeI*; S, *SalI*; and B, *BamHI*. (B) Southern analysis of DNA (treated with *SpeI* and *SalI*) isolated from meiotic cells of DNY86. A *XhoI*-*BglII* fragment of the *HIS4* coding region was used as a probe (Fig. 1A). The numbers on the top indicate the time points (in hours) of sample collection, and the recombinant band is indicated.

detected after about 6 h of sporulation (Fig. 5B) and had approximately the same kinetics of appearance as that observed for the heteroduplex band (Fig. 4A). As observed for heteroduplexes, the level of the 7-kb crossover (maximum level of 5 to 7% of total DNA) product declined as spores formed. As discussed below, we attribute this decline to a small fraction of cells that do not undergo meiosis. Gene conversion events also can result in the formation of a 7-kb recombinant band; in the Discussion, we calculate that crossovers and conversion events contributed approximately equally to formation of the 7-kb band.

(iii) **Timing of meiosis I and sporulation.** The meiosis I division was assayed by examining the frequency of binucleate cells by fluorescence microscopy. As shown in Fig. 4B, the maximum level of binucleate cells was observed between 12.5 and 17.5 h. The broadness of this peak probably reflects a continued increase in the level of binucleate cells, coupled with loss of binucleate cells as a consequence of the second meiotic division. The kinetics of appearance of binucleated cells is similar to the kinetics of heteroduplex formation, presumably because of the asynchrony of the culture. After the premeiotic DNA replication, the homologs segregate to separate poles during the first meiotic division.

FIG. 6. Physical analysis of heteroduplex DNA in Rec^- mutants. Southern analyses were performed as described in the legend to Fig. 2. The numbers indicate the time in hours of sample collection after the induction of meiosis. The expected position of the heteroduplex DNA (hDNA) is indicated. (A) Diploid strain DNY90 (homozygous for *rad50* null mutation); (B) diploid strain DNY93 (homozygous for *rad50S* mutation); (C) diploid strain DNY102 (homozygous for *rad52* null mutation).

Spore formation begins at about 17 h after induction of meiosis, and the maximum level is reached at 32 h (Fig. 4B).

Heteroduplex formation in Rec^- mutants. A large number of mutants have been isolated that reduce the frequency of meiotic recombination (reviewed in reference 20). These mutations greatly reduce spore viability, a result that is interpreted as indicating that recombination is necessary for proper chromosome disjunction at the first meiotic division (20). The *spo13* mutation leads to bypass of the first meiotic division (14). The construction of strains with both the *spo13* mutation and a Rec^- mutation revealed that there are two classes of Rec^- mutants, those that yield viable spores in combination with *spo13* (class 1) and those that do not yield viable spores (class 2). The accepted interpretation of this result is that class 1 mutants represent early blocks in the recombination process (for example, formation of the DNA lesion that initiates recombination), whereas class 2 mutants affect later steps (for example, resolution of Holliday junctions).

It would be useful to determine by physical techniques the steps of recombination that are defective in the Rec^- mutants. Two physical parameters that have been examined for some mutants are the ability to form mature crossovers and the ability to form a double-strand break. Borts et al. (3) showed that the *rad6*, *rad52*, and *rad57* mutants were not defective in formation of mature crossovers but that the *rad50* and *spo11* mutants were. Since *rad50* and *spo11* were previously classified as class 1 mutants, and *rad52* and *rad57* were classified as class 2 mutants, these physical studies were consistent with genetic predictions. Cao et al. (4) found that *rad50* null mutations prevented formation of a double-strand break, whereas the *rad50S* mutation accumulated unprocessed double-strand breaks. An unprocessed break represents a double-strand break in which the broken ends have not been degraded into single-stranded tails. It has been

suggested that these single-stranded ends are required for heteroduplex formation (28).

We examined heteroduplex formation in a *rad50* null mutant, *rad50S*, and *rad52* (Fig. 6). We found that the *rad50* null mutant had no detectable heteroduplex. The *rad50S* mutant had a very small amount of heteroduplex (less than 10% wild-type levels). In contrast, strains with the *rad52* mutation (Fig. 6C) had levels of heteroduplex formation that were only slightly (less than twofold) reduced relative to wild-type strains. In addition, heteroduplex formation was slightly delayed in the *rad52* strain compared with the wild-type strain. This delay could reflect the slower growth rate of the *rad52* strains.

In summary, our analysis of these mutants is consistent with earlier conclusions based on genetic and physical studies. The *rad50* mutant is blocked before heteroduplex formation, and the *rad52* mutant is blocked at some stage after heteroduplex formation. The absence of the 2.4-kb *PvuII* band in the *rad50* strain also supports our conclusion that the presence of this band in wild-type strains does not represent a product of partial digestion with restriction endonucleases.

Double-strand breaks at *HIS4*. At two loci in *S. cerevisiae*, meiosis-specific double-strand breaks have been detected, and it has been suggested that these breaks represent the initiating lesion for meiotic recombination (4, 28). In our genetic background, despite the high level of *HIS4* recombination, the level of double-strand breaks is low (Fig. 7A). We monitored the double-strand break by hybridizing a *HIS4*-specific probe to a *BglIII* digest of meiotic DNA. There is a faint discrete band (about 1.9 kb in size) prior to sporulation. This band becomes more intense and more fuzzy during meiosis. In an isogenic *rad50S* strain, the band is more discrete (Fig. 7B); the maximum level of the band is 2.4% of the *HIS4*-specific DNA. Double digests with *BglIII*

FIG. 7. Double-strand breaks at the *HIS4* locus in wild-type and *rad50S* strains. DNA was isolated from cells after induction of meiosis and treated with *BglIII*. The separated fragments were hybridized to the *XhoI-BglIII* fragment of *HIS4*. The sizes of the fragments (in kilobases) are indicated. (A) Diploid DNY112 (homozygous *HIS4* and *RAD50*); (B) diploid DNY115 (homozygous *HIS4* and *rad50S*).

and *PvuII* indicated that the position of the break is 1.9 kb from the *BglII* site in *HIS4*. The location of the break is at the RAP1 binding site (± 75 bp). Previous studies indicate that mutations at this site significantly reduce meiotic recombination at *HIS4* (31).

DISCUSSION

We describe above a method for detecting heteroduplex formation that should be generally useful for any locus in *S. cerevisiae* that has a high level of recombination. We used this method to examine the kinetic relationship between heteroduplex formation and other meiotic events and to examine heteroduplexes in several meiotic mutants. In addition, we found a meiosis-induced double-strand DNA break located upstream of the *HIS4* locus. The implications of these results are discussed below.

In our studies, commitment to heteroallelic recombination occurred before heteroduplex formation or formation of crossover products; the latter two events occurred at approximately the same time. In the crossover experiment, the 7-kb *SalI-SpeI* fragment was used as an indication of crossovers in the *HIS4* region; approximately 5 to 7% of the total meiotic DNA was in this fragment. Gene conversion events involving either the heterozygous *SalI* or *SpeI* sites could also result in a 7-kb fragment. To obtain the 7-kb fragment by gene conversion, one would need either a 6:2 conversion event involving the *BIK1* marker or a 2:6 event involving the *URA3* gene. The observed frequency of 6:2 conversion of the *BIK1* marker is 9% (21), and 6% of the tetrads had a 2:6 conversion for the *SpeI::URA3* marker. Since only one spore of each tetrad is affected by these events, the expected fraction of meiotic chromosomes with a 7-kb fragment resulting from conversion is about 4%. Thus, about 60% of the events that produced the 7-kb *SalI-SpeI* fragment were likely to be conversion events, and about 40% were crossovers.

The observation that heteroduplex formation occurred at approximately the same time as the conversions and crossovers has two interpretations. One interpretation is that heteroduplexes are an intermediate in the production of the other products and that this intermediate is quickly processed. Alternatively, it is possible that heteroduplexes and crossovers represent two different classes of recombination events. Intrachromosomal gene conversion events between repeated sequences are rarely associated with crossing over (15). In addition, some yeast mutations differentially affect gene conversion and crossing over (7). Since it is difficult to formulate a model of crossing over that does not require some level of heteroduplex, we prefer the first interpretation.

We observed a decline in the level of heteroduplexes late in meiosis. This decline can be explained in two ways. First, it may reflect an artifact of the difficulty in isolating DNA from spores. In our experiments, about 50% of the cells sporulate. If some fraction (or all) of the nonsporulating cells fail to undergo meiotic levels of heteroduplex formation and if DNA is more difficult to extract from spores than from unsporulated cells, then the fraction of DNA in the heteroduplex band would decline as spores are formed. Alternatively, the decline in heteroduplexes could reflect mismatches that are repaired during meiosis leading to gene conversion events. We favor the first alternative for several reasons. First, we find that the yield of DNA declines as spores are formed (data not shown). Second, the decline in heteroduplexes (Fig. 4A) occurs at the same time as spores

begin forming (Fig. 4B). Third, we observe a similar decline in the level of conversions and crossovers whose formation is not dependent on mismatch repair.

About 7% of the spore colonies in DNY86 were sectored. If all the cells in the sporulating culture underwent meiotic recombination, we would expect that at least 7% of the meiotic DNA should be in the heteroduplex band. If only half of the cells underwent meiotic recombination and if all cells had the same DNA content, then only 3 to 4% of the DNA would be in the heteroduplex band. Since most of the unsporulated cells were unbudded, however, it is likely that the meiotic cells contain twice the amount of DNA as the unsporulated cells. Consequently, based on a 7% sectored colony frequency and assuming that only half of the cells underwent meiotic recombination, we calculate that about 5% of the DNA in DNY86 should be in heteroduplexes. This value is not significantly different from the observed level of heteroduplex (6%).

Heteroduplex formation, as expected, precedes spore formation. Heteroduplex formation also initiates prior to meiosis I, although there is considerable overlap (presumably due to asynchrony) between cells that are initiating heteroduplexes and those that are completing meiosis I. C. Goyon and M. Lichten (11) have also performed experiments comparing the kinetics of heteroduplex formation (measured with denaturing gels) and other meiotic events. They find the same relative order of events at *ARG4* as we find at *HIS4*.

One advantage of physical methods of detecting recombination events is that it allows the analysis of recombination in strains that do not produce viable meiotic products. We found that *rad50* mutants were defective in heteroduplex formation, whereas *rad52* mutants had nearly wild-type levels of heteroduplex. These results are consistent with previous studies indicating *rad50* mutants were blocked at an early step in recombination and *rad52* mutants were blocked at a later stage (reviewed in reference 20). In addition, our results support earlier conclusions based on a physical analysis of crossover products (3).

As with several other meiotic recombination hotspots in yeast cells (4, 28), we have found evidence for a double-strand break; in two previous studies (11, 33), a double-strand break was also observed near *HIS4*. The band representing the broken chromosomes is diffuse in wild-type cells and discrete in the *rad50S* strain. The position of this break is close to the RAP1 binding site located between *HIS4* and *BIK1*. Previous studies indicate that the binding of the RAP1 protein to the RAP1 binding site is required for the highest level of meiotic recombination at both *HIS4* and *BIK1* (31).

There are several observations that indicate that this break may not represent the sole initiating lesion for recombination at *HIS4*. First, the break is not meiosis specific (Fig. 7A). Second, although genetic experiments indicate that the site located between *HIS4* and *BIK1* stimulates recombination at both loci (6, 31), in strains heterozygous for markers in both loci, most of the recombinant tetrads involve either *HIS4* or the *BIK1* locus (21). This pattern indicates that the initiating lesion is not likely to represent a symmetrically processed double-strand break located between the two loci, although a model involving an asymmetrically processed double-strand break is not ruled out. Third, the maximum level of the break (2.4%) in the *rad50S* strains is less than the observed level of heteroduplex formation (6%) in wild-type cells. This last conclusion has the caveats that we cannot rule out some processing of the double-strand break in the *rad50S* strains and we cannot be sure that the fraction of

cells entering meiosis was identical in the wild-type and the *rad50S* strains.

Since heteroduplexes can be detected either by the method described above or by denaturing gels, one final issue is the comparative advantages of the two methods. For both methods, mutant alterations that lead to poorly repaired mismatches must be introduced into the locus of interest. We have found that palindromic insertions do not alter the pattern of recombination when inserted at a number of different positions in the *HIS4* gene (6, 19). One disadvantage of the denaturing gel system is that the ease of detection of mismatches depends on the context of the mismatch (18). In addition, our system employs standard methods of gel analysis. One disadvantage of our method is that partially digested DNA products can be confused with heteroduplexes. Presumably, the relative advantages of the two methods will be clarified as other laboratories begin using these procedures.

In summary, we describe a general physical method of demonstrating heteroduplex formation in *S. cerevisiae*. We find that heteroduplex formation and mature crossovers occur at approximately the same time, and we show that heteroduplex formation is defective in *rad50*, but not *rad52*, mutant strains. Finally, we observe a double-strand break that occurs near the RAP1 binding site between *HIS4* and *BIK1*.

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