The Mechanism of Mitotic Recombination in Yeast

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the Department of
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ABSTRACT

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Abstract

A mitotically dividing cell regularly experiences DNA damage including double-stranded DNA breaks (DSBs). Homologous mitotic recombination is an important mechanism for the repair of DSBs, but inappropriate repair of DNA breaks can lead to genome instability. Despite more than 70 years of research, the mechanism of mitotic recombination is still not understood. By genetic and physical studies in the yeast *Saccharomyces cerevisiae*, I investigated the mechanism of reciprocal mitotic crossovers. Since spontaneous mitotic recombination events are very infrequent, I used a diploid strain that allowed for selection of cells that had the recombinant chromosomes expected for a reciprocal crossover (RCO). The diploid was also heterozygous for many single-nucleotide polymorphisms, allowing the accurate mapping of the recombination events.

I mapped spontaneous crossovers to a resolution of about 4 kb in a 120 kb region of chromosome V. This analysis is the first large-scale mapping of mitotic events performed in any organism. One region of elevated recombination was detected (a "hotspot") and the region near the centromere of chromosome V had low levels of recombination ("coldspot"). This analysis also demonstrated the crossovers were often associated with the non-reciprocal transfer of information between homologous chromosomes; such events are termed "gene conversions" and have been characterized in detail in the products of meiotic recombination. The amount of DNA transferred during mitotic gene conversion events was much greater than that observed for meiotic conversions, 12 kb and 2 kb, respectively. In addition, about 40% of the conversion

events had patterns of marker segregation that are most simply explained as reflecting the repair of a chromosome that was broken in G1 of the cell cycle.

To confirm this unexpected conclusion, I examined the crossovers and gene conversion events induced by gamma irradiation in G1- and G2-arrested diploid yeast cells. The gene conversion patterns of G1-irradiated cells (but not G2-irradiated cells) mimic the conversion events associated with spontaneous reciprocal crossovers (RCOs), confirming my hypothesis that many spontaneous crossovers are initiated by a DSB on an unreplicated chromosome. In conclusion, my results have resulted in a new understanding of the properties of mitotic recombination within the context of cell cycle.

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1. Introduction

A necessary condition to long-term survival of all organisms is the faithful transfer of genetic information between generations. DNA damage, therefore, must be repaired efficiently. Both prokaryotes and eukaryotes have multiple systems designed to repair multiple types of DNA damage and DNA mutations. Some of these types of DNA damage/mutations include misincorporation errors during DNA replication, DNA polymerase slippage events, base damage (for example, oxidation of guanine), pyrimidine dimers caused by ultraviolet light, single-stranded DNA nicks, and double-stranded DNA break (Shaughessy and Demarini, 2009). One particularly potent type of DNA damage is the double-stranded DNA break (DSB). In yeast, it has been calculated that one unrepaired DSB is often lethal (Resnick and Martin, 1976; Frankenberg-Schwager and Frankenberg, 1990). In *Saccharomyces cerevisiae*, homologous mitotic recombination is the primary pathway for the repair of DSBs (Paques and Haber, 1999). Despite the importance of mitotic recombination as a mechanism for DSB repair, many of the mechanistic details of mitotic exchange are not understood. In this thesis, I investigate a number of important features of spontaneous and induced mitotic crossovers in yeast.

In Chapter 1, I describe the background relevant to my study. In Chapter 2, I present my analysis of spontaneous mitotic crossovers and associated gene conversion events. In Chapter 3, I address the properties of mitotic crossovers induced by gamma rays in yeast cells synchronized in G1 or G2. In the last chapter, I summarize the major findings of my research and how these findings contribute to our current understanding

of DNA repair and recombination. In this last chapter, I will also briefly discuss potential future research directions.

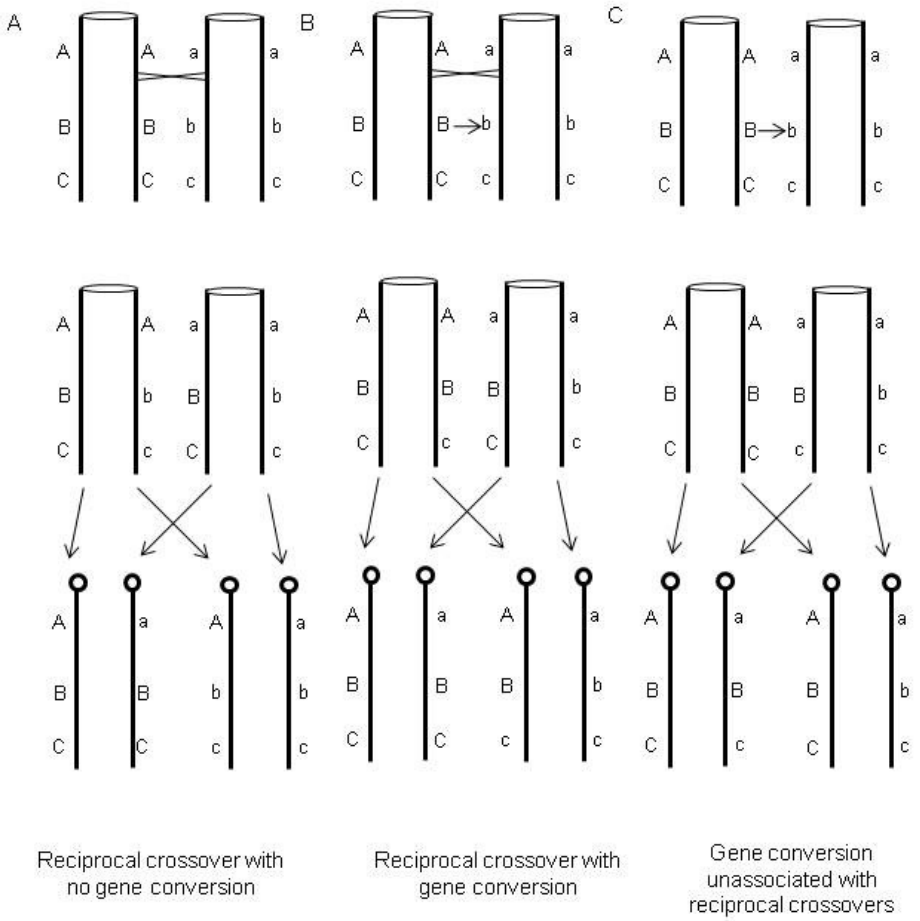
The topics that will be discussed in this chapter include: 1) Systems for detecting mitotic crossovers and gene conversion, 2) DNA lesions, DNA structures, and cellular conditions associated with spontaneous or induced mitotic recombination, 3) repair of DNA damage by homologous recombination (HR), and 4) repair of DNA damage by non-homologous end joining (NHEJ).

1.1 Systems for detecting mitotic crossovers and gene conversion

One defining characteristic of mitotic crossovers is that markers that are centromere-distal to the site of the exchange that are originally heterozygous in the diploid become homozygous (Fig. 1A). It should be noted that only half of the crossovers result in the marker becoming homozygous (as shown in Fig. 1A), because if both recombinant chromatids co-segregate, the markers retain heterozygosity, although the coupling relationships are changed.

In addition to reciprocal crossovers (RCOs), mitotic gene conversion events have also been observed. In a gene conversion event, DNA sequences are transferred non-reciprocally from one homologue to another. In the example shown in Fig. 1B and 1C, the sequences of the “B” allele replace the sequences of the “b” allele. As will be discussed elsewhere in the Introduction, the amount of DNA transferred during gene conversions in meiotic recombination is usually about 1 kb. The mechanism of transfer (also discussed below) often involves heteroduplex formation between the two

homologues followed by correction of mismatched bases by the mismatch repair system (Petes *et al*. 1991). As shown in Fig. 1B, gene conversion events are sometimes associated with an RCO event adjacent to the conversion tract. Alternatively, gene



conversion events can occur without an associated RCO (Fig. 1C). For mitotic conversion events, about 1-10% are associated with RCOs (Petes *et al*. 1991).

Figure 1 Mitotic reciprocal crossovers and crossover-associated gene conversions

(A) In half of the mitotic crossover events during a G2-recombination event, markers that are originally heterozygous and centromere-distal to the exchange junction become homozygous in both of the daughter cells. (B and C) In addition, gene conversion (the non-reciprocal transfer of information from one chromosome to another) can occur; in this figure, gene conversion is shown as the replacement of the "b" allele with the "B" allele. Gene conversion can be associated with reciprocal crossovers (B), or not (C).

than the frequency of meiotic exchanges for the same physical interval (Barbara and Petes, 2006). Thus, selective methods have been developed to allow the detection of these events. The "classic" procedure for detecting crossovers is shown in Fig. 2A. Diploids are constructed that are heterozygous for the recessive marker *can1*. Cells that are heterozygous for this mutation are sensitive to canavanine. If a crossover occurs between the heterozygous mutation and the centromere of chromosome V, half of the events will produce a *can1*/*can1* (canavanine-resistant) derivative. Although a canavanine-resistant derivative could also be produced by gene conversion, since the distance between *CEN5* and the *CAN1* locus is 120 kb and conversion tracts are thought to be small, it is assumed that most Can^R derivatives reflect RCOs rather than conversions.

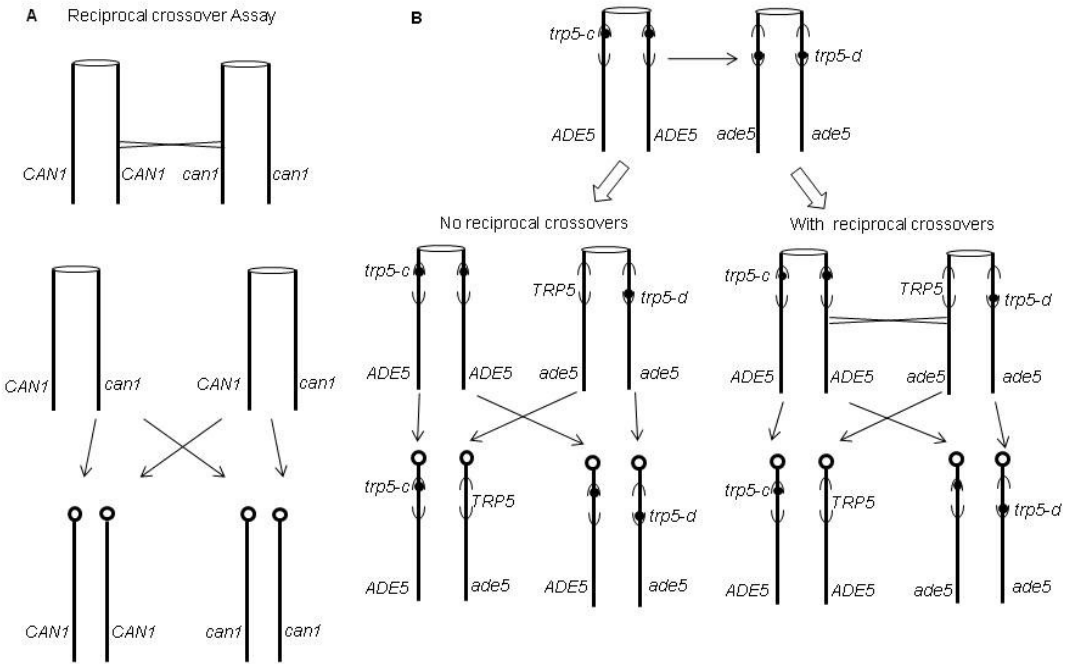


Figure 2 Classical assays to select reciprocal crossovers and gene conversions in yeast

(A) The "classical" system utilizes a diploid strain heterozygous for *CAN1*/*can1* allele to select for reciprocal crossovers. The starting diploid is canavanine sensitive, but can give rise to a canavanine-resistant daughter cell through reciprocal crossing over between the centromere and the heterozygous *can1* marker. (B) In this example of a genetic system to select for gene conversions, the starting diploid contains two non-complementing *trp5* heteroalleles and is, therefore, Trp^-, it is also heterozygous for the *ade5* mutation, located centromere-distal to the *trp5* heteroalleles. The replacement of the *trp5-d* sequence with the wild-type information derived from the *trp5-c* gene can generate a Trp^+ daughter cell that remains heterozygous for the *ade5* markers (left side of B). Alternatively, a crossover may be associated with the conversion event, producing a Trp^+ daughter cell that is homozygous for the *ADE5* allele (right side of B).

The classic mitotic gene conversion assay is shown in Fig. 2B. In this example, the diploid has non-complementing *trp5* heteroalleles and a heterozygous centromere-distal marker (*ade5*/*ADE5*). In the example shown, a portion of the *trp5-c* gene that contains the wild-type sequence in the region of the *trp5-d* is substituted for the *trp5-d* sequence, generating a wild-type *TRP5* gene. It was shown previously that generation of a wild-type gene from a heteroallelic pair was almost always a consequence of gene conversion rather than reciprocal exchange between the two mutations (reviewed by Petes *et al*. 1991). Thus, in this type of experiment, the frequency of Trp^+ cells is a measure of the frequency of mitotic gene conversion, and the *ADE5*/*ade5* markers are examined to determine the frequency of associated RCOs. It should be noted that this method of measuring gene conversion events is quite restrictive. A conversion event in which the entire *trp5-c* allele replaces the *trp5-d* allele would not produce a *TRP5* gene and would not be detected.

Another problem with existing systems for monitoring RCOs and gene conversion events is that these methods select for the recombinant products of only one of the two daughter cells. In Chapter 2, I will describe a system developed recently in our lab (Barbera and Petes, 2006) that allows selection of both recombinant products. This system is the one used in my studies.

1.2 DNA lesions, DNA structures, and cellular conditions associated with spontaneous or induced mitotic recombination

Since spontaneous mitotic recombination events are, in general, very infrequent, evidence for the nature of the DNA lesion that initiates exchange is indirect. Most of the evidence concerning the likely nature of the recombinogenic DNA lesion is based on the use of exogenous DNA-damaging agents (ionizing radiation or ultraviolet light) or site-specific endonucleases (such as HO). Below, I describe some conclusions from these types of studies.

1.2.A Recombinogenic DNA lesions

Two types of DNA lesions are likely to be relevant to recombination: 1) DNA double-strand breaks (DSBs) and 2) DNA single-strand nicks. If two strands are nicked on opposite strands of the duplex within 10-20 base pairs, a DSB can be generated. The evidence that DSBs can induce recombination is based on several lines of evidence (summarized in Paques and Haber, 1999). First, exogenous agents known to induce DSBs (such as X-rays and gamma rays) stimulate recombination (Nakai and Mortimer, 1967). Second, mutants defective in the repair of DSBs have reduced rates of recombination (reviewed by Petes *et al*. 1991). Third, induction of a DSB by a site-specific endonuclease greatly elevates the frequency of mitotic recombination (reviewed by Paques and Haber, 1999). Fourth, Spo11-mediated DSBs are the initiating lesion for meiotic recombination (reviewed by Paques and Haber, 1999).

Since I used gamma rays to stimulate mitotic recombination in the studies reported in Chapter 3, I will review the recombinogenic properties of ionizing radiation in

detail. Ionizing radiation can directly deposit energy into the DNA molecule to produce single-strand nicks and DSBs, or indirectly damage DNA through reactive oxygen species (ROSs) generated by ionization of water molecules surrounding the DNA (Ward, 1988). Under laboratory conditions, ionizing radiation produces 25-fold more nicks than DSBs (Elkind and Redpath, 1977), and X-ray-induced ROSs generate three orders of magnitude more nicks than DSBs (Bradley and Kohn, 1979). High concentrations of ROSs are known to produce closely-spaced lesions, and these lesions include damaged bases and deoxyribose residues, in addition to nicks and DSBs (Ward, 1991). The repair of ionizing radiation induced-damaged nucleotides is also implicated in formation of DSBs. Experiments in both prokaryotic and yeast cells show that the repair of clustered damaged nucleotides through base-excision repair generates DSBs (Blaisdell *et al*., 2001; Kozmin *et al*., 2009). Recently, it has been shown that nucleotide excision repair of DNA cross-links in these damage clusters also can lead to DSB formation in prokaryotes (Sczepanski *et al*., 2009).

The evidence that single-stranded nicks directly stimulate recombination is weak. First, a site-specific nicking enzyme derived from the bacteriophage f1 stimulates mitotic recombination in yeast (Strathern *et al*., 1991). Second, yeast cells treated with ultraviolet light have elevated levels of recombination, if the cells transit the S-period (Galli and Schiestl, 1999). It is not clear at present whether the nicked DNA molecules are directly involved in recombination or whether the nicked molecules need to be replicated to generate a recombinogenic DSB. The results of Galli and Schiestl (1999) are most consistent with the second alternative. Although the DNA lesions for

spontaneous events have not been detected directly, from the evidence cited above, DSBs are likely to be the recombinogenic lesion, although a role for nicks cannot be excluded. My own analysis described in Chapter 2 argues that DSBs initiate at least some spontaneous recombination events.

1.2.B DNA structures associated with elevated levels of mitotic recombination

Two types of unusual DNA structures have been associated with elevated levels of mitotic recombination and elevated frequencies of DSBs: inverted repeats and trinucleotide microsatellites. In two studies, it has been shown that closely-spaced inverted repeats are hotspots for DSBs that stimulate mitotic recombination. Lobachev *et al*. (2002) showed that inverted copies of the 320 bp Alu repeat (derived from the human genome) greatly stimulate mitotic recombination in yeast; they found a DSB that localized at the position of the repeats. Lemoine *et al*. (2005) observed that an inverted pair of Ty elements on chromosome III greatly stimulated mitotic recombination in yeast, particularly when cells had low amounts of DNA polymerase alpha. As in the Lobachev study, a DSB was observed at the position of the inverted repeats. Lemoine *et al*. suggested that, under conditions of low DNA polymerase, “hairpin” structures are formed by the inverted repeats on the lagging strand during DNA replication. They hypothesized that these secondary structures are cleaved by a structure-specific endonuclease, leading to the observed DSB.

A similar type of mechanism may account for the properties of certain trinucleotide repeats. Certain trinucleotide tracts (particularly CCG/CGG, CTG/CAG, and AAG/CTT) are subject to expansion in human cells, producing a variety of genetic diseases (myotonic dystrophy, Huntington's disease, various types of ataxia, and fragile X syndrome; Reddy and Housman, 1997). When CTG/CAG or CCG/CGG tracts are put into yeast, they result in elevated mitotic recombination and high rates of DSB formation (Freudenreich *et al*., 1998; Balakumaran *et al*., 1998). Since both of these trinucleotides are capable of forming "hairpin" structures, it is likely that DSB formation is related to the processing of these structures by structure-specific endonucleases. Recently, Kim *et al.* (2008) showed that AAG/CTT repeats are also a preferred site for DSB formation and that these DSBs elevate the frequency of recombination. Although, AAG/CTT repeats cannot form hairpins but, they can form triplex structures that are also likely substrates for cellular endonucleases. It is clear that mitotic recombination can also occur in regions of the genome that do not have inverted repeats or other unusual structural motifs. Below, I will describe cellular conditions that affect the frequency of mitotic recombination.

1.2.C Cellular conditions affecting the rate of mitotic recombination

One possible source of recombinogenic DNA damage is lesions generated during DNA replication. As discussed above, replication of a nicked DNA template could result is the formation of a DSB (Kuzminov, 2001). Also, if DNA replication is halted by a damaged base, the fork may break, generating a DSB. Third, during DNA replication,

DNA topoisomerase II acts to remove supercoils from the replicating DNA molecule (Champoux, 2001). DSBs could be formed if DNA helicase displaces the DNA strands from the topoisomerase II-DNA complex (Howard *et al*., 1994). Finally, as discussed above, conditions that perturb DNA replication (low levels of DNA polymerases, for example) elevate structure-dependent recombination events.

Another cellular function associated with elevated rates of recombination is transcription. In a screen for yeast sequences that stimulated recombination, Voelkel-Meiman *et al*. (1987) identified *HOT1*, a *cis*-acting recombination hotspot. They found that *HOT1* was the RNA polymerase I promoter. In subsequent experiments, it was shown that elevated levels of RNA polymerase II transcription also stimulate both mitotic crossovers and gene conversions (Thomas and Rothstein, 1989; Bratty *et al*., 1996; Nevo-Caspi and Kupiec, 1994; Saxe *et al*., 2000).

The mechanism of transcription-induced recombination was examined in a study in which recombination events stimulated by the HO endonuclease were compared with those stimulated by high rates of transcription (Gonzalez-Barrera *et al*., 2002). It was shown that both types of recombination had a similar dependence on Rad52p and other recombination proteins (Gonzalez-Barrera *et al*., 2002). Furthermore, the highly transcribed DNA sequence is usually the recipient of information during recombination (Saxe *et al*., 2000; Gonzalez-Barrera *et al*., 2002), as observed in HO-induced recombination, supporting the hypothesis that high rates of transcription are associated with the formation of DSBs.

It is unclear why elevated transcription leads to elevated levels of DNA lesions. Among the possibilities are: 1) highly-transcribed genes have single-stranded regions that are subject to recombinogenic DNA damage, 2) high levels of transcription create a recombinogenic chromatin structure, 3) high levels of transcription are associated with high levels of topoisomerases, and 4) high levels of transcription block passage of the replication fork. Although strong support for the last of these possibilities has been obtained recently (Prado and Aguilera, 2005), it is likely that more than one mechanism is responsible for transcription-activated recombination.

1.3 Repair of DNA damage by homologous recombination (HR)

Much of what we know about the mechanism and proteins involved in homologous mitotic recombination in yeast comes from studies of meiotic recombination. Since all of the chromatids involved in meiotic recombination can be recovered and analyzed by tetrad analysis, inferences about the mechanism of meiotic exchange are more direct than in studies of mitotic exchange. In particular, gene conversion was discovered by analyzing the meiotic segregation of markers. In this section of the Introduction, I will first describe the general features of the three different pathways by which DSBs are repaired by homologous recombination (1.3.A). I will then discuss mechanisms of gene conversion (1.3.B), the template choice for the repair of DSBs (1.3.C), and the regulation of recombination and DNA repair in the cell cycle (1.3.D).

1.3.A Pathways of DSB repair by homologous recombination

In this section, I will describe the basic mechanisms of three homology-mediated recombination repair pathways: the "classic" DSB-repair pathway (DSBR), synthesis-dependent strand-annealing (SDSA), and break-induced replication (BIR). These pathways are diagrammed in Fig. 3. In my discussion, I will emphasize the DSBR pathway because this pathway is the only one that can produce reciprocal crossovers (RCOs), the focus of my thesis.

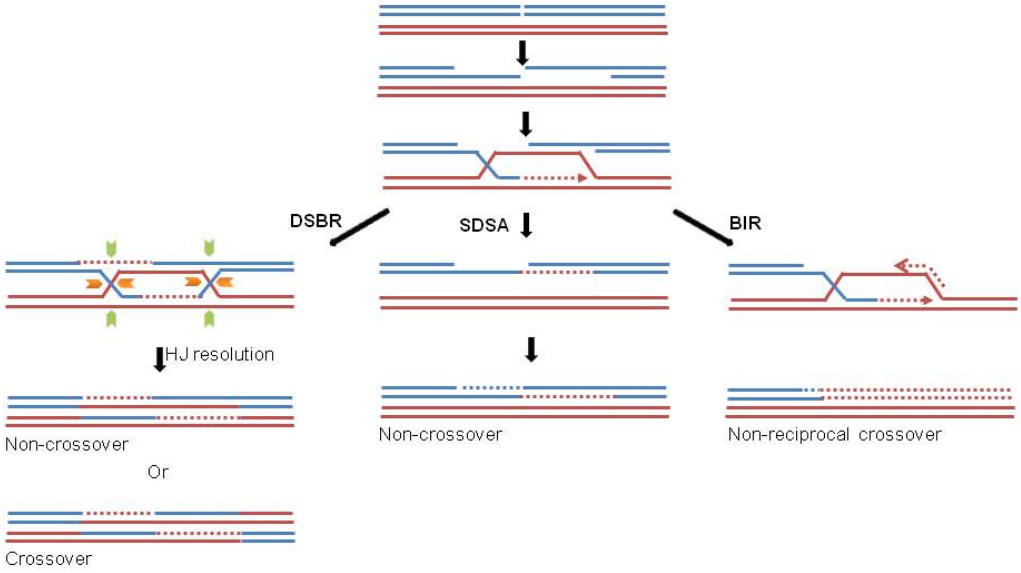


Figure 3 Three homology-mediated DSB repair pathways

A DNA double-strand break (DSB) can be repaired via three related homology-mediated recombination pathways. In the double-strand break repair (DSBR) pathway, one strand at each of the broken ends is excised 5' to 3' in order to generate single-strand tails, which then invade an intact homologous sequence. The region in which two strands derived from different chromosome are paired is a heteroduplex, which may contain mismatched bases. The "capture" of the second end subsequently results in a double Holiday junction (dHJ). The dHJ can be resolved by endonucleases to yield non-crossover or crossover products. Althernatively, the dHJ can be dissolved, resulting in a non-crossover by the the action of the Sgs1-Top3p-Rmi1p complex. In the second pathway (synthesis-dependent strand-annealing, SDSA), the first steps are identical to those of the DSBR pathway. However, before the capture of the second end, the invading strand is displaced. Consequently, this pathway leads to gene conversion without an associated crossover. In the third pathway (break-induced replication, BIR), one portion of the chromosome is lost and the other initiates a replication fork that copies the donor chromosome from the point of invasion to the end of the chromosome.

1.3.A.1 Double-strand-break-repair (DSBR) pathway

The basic features of the DSBR pathway are shown on the left side of Fig. 3, a slightly modified version of the model by Szostak *et al*. (1983). In meiosis, in yeast and most eukaryotes, the DSB that initiates recombination is generated by Spo11p (a modified topoisomerase) and about ten other associated proteins (Aguilera and Rothstein, 2007); as discussed above, the enzyme(s) involved in generated DSBs in mitotic cells are not known. For the homologous recombination pathways (distinct from NHEJ), the broken ends are processed by 5' to 3' excision of one strand of each broken end. This excision event requires a number of proteins including the Mre11p/Rad50p/Xrs2p (MRX) complex, Sae2p, Sgs1p, Exo1p, and Dna2p (Mimitou and Symington, 2009). The resulting single-stranded DNA is initially coated with RPA that is subsequently displaced by Rad52p, Rad51p, Rad55p, and Rad57p (Aguilera and Rothstein, 2007). Rad51p is the primary strand transfer protein and mediates the formation of a heteroduplex with the homologous chromosome.

In the DSBR model, following the initial strand invasion of one broken end in the homologous chromosome and DNA synthesis, the second end is "captured", resulting in the double Holliday junctions (HJ) shown on the left side of Fig. 3. In this structure, there are two regions of heteroduplex (shown as regions in which a blue strand is paired with a red strand). As discussed further below, repair of mismatches within this heteroduplex can produce gene conversion events. Separation of the two recombining molecules requires the resolution of both HJs. The resolution can occur through two different pathways. First, the dHJs can be reversed by the action of the Sgs1-TopoIII-Rml1 complex, leading to non-crossover products (Aguilera and Rothstein, 2007). Alternatively, the HJs can be cut by nucleases. Cutting all four strands at the positions of the green or orange arrows in Fig. 3 would separate the two DNA molecules without a crossover. Cutting one junction at the positions of the green arrows and the other at the position of the orange arrows would separate the molecules in the recombined configurations. The details of these enzymatic cleavages are not yet completely worked out, but the enzymes Yen1p and Mus81p-Eme1p are likely to be involved (Lisby and Rothstein, 2009; Krough and Symington, 2004; Symington and Holloman, 2008). In addition to the proteins described above that are directly involved in repair of the DSBs, there are other proteins that will be discussed later that are needed for the DNA damage checkpoint.

In meiotic recombination in yeast, about half of the conversions are associated with reciprocal crossovers and about half are not (Petes *et al*., 1991). In the context of the DSBR model, it was suggested that this ratio was controlled by the HJ resolvases.

Subsequently, it has been shown that there is a pathway of gene conversion that is not associated with crossing over. Allers and Lichten (2001) showed that *ndt80* mutants have normal levels of gene conversion but no meiotic crossovers. In addition, for mitotic recombination, only a small fraction (<10%) of the conversion events are associated with crossovers (Petes *et al*., 1991). One pathway that yields only gene conversion events is the synthesis-dependent strand-annealing (SDSA) pathway shown in Fig. 3 (Prado *et al*., 2003; Krogh and Symington, 2004).

1.3.A.2 Synthesis-dependent strand-annealing (SDSA) pathway

SDSA shares the early steps of DSBR as shown in Fig.3. However, before second-end capture to form the double HJ, the invading strand is displaced and reassociates with the other broken end (Prado *et al*., 2003; Krogh and Symington, 2004). In addition to the lack of an associated crossover, the SDSA model has another important distinction compared to the DSBR model. Heteroduplex DNA is restricted to one side of the initiating DSB in the SDSA model.

1.3.A.3 Break-induced replication (BIR) pathway

The third pathway of homologous recombination, break-induced replication (BIR), is an infrequently used pathway in meiotic recombination events, although meiotic BIR events have been observed (Merker *et al*., 2003; Mancera *et al*., 2008). In this pathway, one chromosome fragment resulting from the DSB is lost, and the other broken end sets up a unidirectional DNA replication fork that copies the template from the site of the DSB to the end of the chromosome.

The first demonstration of BIR was from a study in which a linear DNA fragment containing a replication origin and centromere was transformed into yeast (Morrow *et al*., 1997). Analysis of the transformants showed that the end of the fragment with homology to the yeast chromosome invaded the chromosome and copied it to the end. The net result of such events, if the BIR events involve homologous chromosomes in a diploid, is a very long gene conversion event that extends to the telomere. Although most mitotic gene conversion tracts are not very long, a minority of conversion events co-convert markers more than 200 kb apart (reviewed in Prado *et al*., 2003); such events are likely to reflect BIR. In other yeast studies, it was found that DSBs in repeated genes could lead to translocations by BIR events that utilize repeats on non-homologous chromosomes (Bosco and Haber, 1998; Lemoine *et al*., 2005). Since my research is restricted to mitotic reciprocal crossovers, BIR events are not likely to be involved.

1.3.B Mechanisms of gene conversion

As discussed above, adjacent to meiotic and mitotic crossovers, there is often a region in which markers are transferred between chromosomes by a non-reciprocal mechanism. Such events are called “gene conversions.” There are two mechanisms that can produce gene conversion: repair of mismatches within a heteroduplex and repair of a double-stranded DNA gap. These mechanisms will be discussed below.

1.3.B.1 Gene conversion as a consequence of correction of mismatches within a heteroduplex

Most of what we know about the mechanism of gene conversion comes from studies of meiotic gene conversion in yeast. When a diploid strain that is heterozygous

for a marker (alleles A and a) is sporulated, most tetrads segregate 2A:2a spores. At a low frequency (usually 1-10%), tetrads that segregate 3A:1a or 1A:3a spores are observed. These conversion events are non-randomly associated with crossovers; about 50% of the meiotic conversions are associated with a crossover (Petes *et al.*, 1991). Holliday (1964) suggested that gene conversion events reflect an early step in recombination, heteroduplex formation. If the two homologues have one or more base alterations within the region of the heteroduplex, there will be a mismatch within the heteroduplex. Correction of the mismatch can produce the gene conversion event (Fig. 4A). Although, at the time the Holliday model was proposed, there was no direct evidence of a system for mismatch repair, this type of activity has subsequently been demonstrated in prokaryotes and eukaryotes (Modrich and Lahue, 1996).

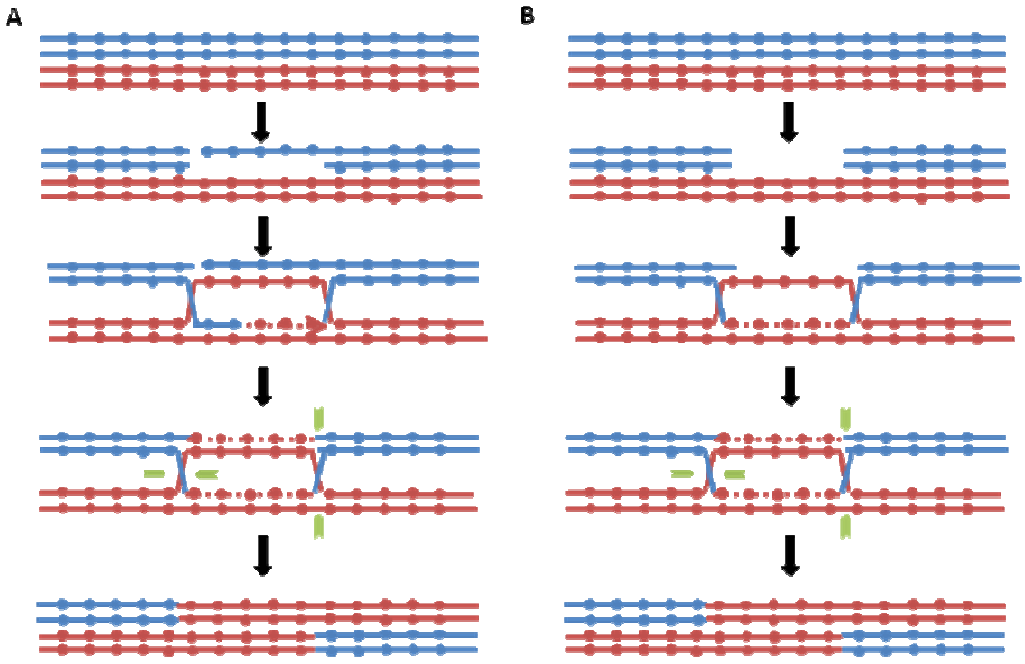


Figure 4 Two mechanisms leading to gene conversion

(A) Gene conversion through mismatch repair (MMR) correction of heteroduplexes associated with crossing over of the flanking markers. The amount of DNA transferred is dependent on the length of heteroduplex tracts and the pattern of mismatch correction. (B) In conversion events resulting from gap repair, the ends of the break are processed into a double-stranded DNA gap. Consequently, the conversion event primarily reflects DNA synthesis rather than mismatch repair.

There are both physical and genetic experiments that support the model shown in Fig. 4A as an explanation of meiotic recombination. In one such experiment, meiosis-specific DSBs were detected associated with the meiotic recombination hotspot at the *ARG4* locus (Sun *et al*., 1991). It was also shown that the broken ends underwent 5' to 3' resection. The highest level of single-stranded DNA was located adjacent to the DSB with a lower level of single-stranded DNA extending about 1 kb from the DSB site. This gradient correlated with the frequency of gene conversion for different sites within the *ARG4* gene. In addition, in a number of studies (reviewed in Petes *et al*., 1991), it was shown that mutations in the genes required for mismatch repair (MMR) reduced the frequency of gene conversion and elevated the frequency of post-meiotic segregation. Post-meiotic segregants are events in which one spore segregates two different alleles; these events reflect cells that contain a heteroduplex with an unrepaired mismatch.

The same MMR system corrects mismatches in heteroduplexes and mismatches resulting from bases misincorporated during DNA replication (Modrich and Lahue, 1996). In *E. coli*, two of the important MMR proteins are MutS and MutL. *S. cerevisiae* has six MutS-related proteins with Msh2p, Msh3p, and Msh6p having clear roles in the nuclear MMR (Surtees *et al*., 2004; Marsischky *et al*., 1996; Reenan and Kolodner, 1992). Yeast has four MutL homologues: Pms1, Mlh1, Mlh2, and Mlh3 (Kramer *et al*., 1989; Flores-Rosas and Kolodner, 1998; Prolla *et al*., 1994). The interaction between an Msh2p–

Msh6p heterodimer and the Mlh1p–Pms1p complex is important for the repair of base–base mismatches. MMR is initiated by the binding of Msh2p–Msh6p to the mismatch, which then recruits the Mlh1p–Pms1p heterodimer. The function of Mlh1p–Pms1p is hypothesized to be relay of the mismatch recognition signal generated by Msh2p–Msh6p to downstream effectors responsible for removal of the mismatch and adjacent DNA sequences (Surtees *et al*., 2004). In yeast, most mismatch excision proteins were identified through genetic studies. Thus far, four nucleases have been implicated in MMR repair: Exo1p, Rad27p, and the 3' exdonucleases associated with Polε and Polδ (Kunkel and Erie, 2005). Exo1p has been most clearly implicated in MMR, with strong genetic evidence of its interaction with yeast MutS and MutL homologues (reviewed in Kunkel and Erie, 2005). In addition, the human Pms2p (equivalent to Pms1p from yeast) has an endonucleolytic function (reviewed in Modrich and Lahue, 1996).

The amount of DNA transferred in a single conversion event (the conversion tract) is a function of the length of the heteroduplex and the pattern of mismatch correction. In heteroduplexes with multiple mismatches, most of them are repaired in the same direction (Detloff and Petes, 1992). From a large number of studies, the amount of DNA transferred in a single meiotic gene conversion event varies between several hundred bases to a few kb (Petes *et al*., 1991; Paques and Haber, 1999; Mancera *et al*., 2009).

In addition to its role in the correction of DNA mismatches, the MMR system has an antirecombination activity that reduces the frequency of recombination between

diverged sequences (Harfe and Jinks-Robertson, 2000). The MMR system reduces both the frequency of recombination between diverged repeats and the lengths of associated gene conversion tracts (Datta *et al.*, 1996; Chen and Jinks-Robertson, 1998). The mechanisms involved in the antirecombination activity and the mismatch repair activity of the MMR systems are somewhat different, since some mutant alleles of MMR genes affect one activity more than the other (Welz-Voegele *et al.*, 2002).

1.3.B.2 Gene conversion as a consequence of gap repair

Although most evidence argues that meiotic gene conversion events reflect heteroduplex formation followed by MMR, it is clear that DNA molecules with a double-stranded DNA gap can be repaired to generate a gene conversion (Fig. 4B). In the first demonstration of gap repair, Orr-Weaver and Szostak (1983) transformed a plasmid with replication origin and a selectable marker into yeast. The region of homology to the yeast genome contained a small gap. They showed that gap could be repaired in two different ways, either integrating the plasmid or without integration. They found that repair of the gap (the conversion event) was associated with crossing over about 50% of the time. Gene conversion by gap repair occurs primarily through DNA synthesis rather than MMR and, thus, the frequency of this type of conversion would not be expected to be greatly affected by mutations of the MMR genes. Relatively little research has been done to examine the mechanistic details of gene conversion by gap repair.

1.3.C Template choice for the repair of DSBs during mitotic recombination

For haploid cells, DSBs in single-copy chromosomal sequences can be repaired using sister chromatids if the cell is in S or G2; in G1, DSB repair would require non-homologous end-joining or ectopic recombination (described in Section 1.4). A diploid cell has more options. In G1 cells, DSBs can be repaired using the other homologue and, in S and G2 cells, the diploid has the options of the homologue or the sister chromatid. As described below, several experiments argue that the sister-chromatid is the preferred substrate in G2 diploids. This preference can be rationalized as ensuring the more error-free product, since the sister-chromatids are identical.

Studies of X-ray-induced DNA damage in yeast by Fabre *et al*. (1984) and Kadyk and Hartwell (1992) showed that irradiation of G1-synchronized cells stimulated heteroallelic recombination much more efficiently than irradiation of G2-synchronized cells. These researchers suggested that the smaller recombinogenic effect in G2-irradiated cells reflected a preference for the use of sister chromatids in the repair of DSBs. Gonzalez-Barrera *et al*. (2003) examined the repair of an HO-induced DSB on a plasmid with heteroalleles and showed that the sister-chromatid was the preferred substrate for repair. They showed that this preference required cohesion between the sister chromatids (Cortes-Ledesma and Aguilera *et al*., 2006). The effect of cohesion on DSB repair will be discussed further below.

In eukaryotic cells, the cohesin proteins bind the newly duplicated sister chromatids together from the onset of DNA replication until anaphase (Guacci *et al*., 1997; Michaelis *et al*., 1997). In dividing yeast cells in the absence of DNA damage,

cohesins are distributed along the chromosome arm at 15 kb intervals, usually within AT-rich sequences situated between convergently transcribed genes; there is an enrichment of cohesin binding near the centromeres (Glynn *et al*., 2004). Cohesin binding shows a negative correlation with meiotic recombination, and it is influenced by transcription (Blat and Kleckner, 1999; Glynn *et al*., 2004; Laloraya *et al*. 2000, Lengronne *et al*., 2004). Cohesin subunits are loaded onto chromosomes prior to DNA replication, during the G1/S phase, in a non-cohesive state (Guacci *et al*., 1997; Michaelis *et al*., 1997; Ciosk *et al*., 2000). Cohesion between the sister-chromatids is achieved after S phase, mediated by the Eco1p co-factor (Toth *et al*., 1999; Skibbens *et al*., 1999; Haering *et al*., 2004; Lengronne *et al*., 2006; Strom *et al*., 2004; Uhlmann and Nasmyth 1998).

In addition to the cohesin proteins observed in cycling yeast cells in the absence of DNA damage, the cohesin proteins accumulate around the site of an HO-induced DSB during G2/M phases of the cell cycle. Mec1p, Tel1p, and phosphorylated H2A (the yeast equivalent of H2AX) are all essential for the efficient induction of DSB-induced cohesion (Strom and Sjogren, 2005). This recruitment of cohesin is important for DSB repair between sister-chromatids but does not affect intrachromatid recombination (Strom *et al*., 2004; Unal *et al*., 2004). Later studies showed that DSBs induce cohesion throughout the genome in addition to the localized deposition near the site of the DSB, and that Eco1p is required for the DNA replication-independent *de novo* establishment of cohesion in response to DSBs in G2 yeast cells (Strom *et al*., 2007; Unal *et al*., 2007).

1.3.D Regulation of the timing of recombination and DSB repair during the cell cycle

As described above, mitotic recombination can be induced at various times during the cell cycle. In this section of the Introduction, I will address several different issues relating to the timing of recombination and DSB repair: 1) the timing of spontaneous mitotic recombination events, 2) the cell-cycle-mediated regulation of the processing of broken DNA ends, and 3) DNA damage checkpoints.

1.3.D.1 Timing of spontaneous mitotic recombination events

The first study of mitotic recombination was conducted in *Drosophila melanogaster* more than 70 years ago (Stern, 1936). Stern examined females that were heterozygous for two recessive markers on the X chromosome: *y* (yellow body) and *sn* (singed bristles). The recessive alleles were in repulsion (Fig. 5A). Without mitotic recombination, all females of the y^+ *sn/ y* sn^+ genotype are expected to be phenotypically wild-type for body color and bristle morphology. Stern found, however, that some females had a patch of a yellow tissue adjacent to a patch of singed bristles (a twin spot). He argued that these events were likely to reflect a mitotic crossover in G2 between the *sn* marker and the centromere. Since a G1 crossover would not produce a twin patch (Fig. 5B), Stern suggested that spontaneous recombination occurred in G2. In retrospect, there are two criticisms of Stern's conclusion. First, his system could not detect a G1 event and, therefore, one cannot make any conclusion about the relative frequency of G1 and G2 events. Second, I will present evidence in Chapter 2 that many spontaneous recombination events initiate with a DSB in G1. The broken chromosome

replicates, producing two broken chromatids, that are subsequently repaired in G2. This model is also consistent with Stern's data, although not his interpretation of his data.

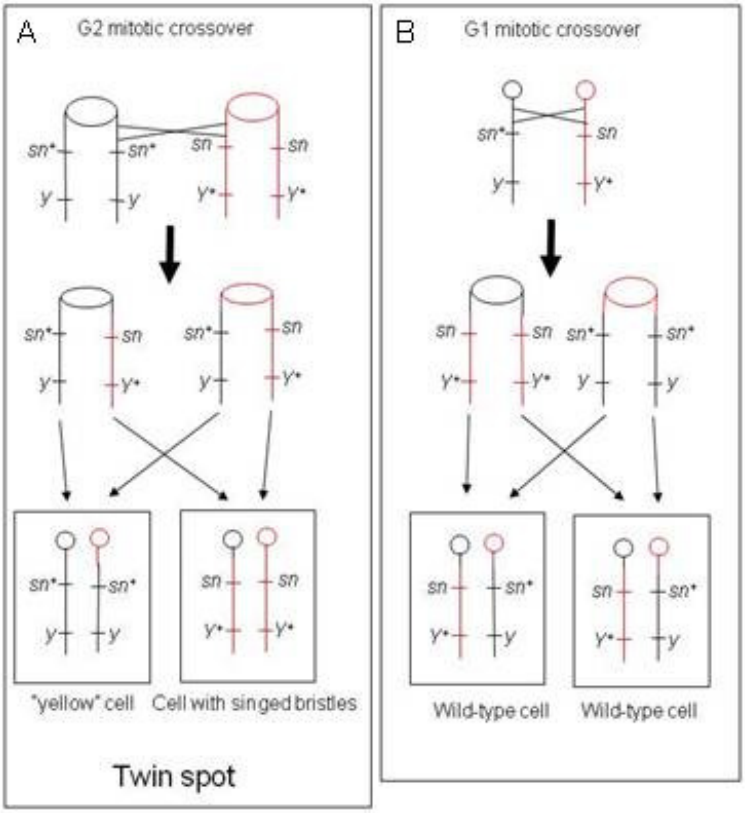


Figure 5 First example of mitotic recombination through the discovery of "twin spot"

Analysis of mitotic recombination in *Drosophila melanogaster* (Stern,1936). Female flies were constructed that were heterozygous for two linked markers, one affecting body color (*y*) and one affecting bristle morphology (*sn*). Although most females of this genotype were wild-type, Stern occasionally observed females with spots of yellow tissue next to spots of singed bristles. He argued that such twin spots could be formed by a mitotic crossover in G2 (A), but not a mitotic crossover in G1 (B).

occur in G1 yeast cells using the system shown in Fig. 6. The diploid strain used in his

study had *trp5* heteroalleles and was heterozygous at the *ade5* locus. He observed Trp^+ colonies that had *ade5*/*ade5* and *ADE5*/*ADE5* sectors. He suggested that such colonies could be generating by forming a heteroduplex in G1 that was repaired in a “patchy” manner to generate a wild-type *TRP5* gene. By replicating the resulting Holliday junction, he argued that a Trp^+ colony that was sectored for the *ade5* marker could be obtained. His model has a number of unappealing features (patchy MMR and replication through a Holliday junction), but his observations can also be explained by a model that I will present in Chapter 2.

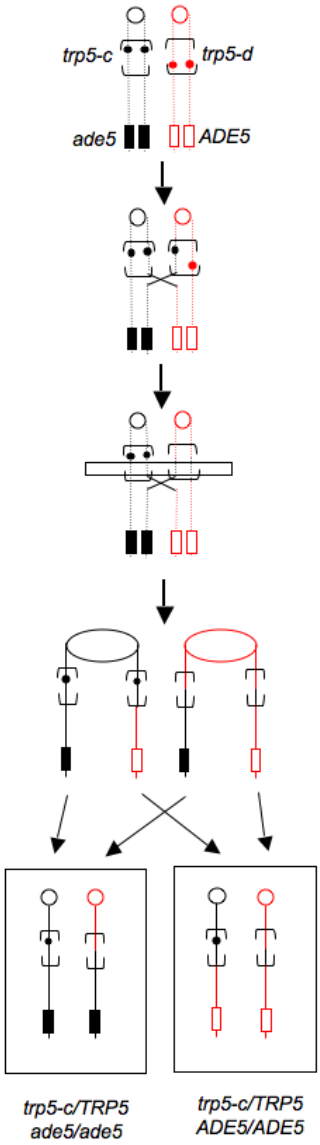


Figure 6 Mitotic recombination can occur during G1 in yeast

This figure illustrates a model of recombination proposed by Esposito (1978). In this selection system, a diploid yeast strain is heterozygous for the *ADE5*/*ade5* markers located centromere-distal to the *trp5* heteroalleles. Trp^+ colonies that were sectored for the distal *ade5*/*ADE5* markers were isolated. Esposito suggested that such colonies were the result of "patchy" repair of a heteroduplex formed in G1, generating two wild-type *TRP5* genes. He argued that replication through the resulting Holiday junction led to the sectoring of the *ADE5*/*ade5* markers.

1.3.D.2 Cell-cycle-dependent processing of broken DNA ends

Following the formation of broken DNA ends, one important early step in DNA repair/recombination is processing of the broken ends. This processing is important in two ways. First, the production of a single-stranded "tail" is necessary to initiate the invasion of the broken end into the intact homologous template. Second, as discussed in Section 1.3.D, the recognition and processing of the broken DNA ends is an important component of the DNA damage checkpoint response. Broken ends produced at different times during the cell cycle are processed somewhat differently, and some features of the processing are regulated, at least indirectly, by the cyclin-dependent kinase Cdk1p. In *S. cerevisiae*, there is a single cyclin-dependent kinase, Cdk1p (encoded by *CDC28*). Cdk1p is responsible for the progression of the cell cycle, and its various activities are determined by its binding to different activating subunits, or cell cycle-specific cyclins (Tyers *et al.*, 1993). *S. cerevisiae* has three G1 cyclins (Cln1p, Cln2p, and Cln3p), and the formation of the Cdk1p-Cln complex is necessary for the yeast cells to enter the cell cycle (Tyers *et al.*, 1993). The Cdk1p-Cln complexes also promote transcription of S- and G2-specific cyclins, also known as the B-type cyclins (Clb1-5p).

If a DSB is induced in G1-arrested cells, the resulting broken ends are processed to a very limited extent because the Cdk1p-Clb complexes are required for this processing (reviewed by Harrison and Haber, 2006). The Cdk1p-Clb complex regulates DSB end-resection through activation (by phosphorylation) of the endonuclease Sae2p. This endonuclease was identified in a genetic screen for mutants that had low DSB

repair fidelity (Rattray *et al*., 2001). The Sae2p binds single-stranded DNA independently of the MRX complex (Lengsfeld *et al*., 2007). Sae2p-dependent resection of DSB ends primarily occurs during G2; mutant *sae2Δ* cells that resect DSB ends in G1 show an increased sensitivity to gamma radiation, indicating that erroneous homologous recombination repair initiated by DSB resection in G1 may be detrimental to cell viability (Huertas *et al*., 2008).

Presumably as a consequence of the limited resection of DNA ends, DSBs induced in G1 by the HO endonuclease do not recruit Rad52p to the resulting broken ends (Lisby *et al*., 2001). If the activity of the Cdk1-Clb complexes is inhibited, single-stranded DNA binding proteins, such as RPA and Rad51, are not recruited to the broken ends in G2 (Ira *et al*., 2004). In addition to its interaction with the homologous recombination proteins, Cdk1p suppresses the recruitment of NHEJ proteins to the DSB site during G2 of the cell cycle (Zhang *et al*., 2009). In summary, during the cell cycle, the cyclin-dependent activities of Cdk1p regulate repair and recombination at several steps: DSB end-resection, the binding of proteins to single-stranded ends, and the repression of the NHEJ pathway.

1.3.D.3 DNA damage checkpoints

The concept of the DNA damage checkpoint was first elucidated in lab of Hartwell. Weinert and Hartwell (1988) showed that there were two types of DNA repair proteins in yeast: those (such as Rad52p) that were directly involved in the enzymology of DNA repair and those (such as Rad9p) that were required to arrest the cell cycle to

allow sufficient time for DNA repair. In response to DNA damage, there are three cell cycle checkpoints in *S. cerevisiae*: the G1/S, intra-S, and G2/M checkpoints. For purposes of this Introduction, I will focus mainly on the G1/S and G2/M checkpoints (hereafter referred to as the G1 and G2 checkpoints) and the cellular response to DSBs rather than other types of DNA damage.

The first step in the checkpoint response is the binding of protein complexes to the broken ends (Putnam *et al*., 2009). The binding of Mec1p and Tel1p, two related kinases, is particularly important in the checkpoint response. Tel1p and Mec1p are related to the human ATM and ATR proteins, respectively. Tel1p is recruited to unprocessed broken ends by the MRX complex (Nakada *et al*., 2003), whereas Mec1p is recruited to processed broken ends by its attachment to Ddc2p that is, in turn, recruited by RPA at processed broken ends (Zou *et al*., 2003).

Although Tel1p and Mec1p are somewhat functionally redundant, Mec1p is more important for the checkpoint response in S and G2 (Morrow *et al*., 1995; Putnam *et al*. 2009). The Tel1p is more important than the Mec1p in the establishment of γ-H2AX histones flanking the DSB site during G1 in yeast cells (Ira *et al*., 2004; Shroff *et al*., 2004; Mantiero *et al*., 2007). As described previously, γH2AX, (histone H2A with phosphorylated Ser129 residue) is found flanking the 10-100 kb interval around a DSB (Downs *et al*., 2004; Shroff *et al*., 2004). This phosphorylated histone is important for the recruitment of cohesin proteins to the DSB site (Unal *et al*., 2004).

Following their recruitment to broken ends, both Tel1p and Mec1p phosphorylate and activate a variety of proteins required for the repair of DNA damage and/or to arrest

the cell cycle. In addition to histone H2A, important substrates include Rad53p, Chk1, Rad9p, Dun1p, Pds1p, and Cdc5p (Putnam *et al*., 2009); for most of these substrates, it is not clear whether they are directly phosphorylated by Tel1p and Mec1p. Cell cycle arrest in response to DNA damage is regulated in part by the Mec1p- and Chk1p-dependent hyperphosphorylation and stabilization of Pds1p; degradation of Pds1p (securin) is required for cells to enter anaphase. In addition, the cell cycle is blocked by a Rad53p-dependent inhibition of Cdc5p (Harrison and Haber, 2006). Following the repair of DNA damage, the checkpoint response is turned off (checkpoint recovery) and the cell cycle continues. The recovery requires the phosphatases Ptc2p and Ptc3p (Harrison and Haber, 2006).

Data from numerous experiments indicate that the G1 checkpoint in *S. cerevisiae* is weak compared to the G2 checkpoint. For example, a single HO-induced DSB is sufficient to trigger Rad53 phosphorylation in G2/M cells, but not in G1 cells (Pellicioli *et al*., 2001). To elicit G1 checkpoint responses, multiple DSBs, induced either with gamma radiation or with HO endonuclease, are required (Lisby *et al*., 2004; Zierhut and Diffley, 2008). Even in the event of G1 checkpoint activation, little DNA 3' end-resection is observed (Zierhut and Diffley, 2008), an observation that is in agreement with the Cdk1p studies described in the previous section. It is likely that this weak G1 DNA damage checkpoint allows cells with a broken chromosome to undergo DNA replication, producing two broken chromatids. As will be discussed in Chapter 2, this mechanism is critical for the production of mitotic crossovers.

1.4 Repair of DNA damage by non-homologous end-joining (NHEJ)

As the name suggests, NHEJ events are the joining of two broken ends without homology or with very limited (< 4 base pairs) homology. NHEJ events often involve loss of several bases from the broken ends and, therefore, the NHEJ pathway is error-prone relative to the HR pathway. Most of the enzymes used by NHEJ are different from those used for HR (Krogh and Symington, 2004). The yeast proteins that are important for NHEJ are the MRX (Mre11p-Rad50p-Xrs2p) complex, the Yku70p/Yku80p heterodimer, and DNA ligase IV complex (Nej1p-Dnl4p-Lif1p) (Dudasova *et al*., 2004; Daley *et al*. 2005). Although not directly relevant to the focus of this thesis (which concerns RCOs), the early steps of the NHEJ are in competition with HR in haploid strains or in diploids that express only one mating type; therefore, NHEJ will be briefly discussed below.

The first step in NHEJ is the recognition of the broken DNA ends. This recognition is carried out independently by the MRX complex and the Yku70p/80p heterodimer shortly after induction of the DSB (Martin *et al*., 1999; Wu *et al*., 2008; Lisby *et al*., 2004). The MRX complex and Yku70p/80p heterodimers compete for the DSB-generated DNA ends (Wu *et al*., 2008). The binding of the Ku complex and MRX likely protects the ends and keeps them in close proximity. *In vivo* data show that the presence of the Nej1p-Dnl4p-Lif1p ligase complex, in addition to ligating the two broken ends, stabilizes the binding of Yku70p/80p to the broken DNA ends prior to ligation (Zhang *et al*., 2007). For certain types of processed DNA ends, NHEJ also requires the specialized DNA polymerase Pol4p (Aguilera and Rothstein, 2007).

The efficiency of NHEJ is reduced in *MATa/MATα* diploid yeast cells because the Mata1-Matα2 repressor inhibits the expression of *NEJ1* (NHEJ regulator 1) (Astrom *et al*., 1999; Kegel *et al*., 2001; Valencia *et al*., 2001). Thus, *MATa/MATα* diploid yeast cells repair DSBs exclusively by homologous recombination. In yeast, even in haploid cells, DSBs are primarily repaired by HR rather than NHEJ. Haploid cells lacking Yku70p are much less sensitive to X-rays than cells lacking Rad52p (Siede *et al*., 1996). Furthermore, the NHEJ pathway is regulated in a cell-cycle dependent manner in haploids. G1 haploids primarily utilize NHEJ to repair DSBs, whereas S-phase and G2 cells primarily utilize HR in the repair of DSBs (Zhang *et al*., 2009; Mimitou and Symington, 2009; Lisby and Rothstein, 2009). My experiments concern reciprocal crossovers, and it is likely that NHEJ has a negligible contribution to this type of event.

2. A fine-structure map of spontaneous mitotic crossovers in the yeast *Saccharomyces cerevisiae*

Most higher organisms have two copies of several different types of chromosomes. For example, the human female has 23 pairs of chromosomes. Although the chromosome pairs have very similar sequences, they are not identical. Members of a chromosome pair can swap segments from one chromosome to the other; these exchanges are called “recombination.” Most previous studies of recombination have been done in cells undergoing meiosis, the process that leads to the formation of eggs and sperm (gametes). Recombination, however, can also occur in cells that are dividing mitotically. In our study, we examine the properties of mitotic recombination in yeast. We show that mitotic recombination differs from meiotic recombination in two important ways. First, the sizes of the chromosome segments that are non-reciprocally transferred during mitotic recombination are much larger than those transferred during meiotic exchange. Second, in meiosis, most recombination events involve the repair of a single chromosome break, whereas in mitosis, about half of the recombination events appear to involve the repair of two chromosome breaks.

2.1 Introduction

Although mitotic recombination between homologous chromosomes was first described in 1936 (Stern, 1936), our understanding of the mechanism of spontaneous mitotic recombination is still limited for two related reasons. First, spontaneous mitotic recombination events are very infrequent compared to meiotic exchanges. In *S. cerevisiae*, mitotic crossovers and conversions are about 10^4 to 10^5-fold less frequent than meiotic events (Petes *et al.*, 1993; Paques and Haber, 1999) and usually require a selective system for their detection. Second, these systems, in general, do not allow selection of both daughter cells that contain the recombinant chromosomes generated in the mother cell. Reciprocal crossovers (RCOs) between homologous chromosomes that have a heterozygous marker can lead to daughter cells that are homozygous for the marker (loss of heterozygosity, LOH). One selective system in *S. cerevisiae* to detect such events uses the heterozygous drug-resistance marker *can1* (Figure 7). Since diploids heterozygous for this marker are sensitive to the arginine analogue canavanine, a derivative that is homozygous for the mutant allele arising from crossing over can be selected on medium containing canavanine. The daughter cell homozygous for the wild-type *CAN1* allele, however, cannot be selected.

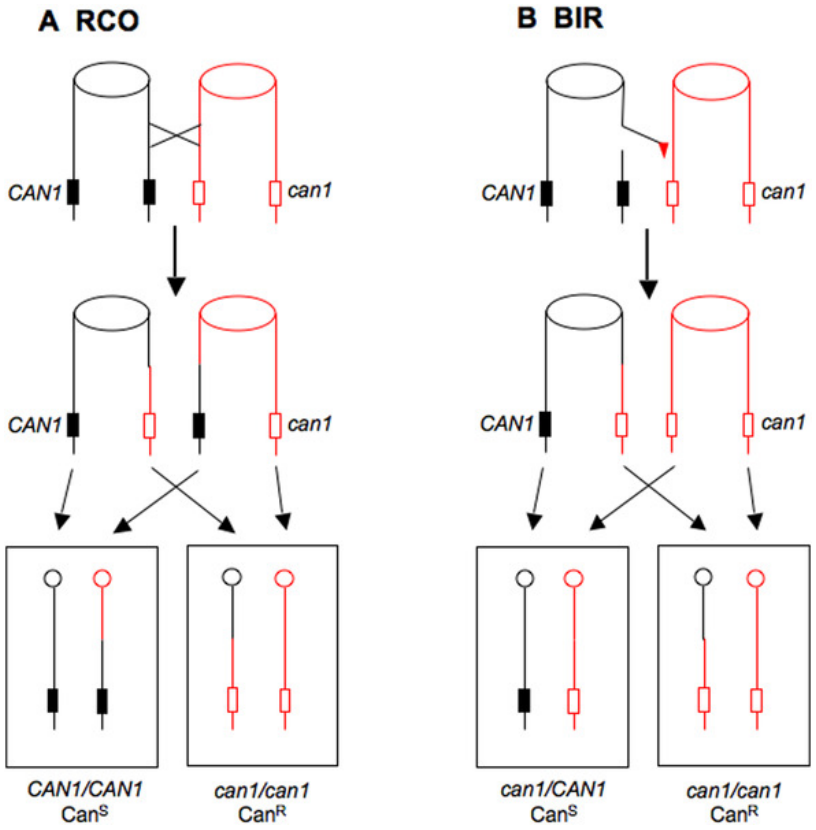


Figure 7 Detection of mitotic recombination events in a diploid heterozygous for the *can1* gene

The two homologues are depicted in G2 with the duplicated chromatids held together at the centromere (shown as ovals). A) Following a reciprocal crossover (RCO), one daughter cell is homozygous for the recessive *can1* allele and is canavanine resistant, whereas the other daughter cell is homozygous for the wild-type allele and is canavanine sensitive. Note that only one of the two possible chromosome disjunction patterns is shown; the other pattern does not lead to the markers becoming homozygous. B) Break-induced replication (BIR) is a fundamentally non-reciprocal process. In this depiction, the black chromatid is broken and the broken end invades the red chromatid, duplicating all the sequences to the end of the chromatid. The net result of this process is one Can^R *can1*/*can1* cell and one Can^S *can1*/*CAN1* cell.

A canavanine-resistant diploid can also be derived from a heterozygous diploid by break-induced DNA replication (BIR) (Llorente *et al.*, 2008). As shown in Figure 7B, a double-strand DNA break (DSB) on the *CAN1*-containing chromosome is repaired by copying the DNA from the *can1*-containing chromosome. Since the only selectable daughter cell in this system is identical for both RCO and BIR, these two mechanisms cannot be distinguished by this system. Two recent studies have examined the relative contributions of RCO and BIR to LOH in yeast. Using a non-selective approach, McMurray and Gottschling (McMurray and Gottschlings, 2003) showed that most loss of heterozygosity (LOH) events in "young" cells (cells that have not undergone many mitotic divisions) represent RCOs, whereas LOH events in "old" cells often involve BIR. Using a selective approach that will be described further below, we found that most spontaneous LOH events are RCOs and recombination events induced by hydroxyurea are both RCO and BIR (Barbera and Petes, 2006).

In mitosis, as in meiosis, gene conversion events are observed and these events are often associated with crossovers (Paques and Haber, 1999). Conversion events are the non-reciprocal transfer of information between homologous DNA sequences and, in meiosis, most conversions reflect heteroduplex formation, followed by mismatch repair (Hoffmann and Borts, 2004). Most studies of mitotic conversion employ strains that are heteroallelic for an auxotrophic marker and heterozygous for a centromere-distal marker (Figure 8). Although a reciprocal crossover between the heteroalleles could produce a prototroph, Roman (1957) showed that most prototrophs were a consequence of a gene conversion event. It should be noted that use of heteroalleles for the detection of gene conversion is rather restrictive. If gene conversion is a consequence of heteroduplex

formation followed by mismatch repair, in order to obtain a wild-type allele by conversion, the heteroduplex must include only one of the two alleles or the repair of the heteroduplex containing both alleles must be “patchy”. As described below, we found that the mitotic conversion tracts associated with RCO in our system are usually very long and continuous.

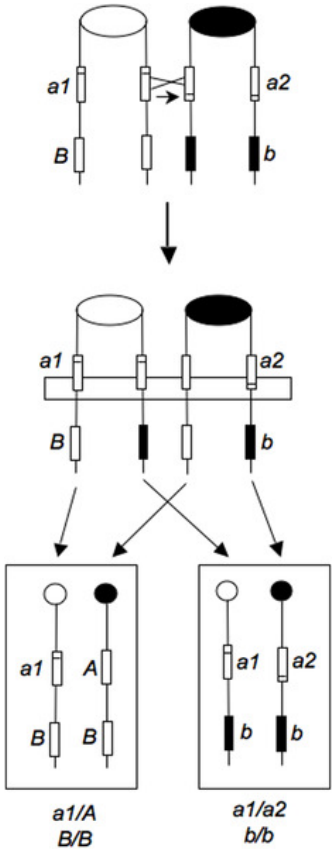


Figure 8 Intragenic mitotic gene conversion associated with crossing over
The two heteroalleles (*a1* and *a2*) are shown as rectangles with the position of the mutation indicated by a horizontal line within the rectangle. In this diagram, wild-type genetic information is transferred (indicated by a short horizontal arrow) from the centromere-distal part of the *a1* allele to the centromere-distal part of the *a2* allele, resulting in a wild-type *A* gene. The horizontal rectangle shows the region of gene conversion (three of the chromatids having wild-type sequences at the distal end of the gene and one having mutant sequences). The wild-type and mutant alleles of the centromere-distal marker are shown as white and black rectangles, respectively.

In numerous studies of the type diagrammed in Figure 8, heteroallelic gene conversion is associated with LOH of a centromere-distal heterozygous marker. The degree of association varies between about 10% and 50% (Petes *et al*., 1991). Based on the expected patterns of segregation following an RCO, one would expect that only half of the RCOs would be detectable by producing cells that have undergone LOH (Figure 7 and 8). Chua and Jinks-Robertson (1991) showed that this expectation is met for *S. cerevisiae*, although in *Drosophila*, the crossover chromatids usually segregate into different daughter cells (Beumer *et al*., 1998).

Stern (1936) argued that mitotic crossovers occur in G2 (as shown in Figure 7 and 8) because a mitotic crossover between unreplicated chromosomes would not result in LOH for heterozygous markers (assuming that the chromosomes undergo an equational division). In *S. cerevisiae*, however, two studies demonstrated that mitotic gene conversion could be induced in G1 cells by ultraviolet light or gamma rays (Wildenberg, 1970; Fabre, 1978). From his analysis of crossovers associated with heteroallelic gene conversion events, Esposito (1978) suggested that spontaneous mitotic exchanges also occur in G1. He argued that Holliday junction intermediates formed in G1 were replicated rather than resolved by junction-cleaving enzymes, generating G2-like crossovers. In the analysis described below, we present evidence that at least 40% of spontaneous RCOs are initiated in G1.

2.2 Results

2.2.A Experimental rationale

We previously described a genetic system (Figure 9) allowing for the selection of both daughter cells containing the reciprocal products of mitotic crossovers in the 120 kb

CEN5-CAN1 interval on chromosome V (Barbera and Petes, 2006). One homologue has the *can1-100* allele, an ochre mutation. On the other homologue, the *CAN1* gene has been replaced with *SUP4-o*, a gene encoding an ochre-suppressing tRNA (Barbera and Petes, 2006). In addition, the diploid is homozygous for *ade2-1*, also an ochre mutation. In the absence of a suppressor, strains with the *ade2-1* mutation require adenine, form red colonies because of the accumulation of a red precursor to adenine, and are canavanine-resistant. The starting diploid strain is canavanine-sensitive (Can^S) and forms white colonies. If an RCO occurs between *CEN5* and the *can1-100/SUP4-o* markers as the cells are plated on canavanine, a red/white sectored Can^R colony will be formed.

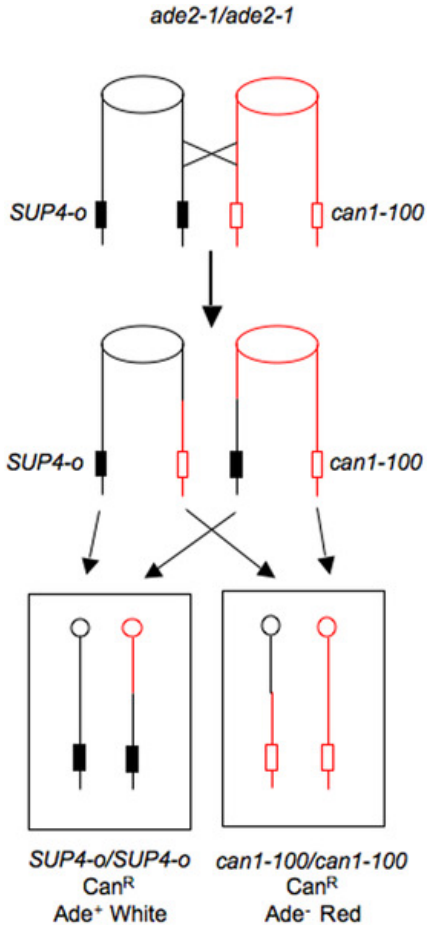


Figure 9 A diploid strain that allows the selection of both products of an RCO

The *SUP4-o* gene encodes a tRNA that suppresses both the *can1-100* and *ade2-1* alleles. Strains that have these mutations in the absence of the suppressor are canavanine resistant, adenine auxotrophs, and form red colonies (because of the accumulation of a pigmented precursor to adenine). In the presence of the suppressor, the strains are canavanine sensitive, adenine prototrophs, and form white colonies. If there is an RCO between the centromere and the *can1-100*/*SUP4-o* markers, two Can^R cells will be produced; subsequent divisions of these cells will result in a red/white Can^R sectored colony.

In our first use of this system, the two homologues were derived from isogenic haploids, resulting in a diploid that had no polymorphisms. In the current study, using standard methods (Guthrie and Fink, 1991), we constructed a diploid by crossing the haploid strains W303A and YJM789. These strains have about 0.5% sequence divergence and, therefore, about 60,000 single-nucleotide differences (Wei *et al*., 2007); S288c and W303A are closely related in sequence (Winzeler *et al*., 2003). By comparisons of the genomic sequences, we identified 34 polymorphisms between W303A and YJM789 in the *CEN5-CAN1* interval and used those polymorphisms to map crossovers and associated gene conversion tracts as described below. The diploids derived from crossing W303a- and YJM789-derived strains were PSL100 and PSL101. These strains are identical except one strain (PSL100) is homozygous for the *ura3* mutation and the other (PSL101) is heterozygous *ura3/URA3*; these strains yielded very similar results.

Each red/white sectored Can^R colony reflects an independent RCO (Figure 9). We isolated genomic DNA from cells purified from the red and white sectors and analyzed the segregation of the polymorphisms by PCR followed by restriction enzyme treatment (details in Section 2.5). For example, one polymorphism distinguishing W303A and YJM789 is located at SGD coordinate 60,163 on chromosome V. A *Hpy188*III site that is present at this position in the W303A genome is absent in the YJM789 genome. We designed primers flanking this site (Table 2) that result in a PCR product of about 520 bp. Thus, if we amplify genomic DNA from a diploid that is homozygous for the W303A form of the polymorphism, treat the amplified product with *Hyp188*III, and analyze the products by agarose gel electrophoresis, we observe two

fragments of about 250 and 270 bp. A strain homozygous for the YJM789 form of the polymorphism produces a single fragment of 520 bp, and a heterozygous diploid produces three fragments of 250, 270, and 520 bp.

The patterns of marker segregation that were expected are shown in Figure 10. For a RCO unassociated with gene conversion (Figure 10A), we expect that markers centromere-proximal to the exchange will be heterozygous in both the red and white sectors. Centromere-distal to the exchange the sectors should be homozygous, the red sector homozygous for the W303A markers and the white sector homozygous for the YJM789 markers. If there is a conversion associated with the RCO (Figure 10B), there will also be a region in which a marker is heterozygous in one sector but homozygous in the other. Such a segregation pattern is analogous to a 3:1 meiotic segregation event.

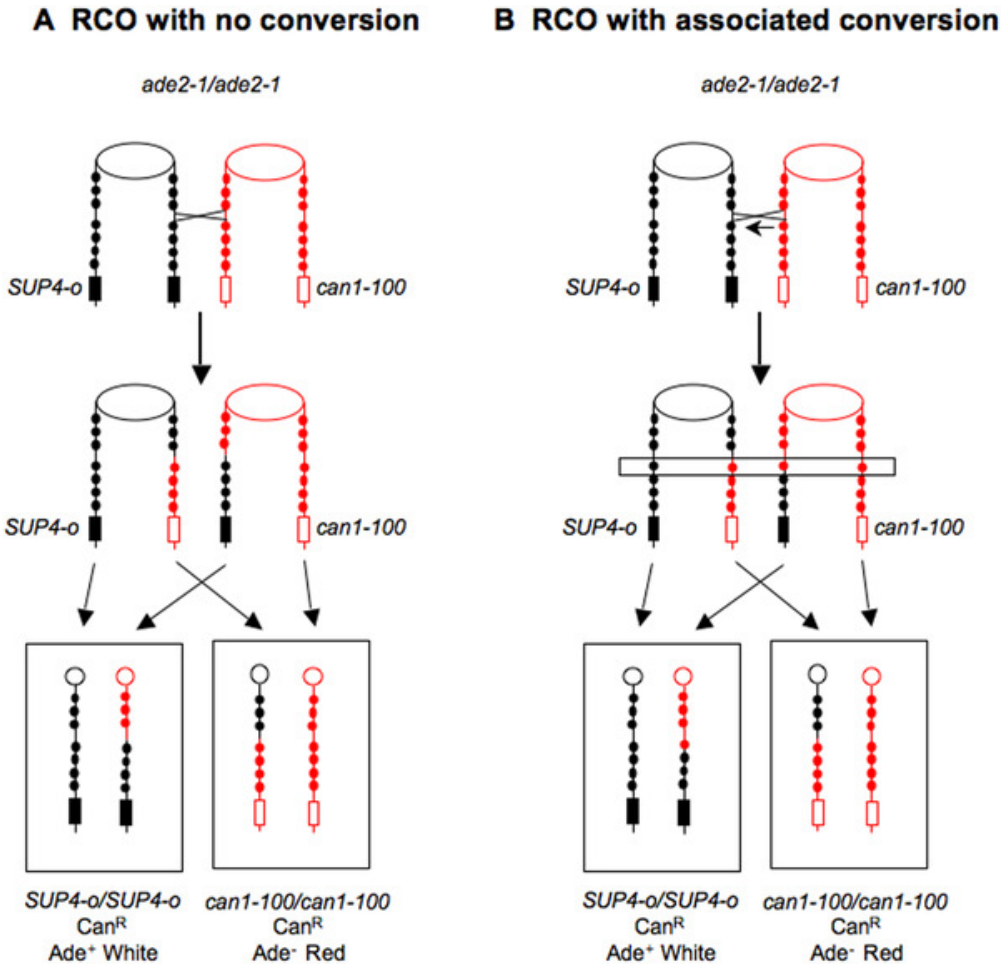


Figure 10 Patterns of heterozygous markers after RCO

In this figure, the heterozygous markers are depicted as circles. Only seven of the 34 heterozygous markers in the *CEN5-CAN1* interval are shown. The red and black colors represent markers derived from the W303A- and YJM789-related chromosomes, respectively. A) Following an RCO that had no associated conversion, both sectors are heterozygous for all markers centromere-proximal to the exchange. Distal to the exchange, the white sector is homozygous for the YJM789 markers and the red sector is homozygous for the W303A markers. B) This diagram shows a conversion event (indicated by the arrow) in which one of the black markers is lost and one of the red markers is duplicated. For this marker (boxed with the horizontal rectangle), three of the chromatids have the red marker and one has the black marker. Proximal to the conversion and associated crossover, the markers in both sectors are heterozygous; distal to the conversion/crossover boundary, the markers are homozygous with the same patterns observed in Figure 10A.

2.2.B Rates of RCOs in PSL100/101 and related strains

Before mapping the crossovers and associated conversion events, we determined the rate of RCOs. In our previous study with a diploid (MAB6) that was constructed from a cross of two W303A-related haploids and had no polymorphisms between *CEN5* and *CAN1* (Barbera and Petes, 2006), we observed Can^R red/white sectors at a rate of $2\pm0.6\times10^{-5}$/division (±95% confidence limits); this analysis was done in cells cultured at 30°C. Since the background growth of Can^S cells on the canavanine-containing solid medium in the W303A/YJM789 diploid used in the present study is strong at 30°, we performed all experiments at 22°. At this temperature, the rate of Can^R red/white sectors in MAB6 was reduced to $2.9\pm0.4\times10^{-6}$/division. The rate of Can^R red/white sectors in PSL101 (the diploid with the hybrid W303A/YJM789 background) was $3.3\pm0.2\times10^{-6}$/division, indicating that the numerous sequence polymorphisms do not significantly affect the rate of RCOs. Since only half of the segregation events in cells with an RCO result in loss of heterozygosity (Chua and Jinks-Robertson, 1991), the calculated rate of RCO in PSL101 (about 7×10^{-6}) is twice the rate of sector formation.

We also examined the rates of Can^R red/white sectors in PG311 and MD457, *MATa/MATαΔ* and *spo11/spo11* derivatives of PSL101, respectively. The rates of sectors were $1.1\pm0.5\times10^{-6}$/division (PG311) and $0.8\pm0.1\times10^{-6}$/division (MD457). Since we previously found no significant effect of heterozygosity at the *MAT* locus on RCOs (Barbera and Petes, 2006) and since Spo11p is not expressed in vegetative cells (Atcheson *et al.*, 1987), the significance of the three-fold reduction in the rate of RCOs relative to PSL101 is unclear. As will be described below, the patterns of segregation of polymorphisms in MD457 and PG311 were very similar to those observed in PSL101.

2.2.C Mapping of mitotic crossovers and gene conversions in PSL100 and PSL101

We mapped crossovers and conversions in 74 Can^R red/white sectored colonies derived from PSL100 and PSL101. The locations of the mapped events are shown in Figure 11. Green Xs indicate crossovers unassociated with gene conversion and the horizontal lines indicate the extent of gene conversion tracts associated with crossovers (red and black lines indicating markers derived from the W303A- and YJM789-derived homologues, respectively). Several generalizations can be made based on our analysis. First, most (59 of 74; about 80%) of the RCOs are associated with adjacent conversion tracts; the conversion tract is adjacent to the crossover in 58 of the 59 tracts. For most conversion events (exceptions to be discussed below), we cannot determine whether the crossover occurred within the tract or at one of the two ends of the tract. Second, most (54 of 59) of the tracts are exclusively red or exclusively black, indicating that only one homologue was the donor in each conversion event. Third, the red and black conversion tracts are not usually interrupted by markers that do not undergo conversion, demonstrating that regions of DNA from one homologue are usually non-reciprocally transferred as a single entity to the other homologue. Fourth, since the numbers of red and black conversion tracts (26 and 28, respectively) are approximately equal, the two homologues are equally capable of donating information during a conversion event. Fifth, although about 20% of the crossovers have no detectable conversion tracts, it is likely that most or all of such crossovers are associated with conversion events that could be detected with a denser array of markers.

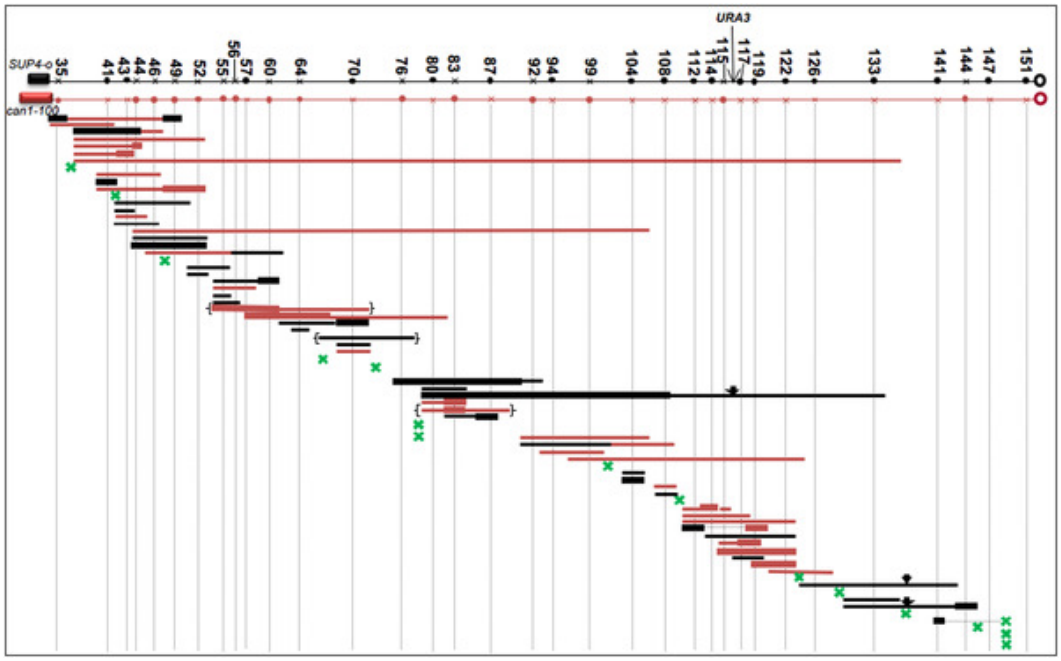


Figure 11 Mapping of RCOs and associated gene conversion tracts in the *CEN5-CAN1* interval.

Thirty-four markers were used to map events in 74 independent red/white Can^R colonies; both sectors were analyzed by methods described in the text. The positions of the markers are shown by circles and X's on the two chromosomes, with the circle indicating that the diagnostic restriction site exists and the X indicating that the site does not exist. The numbers associated with the markers represent approximate SGD coordinates in kb. *CEN5* is located at about SGD coordinate 152,000, and *CAN1* is located at about position 33,000. Green X's show the positions of RCOs that are not associated with a gene conversion tract. Thin horizontal lines show the extent of “normal” 3:1 gene conversion tracts and thick lines show 4:0 conversions. The color indicates whether the markers donated in the conversion event were derived from the homologue with the YJM789 (black) or W303A (red) markers. For example, a thin red line indicates that one sector was homozygous for the markers derived from W303A and the other sector was heterozygous for these markers. For most of the conversion tracts, the crossover maps adjacent to the tract. For those tracts with an arrow above the tract, the crossover occurred within the conversion tract. The tracts in brackets have markers in the unexpected association as discussed in the text. In addition, for two of the tracts, the position of the crossover was separated from the conversion tract; these events are shown with a dotted line connecting the tract and the crossover.

In addition to the “normal” (3:1) gene conversion events shown as thin lines in Figure 11, we detected an unexpected type of conversion. In this class (which we term “4:0” conversion), the same form of the polymorphism was homozygous in the red and white sectors. These conversion tracts are shown as thick lines in Figure 11. Of the 59 conversion events observed, 35 were 3:1 conversions, 7 were 4:0 conversions, and 17 were hybrid 3:1, 4:0 tracts. The 4:0 tracts and hybrid tracts are unlikely to reflect two independent events, since the frequency of these tracts is similar to that observed for the 3:1 tracts. In addition, about 70% of the 4:0 tracts are contiguous with a 3:1 tract and, in 15 of the 17 hybrid tracts, the 4:0 segment of the tract is derived from the same chromosome as the 3:1 tract (Figure 11). Our favored interpretation of the 4:0 conversion events (outlined in detail in Section 2.3) is that they are a consequence of a double repair event of a chromosome that was broken in G1 and replicated to yield two broken chromatids.

There were 48 3:1 or hybrid conversion tracts that involved sequences donated exclusively from W303A or YJM789. In Figure 10B, we show the red chromatid (representing W303A sequences) donating sequences to the black chromatid during the conversion event. For this type of event, we expect that the red sector (homozygous for *can1-100*) will be homozygous for the converted marker(s) and the white sector (homozygous for *SUP4-o*) will be heterozygous for these marker(s). This expected pattern was observed in 42 of the 48 conversion events with a 3:1 or hybrid 3:1/4:0 tract. In three of the 48 events, the patterns of markers in the sector were in the opposite direction (defined as the “unexpected” pattern) and, in three events, the patterns suggested a crossover within the 3:1 conversion tract. These unusual patterns of marker segregation may reflect repair of a G1-associated DSB and are discussed further

in the Section 2.6 (Section 2.6.A, Figs. 16 and 17). For both meiotic and induced mitotic gene conversion events, the chromosome with the DNA lesion that initiates the exchange (for example, a double-strand break) is the recipient of genetic information (Paques and Haber, 1999). Our data do not address this issue for spontaneous mitotic events.

The analysis described above can determine whether the strain is heterozygous or homozygous for markers but does not reveal the coupling of heterozygous markers. Our expectation was that, in sectors with heterozygous markers, the original coupling of these markers was maintained, one chromosome containing the W303A-derived markers and the other the YJM789-derived markers. This expectation was checked for the red and white sectors of nine sectored colonies. Strains derived from each sector were sporulated and we analyzed the segregation of multiple heterozygous markers in the four spores. For the heterozygous markers, we found that two of the spores had markers derived from W303A and two had markers from YJM789, indicating that heterozygous markers usually had the same coupling relationship as in the chromosomes before the mitotic exchange.

We classified 47 of the 59 conversion tracts in our study as “simple” using the following criteria: 1) the tract is continuous and the converted sequences are derived from only one of the two homologues, 2) the crossover is adjacent to the conversion tract, and 3) the 3:1 conversion tract has the expected association (as defined above) with the sector. We included 3:1, 4:0, and hybrid tracts in our analysis. Most of these tracts spanned more than one marker. For each conversion event, we estimated the tract size by averaging the maximum tract size (the distance between markers that flanked the conversion tract) and the minimum tract size (the distance between markers

that were included within the tract); for conversion events that included one site, the minimum tract size was taken to be one bp. The tract size averaged for the 47 events was 11.7±1.6 kb (95% confidence limits); the median track size was 7.6 kb. We also calculated the average tract lengths separately for 3:1 events (12.6±2.4 kb), 4:0 events (6.8±0.8 kb), and hybrid events (11.4±1.2 kb). These tracts are considerably longer than those observed in meiotic cells that average about 1–4 kb (Symington and Petes, 1988; Judd and Petes, 1988; Borts and Haber, 1989; Mancera *et al.*, 2008). The sizes of all conversion tracts for PSL100/PSL101 and the other strains used in this study are in tables in Section 2.6 (Tables 3, 4, 5, and 6).

As discussed above, the mitotic crossovers that had no detectable conversion event are likely to have had a conversion tract that was restricted to the region between the assayed markers. If we assume that these postulated conversion events had tract sizes that were half of the distance between the markers in the interval containing the crossover, then the average mitotic conversion tract for PSL100/PSL101 was 9.4 kb rather than 11.7 kb, still considerably longer than meiotic conversion tracts estimated in other studies. In summary, our analysis of mitotic crossovers indicated two unusual features of the gene conversion tracts associated with the RCO: the tracts were often very long, and about 40% of the tracts were not consistent with the simplest model of a G2-initiated recombination event.

2.2.D Mapping of mitotic crossovers and gene conversions in MD457 and PG311

To ensure that the unusual gene conversion events described above were not a consequence of a sub-set of cells that underwent meiotic levels of recombination, followed by mitotic patterns of chromosome disjunction, we examined mitotic recombination in MD457 (a *spo11/spo11* derivative of PSL101) and PG311 (a

MATa/MATαΔ derivative of PSL101). These strains are incapable of meiotic recombination. The positions of RCOs and their associated conversion tracts (14 from MD457 and 15 from PG311) are shown in Figure 12. The types of conversion events are similar to each other and to those observed in PSL100/PSL101. The gene conversion tracts were very long in the two strains, and we observed 3:1, 4:0, and hybrid 3:1/4:0 tracts in approximately the same proportions as in PSL101. The average conversion tract sizes (average of all three types) were 26.2±5.1 kb for MD457 and 12.8±2.3 kb for PG311; the median track sizes for MD457 and PG311 were 20.1 kb and 6.1 kb, respectively. The average conversion tract size for MD457 is somewhat misleading because one very large tract (103 kb) had a substantial effect on the average. The average tract size for the other tracts in MD457 was 19.2 kb. These results argue that the very long conversion tracts and 4:0 and 3:1/4:0 classes of events observed in PSL101 do not reflect an aberrant type of meiotic recombination.

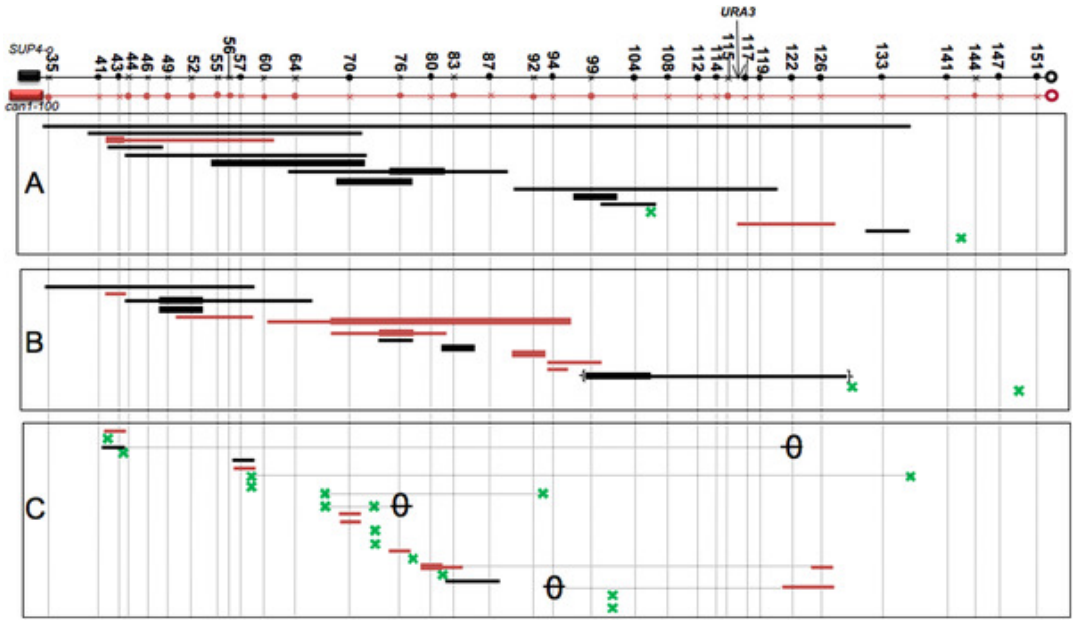


Figure 12 Mapping of mitotic crossovers in meiosis-deficient derivatives of PSL101 (MD457 and PG311) and meiotic crossovers and conversions in PSL101

The depictions of crossovers and conversions are the same as in Figure 11. A) Analysis of crossovers and conversions in 14 sectored colonies derived from MD457, an isogenic *spo11*/*spo11* derivative of PSL101. B) Analysis of crossovers and conversions in 15 sectored colonies derived from PG311, an isogenic *MATa*/*MATαΔ*::*NAT* derivative of PSL101. C) Meiotic crossovers and conversion in PSL101. The diploid was sporulated and the segregation of markers in the spores was examined. Conversion tracts that were unassociated with crossovers are indicated by a horizontal line with a superimposed oval. Multiple events within one tetrad are shown with a connecting dotted line. Two conversion events that include the *can1-100*/*SUP4-o* marker are not shown.

2.2.E Meiotic crossovers and associated gene conversions

Using methods similar to those used to map mitotic crossovers and conversions, we also examined the patterns of meiotic exchanges in 21 tetrads derived from PS101. By examining the segregation of the centromere-linked *trp1* marker and the *can1-100/SUP4-o* markers, we identified tetrads that had at least one crossover in the 120 kb *CEN5-CAN1* interval. The positions of crossovers and the lengths of associated gene conversion tracts in these tetrads are shown in Figure 12C. Eleven of the conversion tracts were associated with crossovers and three were not. Of the eleven tracts

associated with crossovers, eight included only one marker and three included two. None of the conversion sites spanned more than two markers. In striking contrast, of the 47 “simple” conversion events associated with mitotic crossovers in the same strain, as described above, 12 included only one marker, 12 spanned two markers, and 23 involved more than two markers. This difference in the sizes of meiotic and mitotic tracts is very significant (p value of 0.001 by Fisher exact test). In addition, using the same methods to estimate conversion tract length that we used for mitotic tracts, we calculated the average meiotic conversion tract length in PSL101 as 4.7±0.6 kb, significantly ($p<0.05$) less than that observed in mitosis. If we assume that the crossovers with no detectable conversions had tracts that were half of the size of the interval between the markers containing the crossovers, the average conversion tract was 3.2 kb. In summary, these results demonstrate that the long mitotic conversion tracts in PSL101 and related strains are not an artifact generated by the high level of polymorphisms in PSL101 and related diploids, but reflect differences in the mechanisms of meiotic and mitotic recombination.

As expected from many previous studies (Paques and Haber, 1999; Hoffmann and Borts, 2004), most of the meiotic conversion events are 3:1 events (three spores with one form of the polymorphism, one with the alternative form), but one tetrad had a conversion tract with a “4:0” segment adjacent to a 3:1 segment, similar to some of the mitotic conversion tracts described previously. Meiotic conversion events with 4:0 segregation have been seen previously at meiotic recombination hotspots (White *et al.*, 1991) and occur at the frequency expected for two independent conversion events.

In 21 tetrads, we observed 26 crossovers; about 40% (11) were associated with conversion tracts and 60% (15) were not. This association between meiotic crossovers

and conversion is significantly less (p value≤0.001 by Fisher exact test) than observed for mitotic crossovers and conversion in PSL100/101 where 59 of 74 crossovers were associated with a conversion tract. A simple interpretation of this result is that the longer conversion tracts associated with mitotic crossovers make it more likely that an associated conversion event will be detectable in mitotic cells than in meiotic cells.

2.3 Discussion

In this study, we show that most spontaneous reciprocal crossovers are associated with long gene conversion tracts. In addition, we found that about 40% of the conversion tracts had an unusual pattern in which one form of the polymorphism became homozygous in both sectors (4:0 conversion); as described below, we interpret such conversion tracts as representing the repair of a G1-initiated DNA lesion. Below, we discuss: 1) the distribution of mitotic gene conversion events in the *CEN5-CAN1* interval, 2) a comparison of the lengths of mitotic and meiotic conversion tracts, and 3) mechanisms of mitotic recombination.

2.3.A Distribution of mitotic recombination events

Meiotic recombination events in *S. cerevisiae* are distributed non-randomly. Certain chromosomal domains have low levels of exchange (for example, near the centromeres and telomeres) and there are intergenic regions with very elevated rates of recombination (hotspots) correlated with high levels of local meiosis-specific double-strand DNA breaks (Lichten and Goldman, 1995; Petes *et al*., 1991). Although no high-resolution mitotic recombination maps have been constructed previously, several DNA sequence motifs or conditions have been associated with elevated rates of mitotic recombination in yeast including: elevated rates of transcription, replication fork pausing/stalling, and DNA sequences capable of forming secondary structures such as

poly CCG or inverted repeats (Aguilera and Gomez-Gonzalez, 2008). Most of the assays of the recombination-stimulating sequences involve recombination between direct or inverted repeats rather than recombination between homologous chromosomes.

From the patterns of the spontaneous recombination events shown in Figure 11 and (Barbera and Petes, 2006), it is clear that crossovers and conversions are initiated at many sites within the *CEN5-CAN1* interval, although there appear to be more conversion tracts near *CAN1* than near the centromere. This impression is conveyed more clearly in Figure 13A. In this figure, we show the number of times each marker was involved in a conversion event in the strains PSL100/101, MD457, and PG311. If we divide the region into four intervals of approximately the same size and sum the number of events/marker over all markers in each quadrant, we find 124 (Quadrant 1, markers 35 to 55), 112 (Quadrant 2, markers 56–87), 99 (Quadrant 3, markers 92–117), and 43 (Quadrant 4, markers 119–151) events in each quandrant, moving from *CAN1* to *CEN5*. This distribution of events is very significantly different ($\underline{p}$ = <0.0001 by chi-square test) from random. In addition, the number of events in Intervals 1 and 4 are significantly greater and less, respectively, than that expected from a random distribution.

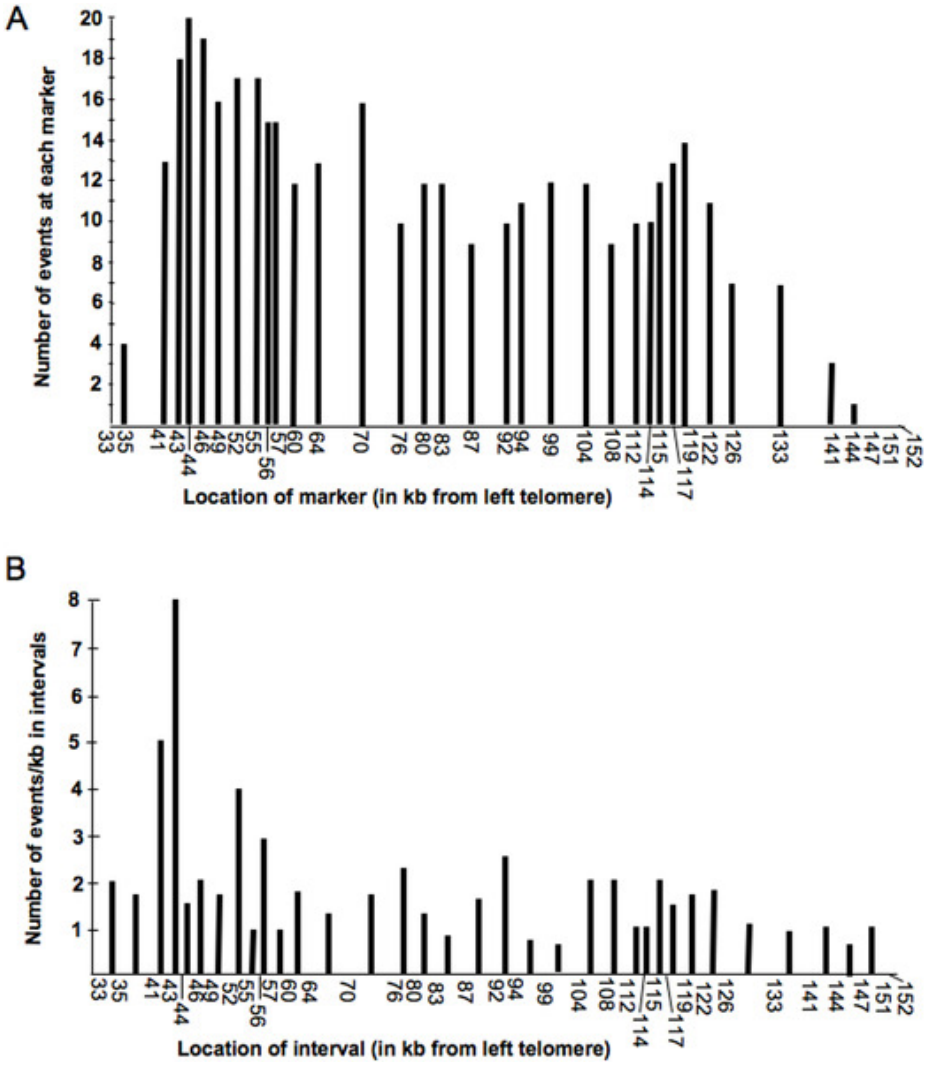


Figure 13 Distribution of mitotic recombination events in the *CEN5-CAN1* interval

This figure is a summary of the distribution of mitotic recombination events in the strains PSL100, PS101, MD457, and PG311. A) For each marker, we summed the conversion events that include the marker over all of the strains. Both simple and complex conversion events were used in this analysis. B) For each interval, we summed the conversion tracts that end in the interval and the crossovers within the interval. We then divided that sum by the length of the interval in kb.

We confirmed this conclusion using two other types of analysis. First, we determined the number of conversion tracts within each quadrant. Only tracts that did not span two different quadrants were included. We found 28, 16, 18, and 4 tracts within the Quadrants 1–4, respectively. This distribution was significantly different from random ($\underline{p}$ = 0.0005). One difficulty in localizing a mitotic recombination hotspot is that the conversion tracts are long and heterogeneous in length. In meiosis, although the initiating DNA lesion stimulates gene conversion tracts bidirectionally, individual gene conversion tracts are propagated unidirectionally from the initiating DNA lesion (Porter *et al.*, 1993). In an analysis of HO-induced mitotic gene conversion tracts (Nickoloff *et al.*, 1999), about 80% of the tracts were bidirectional from the DSB site, although the length of DNA transferred was often much greater on one side of the DSB site than the other. If we assume that individual spontaneous conversion events are propagated predominately in a single direction from the initiating lesion, one of the endpoints of the conversion tract will be near the initiating DNA lesion. Thus, we determined the number of conversion tracts that ended in each of the 35 intervals defined by the polymorphic markers; we also included in this analysis the crossovers within each interval. When these events were summed within each quadrant, we found 65, 54, 38, and 31 events, respectively, in Intervals 1–4. This distribution of events is significantly ($\underline{p}$ = 0.0006) different from random. In Figure 13B, we show the number of events (termini of conversion tracts and crossovers) within each of the 35 intervals, normalized for the size of the interval. A peak between markers 43 (SGD coordinates 43078) and 44 (SGD coordinates 44403) is evident. The observed number of events (8) in this 1.3 kb interval is significantly ($\underline{p}$<0.0001 by chi-square analysis) in excess of that expected based on a random distribution of 188 events in the 119 kb *CAN1-CEN5* interval.

The interval between markers 43 and 44 includes part of the *PCM1* gene and the *SOM1-PCM1* intergenic region. As discussed above, elevated levels of mitotic recombination have been associated with certain types of DNA structures (inverted repeats), microsatellite sequences, or high levels of transcription. There are no obvious structure/sequence elements in the 1.3 kb region, and *SOM1* and *PCM1* are not among the most abundant transcripts in the yeast genome (Holstege *et al*., 1998). We also compared the level of mitotic recombination for each marker (measured as in Figure 13A) with the level of gene expression of the ORF closest to the marker (Holstege *et al*., 1998) by a linear regression analysis; no significant correlation was observed (r^2 = 0.004; $\underline{p}$ = 0.74). An understanding of the nature of mitotic recombination hotspots will probably require identification and analysis of many hotspots.

Several other points should be made concerning the distribution of mitotic events. First, the frequency of gene conversion events near the *CAN1* gene is somewhat underestimated, since a conversion event extending through the *can1-100/SUP4-o* markers would not result in a Can^R red/white sectored colony. Second, in our previous study of mitotic recombination (Barbera and Petes, 2006), we did not observe a reduction of exchange in the 35 kb *URA3-CEN5* interval. In this previous study, however, our estimate of crossovers was based on a relatively small number of events and was insensitive to a small degree of suppression. From our current study, it is possible that mitotic recombination, like meiotic recombination, is reduced close (within 20 kb) to the centromere. This conclusion, however, is tentative until studies of mitotic recombination have been extended to multiple chromosomes. In addition, although mitotic recombination is reduced near *CEN5*, gene conversion events can extend through the centromere (Liebman *et al*., 1988). In summary, our analysis of the

distribution of mitotic recombination events demonstrates that these events can be initiated at many locations in the *CAN1-CEN5* interval, although we have preliminary evidence of one mitotic recombination hotspot.

By a variety of microarray-based procedures, we and others have measured the distribution of meiosis-specific DSBs throughout the yeast genome (Gerton *et al*., 2000; Borde *et al*., 2004; Mieczkowski *et al*., 2006; Buhler *et al*., 2007; Blitzblau *et al*., 2007). We compared the number of mitotic conversion events involving each polymorphic site (Figure 13) with the meiotic recombination activity of the nearest ORF (derived from Table S2) (Mieczkowski *et al*., 2006) by a linear correlation and regression analysis. No significant correlation was observed ($r^2 = 0.021$; $\underline{p} = 0.41$ by two-tailed test). Since meiotic recombinogenic lesions are generated by Spo11p which is not expressed in mitotic cells, this result is not unexpected.

2.3.B Comparison of the lengths of mitotic and meiotic conversion tracts

Before comparing mitotic and meiotic conversion events, we will briefly compare previous studies of mitotic conversions in yeast with our study. In our study, only mitotic conversion tracts associated with crossovers were examined. In a number of studies (Prado *et al*., 2003), it was shown that mitotic conversion tracts associated with crossovers are longer than conversion tracts unassociated with crossovers. Most previous studies of mitotic conversion and crossovers were done using systems in which the length of the conversion was constrained in one of two ways. First, in studies involving inverted or direct repeats, the sizes of the conversion tracts are limited by the size of the repeats. Second, in experiments involving selection of a prototroph from a heteroallelic diploid, the system is biased against long continuous conversion tracts, the type of tract that is most common in our study.

Nickoloff *et al.* (Nickoloff *et al*., 1999) analyzed gene conversion events between homologous chromosomes in which an HO-induced DSB within the *URA3* gene was the initiating lesion. The diploid strain was also heterozygous for markers flanking the HO cleavage site, approximately two kb to one side and 1 kb to the other. Most of the tracts were continuous, and 60% extended outside of the markers on one side or the other; 30% were beyond all of the markers, a minimal distance of 3.4 kb. In an analysis of 51 spontaneous mitotic conversion events *unassociated* with crossovers, Judd and Petes (Judd and Petes, 1988) found 49 that were greater than two kb, and 19 of these 49 were greater than four kb (end points extending beyond the markers). 50 of the 51 tracts in this study were continuous. Using a different approach, Golin and Esposito (Golin and Esposito, 1984) examined co-conversion of heteroalleles located about 30 kb apart on chromosome VII. Although the rate of co-conversion events was 50-fold less than the rates of conversion at one locus or the other, these co-events were 1000-fold more frequent than expected for independent events, arguing the possibility of rare very long mitotic conversion tracts. Although very long conversion tracts could reflect BIR (Llorente *et al*., 2008), co-conversion of two pairs of heteroalleles is unlikely to be a consequence of BIR.

With the exception of the current study, there is only one analysis of meiotic and mitotic conversion events in the same genomic region of the same strain (Judd and Petes, 1988). Of the ten meiotic conversion tracts, eight had two defined endpoints (compared to 11 of 51 mitotic events). The average size of these eight tracts was 2.1 kb, clearly shorter than the mitotic tracts. In two other meiotic studies using similar methods, average conversion tract lengths of 3.4 kb (Symington and Petes, 1988) and 1.5 kb (Borts and Haber, 1989) were observed. Because Borts and Haber (Borts and

Haber, 1989) calculated the minimal tract lengths rather than the average of the minimal and maximal lengths, these two estimates are not significantly different.

The most accurate estimates of meiotic conversion tracts can be obtained in strains with the maximum density of markers with the caveat that the markers themselves could influence the pattern of gene conversion (Borts and Haber, 1987). In a genetic background very similar to one used in our study, Mancera *et al.* (2008) used high-density microarrays to map meiotic crossovers and gene conversions with markers that had a median spacing of about 80 bp. In analyzing several thousand conversion events, Mancera *et al.* found an average tract length of 2.0 kb for conversions associated with crossovers and 1.8 kb for conversions unassociated with crossovers. In summary, our analysis, as well as those of others, demonstrates that meiotic conversion tracts are considerably shorter than mitotic conversion tracts.

2.3 C Mechanisms of mitotic recombination

We will discuss three related aspects of the mechanism of mitotic recombination: 1) the timing of the initiating DNA lesion in the cell cycle, 2) the nature of the initiating DNA lesion, and 3) mechanisms of generating long continuous mitotic conversion tracts.

2.3. C.1 Timing of the initiating DNA lesion in the cell cycle

One very striking feature of our data is the high frequency (about 40%) of crossover-associated conversion tracts in which a marker derived from one homologue in the original diploid has become homozygous in both sectors (4:0 events). Our favored model to explain these tracts is shown in Figure 14. We suggest that one of the two homologues is broken in G1, and the broken chromosome is replicated. As expected from the repair of HO-induced DSBs (Paques and Haber, 1999), the broken chromosome would be the recipient of information during a conversion event. In Figure

14, the first DSB is repaired using the homologue as a template and this conversion event is associated with crossing-over. The second DSB could also be repaired either by using the other homologue or the newly-repaired sister-chromatid as the template to produce the 4:0 tract. This same mechanism would produce a hybrid 3:1/4:0 tract if the length of the first conversion tract is longer than that of the second. For example, if the first tract was 15 kb and the second tract was 5 kb, we would detect a hybrid tract with a 5 kb 4:0 portion and a 10 kb 3:1 portion. The location of the 4:0 region in the hybrid tract would depend on whether the conversion event was unidirectional or bidirectional from the initiating DSBs.

As discussed in the Introduction, two studies showed that mitotic recombination events could be induced in G1-arrested cells by UV damage or X-rays (Wildenberg, 1970; Fabre, 1978), although these findings are not directly relevant to the issue of the timing of spontaneous mitotic recombination events. Based on a complex genetic analysis (described in detail in Section 2.6, Fig. 18), Esposito (Esposito, 1978) concluded that a substantial fraction of spontaneous mitotic recombination was initiated in G1. His model to explain these results involves formation of a single Holliday junction between unduplicated chromosomes and resolution of this junction by DNA replication rather than the action of resolvase (Figure 18). In *S. cerevisiae*, repair of meiotic DSBs is associated with two adjacent Holliday junctions (Szostak, 1983; Hunter, 2007), although in *S. pombe*, crossovers result from resolution of a single Holliday junction (Cromie *et al*., 2006). In our view, the model shown in Figure 14 is a more plausible explanation of the data.

A number of experiments demonstrate that the repair of a DSB generated in G1 has different properties from one induced in S or G2. In haploid yeast cells, DSBs

induced by the HO endonuclease in G1 have very reduced levels of resection (Aylon *et al*., 2004; Ira *et al*., 2004; Clerici *et al*., 2008) and are often repaired by non-homologous end-joining events (Frank-Vaillant and Marchand, 2001). Rad53p is not activated in response to an HO-induced DSB in G1 (Pellicioli *et al*., 2001), and Rad52p is not recruited to the broken DNA ends (Lisby *et al*., 2001). In contrast, broken DNA ends resulting from ionizing radiation treatment of G1 haploids are resected (Barlow *et al*., 2008), although this resection does not result in phosphorylation of Rad53p except at very high doses of radiation (Gerald *et al*., 2002). As observed for the HO-induced DSBs, Rad52p is not recruited to the broken DNA ends (Barlow *et al*., 2008). These results, taken together, suggest that DSBs formed in G1 are unlikely to be repaired by homologous recombination in G1. In addition, since non-homologous end joining of broken ends is suppressed in *MATa/MATα* strains (Frank-Vaillant and Marchand, 2001; Kegel *et al*., 2001; Valencia *et al*, 2001), a chromosome with a spontaneous DSB in G1 would be likely to be replicated rather than be repaired.

It is quite likely that recombinogenic DNA lesions occur throughout the cell cycle. In our study, although we interpret the 4:0 events and 4:0/3:1 hybrid events as representing G1-initiated DNA lesions, 60% of the conversion tracts had the 3:1 pattern expected for S- or G2-initiated DNA lesions. In addition, our system was designed to detect mitotic crossovers between homologous chromosomes. Repair events between sister-chromatids, a preferred pathway for X-ray-induced DNA damage in G2 cells (Kadyk and Hartwell, 1992) are undetectable by our analysis. Thus, a simple interpretation of our data is that many of the recombination events involving homologues are initiated in G1, since DNA lesions occurring in G2 are usually repaired using the sister chromatid as the template. It should be emphasized that we cannot determine the

relative frequency of recombinogenic lesions in various portions of the cell cycle, since we cannot assay sister-chromatid exchanges with our system.

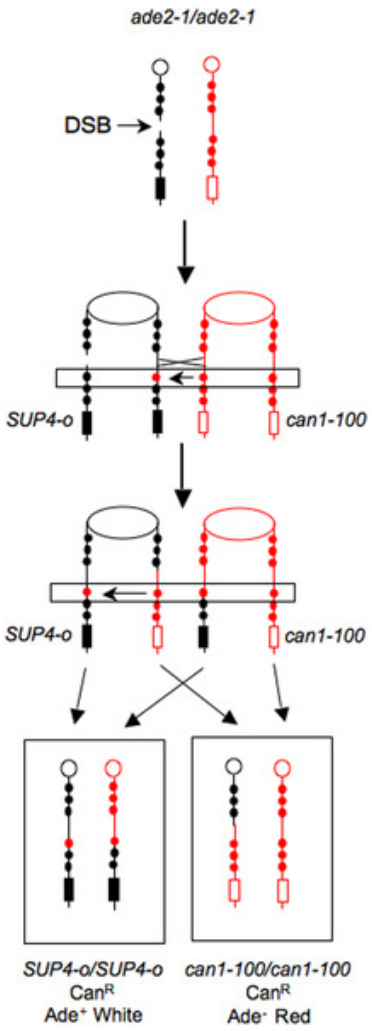


Figure 14 Mechanism to generate a 4:0 conversion event

The recombination event initiates by a DSB in G1 on the black chromosome. The broken chromosome is replicated to yield two broken black chromatids. In gene conversion events initiated by a DSB, the broken chromatid is the recipient of information (Paques and Haber, 1999). Repair of the first broken chromosome is associated with a conversion event in which the red marker is duplicated, and there is an associated crossover. Repair of the second broken chromatid could occur by an interaction with the sister chromatid (as shown) or with one of the two non-sister chromatids. This repair event would produce a second gene conversion, resulting in the 4:0 class of event. If the first repair event had a longer conversion tract than the second, a hybrid 4:0/3:1 conversion tract would be formed.

2.3.C.2. Nature of the DNA lesion that initiates mitotic recombination

Because of the low rate of spontaneous mitotic recombination events, there is no direct physical evidence of the nature of the recombinogenic lesion. As described above, the existence of 4:0 and 3:1/4:0 tracts are most consistent with a G1-associated DSB. The 3:1 tracts could reflect a G2-initiated DSB, replication of a G1-initiated nick to generate a DSB on one chromatid following DNA replication (Galli and Schiesl, 1999), or repair of a DNA molecule with a single-stranded gap (Fabre *et al*., 2002; Mozlin *et al*., 2008).

It is clear that DSBs, induced by X-rays or by site-specific endonucleases, stimulate both mitotic gene conversion and crossovers (Kupiec, 2000). One argument that spontaneous mitotic recombination events are initiated by DSBs is that certain mutants that are incapable of DSB repair (such as *rad52*) are hypo-Rec (Aguilera *et al*., 2000). Arguments in favor of other types of DNA lesions such as single-stranded nicks as recombinogenic include: 1) agents (such as UV) that result in DNA nicks, but not DSBs, are recombinogenic (Kupiec, 2000); 2) a nick-inducing enzyme stimulates mitotic gene conversion (Strathern *et al*., 1991); 3) yeast strains with mutations that eliminate DSB repair grow normally (Fabre *et al*., 2002); 4) certain *rad52* mutants have a strong DSB repair defect, but normal rates of heteroallelic mitotic recombination (Lettier *et al*., 2006). The first two lines of evidence in favor of nick-initiated recombination events are not definitive since the duplication of a nicked chromosome would result in a DSB. Galli and Schiestl (Galli and Schiesl, 1999) showed that cells treated with ionizing radiation in G1 could complete mitotic recombination between direct repeats in G1, whereas G1 cells treated with ultraviolet radiation required transition through the S-period in order to complete the recombination event.

One possibility is that different types of spontaneous DNA lesions initiate different types of mitotic recombination. For example, our studies argue that spontaneous crossovers are likely to involve a DSB. In contrast, Lettier *et al.* (2006) find that heteroallelic gene conversion and direct repeat recombination occur at a wild-type frequency in strains that are incapable of DSB repair. This discrepancy could be resolved by testing the effect of the *rad52* alleles used by Lettier *et al.* in our system. In addition, from an analysis of the effects of *rad51*, *rad55*, and *rad57* mutants on sister and interhomologue recombination, Mozlin *et al.* (Mozlin *et al.*, 2008) argue that most sister-strand recombination reflects the repair of single-strand gaps rather than the repair of DSBs.

2.3.C.3 Mechanisms of generating long continuous mitotic conversion tracts

In our study, as in previous studies, most of the conversion tracts are continuous (sites involved in conversion are not separated by sites not involved in conversion). In one version of the DSB repair model (Figure 15A), one broken end invades the other homologue, priming DNA synthesis that displaces one strand of the invaded duplex. The displaced strand forms a heteroduplex with the other resected end. Mismatches within the heteroduplex are corrected to generate the gene conversion event. One strong argument that most meiotic gene conversions reflect heteroduplex formation followed by mismatch repair is that mutants that inactivate mismatch repair reduce the frequency of gene conversion almost ten-fold and elevate the frequency of post-meiotic segregation (Petes *et al.*, 1991). Similar studies of the effects of mismatch repair mutants on mitotic gene conversion also demonstrate that most mitotic events are a consequence of heteroduplex formation and mismatch repair (Clikeman *et al.*, 2001).

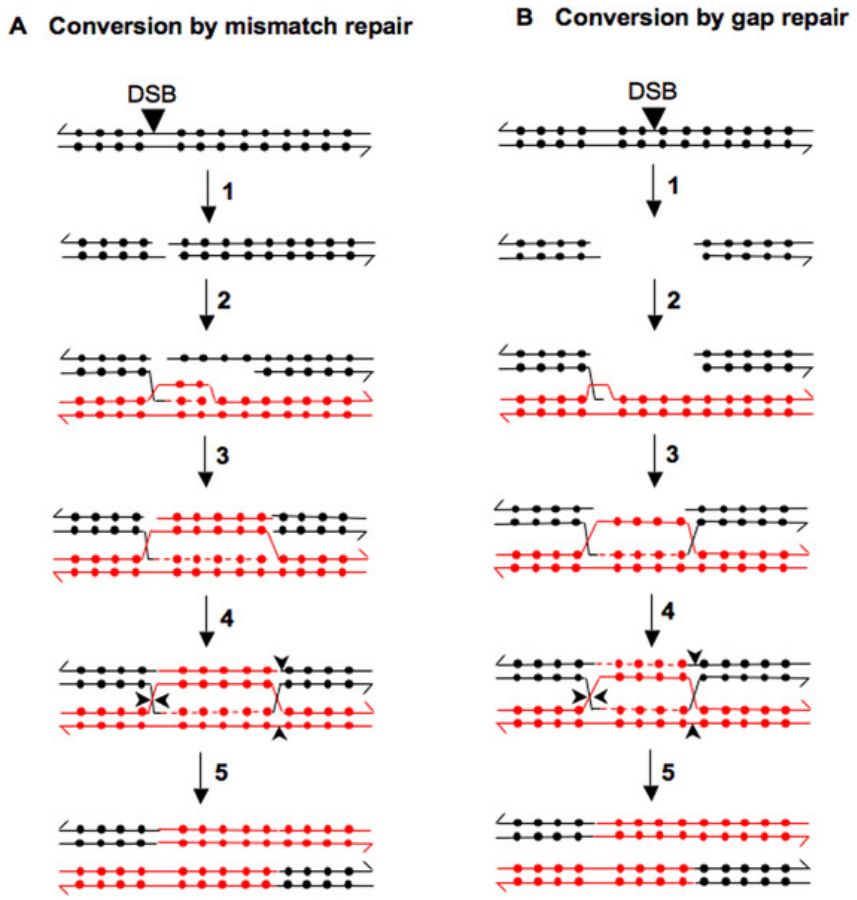


Figure 15 Generation of long conversion tracts by repair of mismatches within a heteroduplex or by gap repair

A) Conversion by mismatch repair. Conversion is initiated by a DSB, followed by 5′ to 3′ resection of the broken ends (step 1). The 3′ strand on one of the broken ends invades the other homologue and the invading strand is used as a primer for DNA synthesis (step 2); the newly-synthesized strand is shown as a dashed line. The broken end that is not used in the initial interaction undergoes more extensive resection. The single strand displaced by DNA synthesis pairs with the extensively-resected end, resulting in a long heteroduplex (step 3). The mismatches within the heteroduplex are converted in the same direction (excision of the black strand) to generate a long continuous conversion tract (step 4). The intermediate with double Holliday junctions is cleaved (cleavage sites indicated by arrows) to generate a conversion event associated with a crossover (step 5). B) Conversion by gap repair. Both strands of the broken ends resulting from the DSB are degraded to yield a gapped molecule (step 1). One of the ends invades the homologous chromosome and initiates DNA synthesis (step 2). The strand displaced by DNA synthesis pairs with the other broken end (step 3), and there is a second round of DNA synthesis (step 4). The intermediate is processed by cleaving the Holliday junctions as in Figure 15A (step 5).

If a heteroduplex involves multiple mismatches, these mismatches must be corrected in the same direction (excision of the mismatches from the same strand) in order to observe a continuous conversion tract. Since the lengths of excision tracts in yeast are about the same as the lengths of meiotic conversion tracts (1 to 2 kb) (Detloff and Petes, 1992), continuous conversion tracts are expected for most events. Some meiotic conversion tracts, however, are greater than 5 kb in length (Symington and Petes, 1988). To explain the existence of long continuous conversion tracts, we suggested that either excision repair is targeted to one strand by some undefined mechanism or the long conversion tracts reflect a different mechanism (for example, repair of a double-stranded DNA gap) than the short tracts (Detloff and Petes, 1992). The same issue is raised by the very long continuous mitotic conversion tracts. One possible explanation is that mitotic gene conversion involves very long excision tracts. This possibility is unlikely based on studies of plasmids with mismatches transformed into yeast cells, demonstrating that most mitotic excision tracts are less than 1 kb (Bishop and Kolodner, 1986).

A second explanation for long continuous gene conversion tracts is that they reflect repair of a double-stranded DNA gap (Figure 15B). Such gaps could arise from a processed DSB or two DSBs on the same chromosome. Although processing of DSBs occurs primarily by 5′ to 3′ degradation of one of the two strands (Paques and Haber, 1999), we suggest that loss of both strands, forming a gap, may occur under certain conditions (for example, a G1-induced DSB in a diploid). Orr-Weaver and Szostak (Orr-Weaver and Szostak, 1983) showed that gapped DNA molecules could be efficiently repaired, resulting in a gene conversion event. Inbar and Kupiec (1999) showed that gene conversion of an HO-induced DSB was efficient even if the DSB occurred in a

large heterologous insertion. One explanation of this result is that the broken ends are processed into a gap, although mechanisms that do not involve a gapped intermediate are also possible (for example, as shown in Figure 4 of Inbar and Kupiec). Finally, Zierhut and Diffley (2008) have recently shown that broken DNA ends that persist into the S-period undergo degradation of both 5′ and 3′ ends, resulting in a gap.

Another mechanism in which gene conversion does not involve extensive heteroduplex formation is BIR (Paques and Haber, 1999; Llorente *et al*., 2008). Since these events extend from the initiating DSB to the end of the chromosome, single BIR events will not lead to reciprocal exchange of a centromere-distal marker and will not produce a Can^R red/white sectored colony (Figure 7B). A model in which two separate BIR events can produce a long conversion tract associated with a crossover is shown in Figure 19. This process is different than the template switching described previously (Smith *et al*., 2007), in which a single end undergoes more than one cycle of strand invasion. Although the model shown in Figure 19 results in a long 3:1 continuous conversion tract, this model does not explain the 4:0 or 4:0/3:1 hybrid events.

One interpretation of our observations is that there are two types of mitotic gene conversion tracts, long tracts that reflect repair of a double-stranded DNA gap and shorter tracts that involve the repair of mismatches in heteroduplex DNA. Our analysis of crossover-associated conversions might be biased toward the first class, whereas studies of heteroallelic recombination might be biased to the second class of conversion. Although more than 90% of the conversion tracts were less than 30 kb in length, six exceeded this size. It is possible that these very long conversion tracts reflect a third mechanism of mitotic conversion.

2.4 Summary

Our analysis of spontaneous mitotic crossing-over in a 120 kb *CEN5-CAN1* interval of yeast chromosome V demonstrates that most crossovers are associated with long continuous gene conversion tracts. Crossovers and conversions occur throughout the whole interval, although these events are reduced in frequency near the centromere and there is one modest hotspot for conversion located near *CAN1*. About 40% of the recombination events have properties indicative of a DSB on one homologue in G1, replication of the broken chromosome, and subsequent repair of the two broken chromatids.

2.5 Method and Material

2.5.A Construction of yeast strains

Most of our analysis was done with two very closely related diploid strains PSL100 and PSL101; the only difference between these strains is that PSL100 is homozygous for the *ura3-1* mutation and PSL101 is heterozygous *ura3-1*/*URA3*. Isogenic diploids that were hemizygous for the mating type locus (PG311) or lacked *SPO11* (MD457) were also analyzed. These diploids are identical except for changes introduced by transformation. Their constructions are described in Section 2.6. All diploids were homozygous for *ade2-1*, heterozygous for *can1-100*, and heterozygous for an insertion of *SUP4-o* at a position on chromosome V allelic to *can1-100*. As explained in Section 2.2, reciprocal crossovers between *CEN5* and *CAN1* can be selected in strains of this genotype. In addition, each diploid was derived by crossing two sequence-diverged haploids (isogenic derivatives of W303A and YJM789), resulting in a diploid with many single-nucleotide polymorphisms (Wei *et al*., 2007). The homologue with the *can1-100* gene had the markers contributed by W303A and the one with the

SUP4-o marker had the markers contributed by YJM789. We used these markers to construct a high-resolution genetic map of the *CEN5-CAN1* region.

2.5.B Genetic analysis and media

Standard yeast procedures were used for mating, sporulation, and tetrad dissection (Guthrie and Fink, 1991). Rich growth medium (yeast extract, peptone, dextrose; YPD) and omission media were also made following standard recipes (Guthrie and Fink, 1991) except the medium contained 10 micrograms/ml of adenine. The solid medium used to select mitotic crossovers lacked arginine (SD-arg) and contained 120 micrograms/ml canavanine.

The diploid strains PSL100, PSL101, MD457, and PG311 were used to analyze mitotic crossovers. These strains were streaked for single colonies on YPD and incubated at 30°C. for 2 days. Individual colonies (about 20/experiment) were resuspended in 400 microliters of water. Each sample was diluted (usually by a factor of 10^5) and plated onto solid medium lacking arginine in order to measure the number of cells per colony; colonies on the control plates were counted after the plates were incubated two days at 30°. 100 microliters of the undiluted samples were plated onto SD-arg medium containing canavanine. These plates were incubated at room temperature for four days, followed by one day of storage at 4° to minimize the background growth of canavanine-sensitive cells and accentuate the red color of colonies that lack the *SUP4-o* gene. We then counted the number of red/white sectored colonies, only counting colonies in which the smallest sector was at least one-eighth of the size of the total colony. Each sector was purified on solid YPD medium for the subsequent analysis described below.

2.5. C Physical analysis of markers in sectored colonies

We isolated yeast DNA from purified red (*can1-100/can1-100*) and white (*SUP4-o/SUP4-o*) sectors by standard procedures (Guthrie and Fink, 1991). The numbers of sectored colonies analyzed for PSL100/101, MD457, and PG311 were 74, 14, and 14, respectively.

As described above, the diploids used in our study were heterozygous for many markers. By comparing the W303A sequence (http://www.sanger.ac.uk/gbrowse/gbrowse/cere_dmc/) and the YJM789 sequence (Wei *et al*., 2007), we identified 34 polymorphisms that changed restriction enzyme recognition sites that were located between *CEN5* (SGD coordinate of 152,000) and *can-100/SUP4-o* (SGD coordinate of about 32,000). The positions of these polymorphisms (SGD coordinates) are shown in Table 2. For each polymorphism analyzed for individual sectors, we PCR-amplified the genomic DNA using the primers that flanked the polymorphism (Table 2) and treated the resulting DNA fragment with the relevant restriction enzyme. The products were analyzed by standard agarose gel electrophoresis. This analysis allowed us to determine whether the strain representing the red or white portion of the sectored colony was homozygous for the YJM789 polymorphism, homozygous for the W303A polymorphism, or heterozygous for the polymorphism. Additional details of our analysis are given in Section 2.6.

2.5. D Physical analysis of markers in meiotic products

The meiotic segregation of markers in the diploid PSL101 was examined in 21 tetrads. All four spores of each tetrad were examined. All 34 markers were analyzed in six of the tetrads; the analysis of the remaining 15 was done by the same approach used

for most of the mitotic sectors. In each tetrad, the crossovers and gene conversion events were mapped to the highest degree of resolution possible with the 34 markers.

2.5. E Statistical analysis

Statistical analyses (Fisher exact test, Chi-square tests, and linear correlation analysis) were done using the VassarStats Website (http://faculty.vassar.edu/lowry/VassarStats.html).

2.6 Supporting information

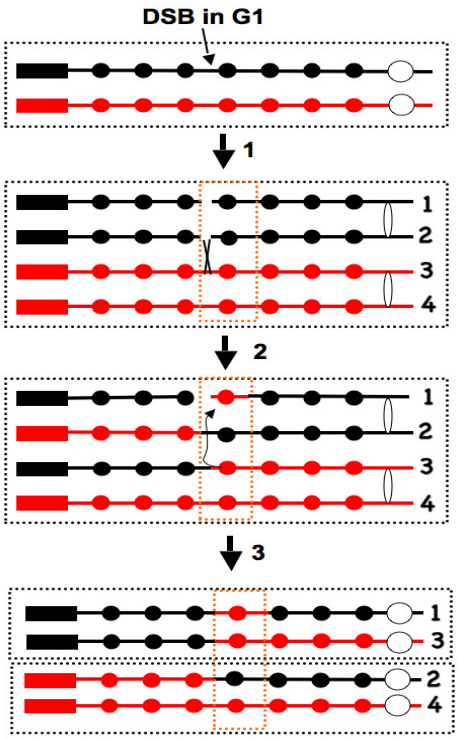


Figure 16 Patterns of conversion and crossing over that generate one of the exceptional classes of sectored colonies

In this diagram, the W303A markers are shown as red circles and the YJM789 markers are shown as black circles; the centromere is shown as a white circle or oval. The direction of conversion is indicated by the small arrow. As explained in the text and as shown in Figure 10, if the W303A-derived chromosome is the donor in a conversion event, at the site of conversion, we expect that the red sector will be homozygous for the W303A-derived marker and the white sector will be heterozygous. About 5% of the sectored colonies had the reverse arrangement (shown at the bottom of this figure). This configuration can be explained by the following sequence of events. One chromosome is broken in G1, and replicated to yield two broken chromatids. The DSB on chromatid 2 is repaired by an interaction with chromatid 3, resulting in a crossover, but no conversion (Step 1). The DSB on chromatid 1 is repaired using sequences derived from chromatid 3 (as shown) or 4; this repair event is associated with a conversion of one marker, but no crossover (Step 2). Chromatids 1 and 3 segregate to one daughter cell, and chromatids 2 and 4 segregate to the other, generating the red/white sectored colony (Step 3).

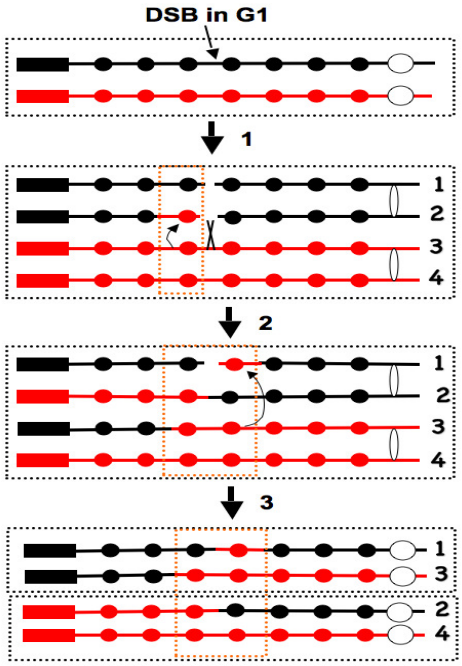


Figure 17 Patterns of conversion and crossing over required to generate a conversion tract with a crossover in the middle of the tract

As in Figure 16, a broken chromosome is replicated to yield two broken chromatids. Chromatid 2 is repaired by an interaction with chromatid 3 associated with a conversion of a centromere-distal marker and a crossover (Step 1). Chromatid 1 is repaired by an interaction with chromatid 3 (as shown) or 4. This repair event is associated with a conversion of a centromere-proximal marker, but no crossover (Step 2). Chromatids 1 and 3 co-segregate, as do chromatids 2 and 4 (Step 3).

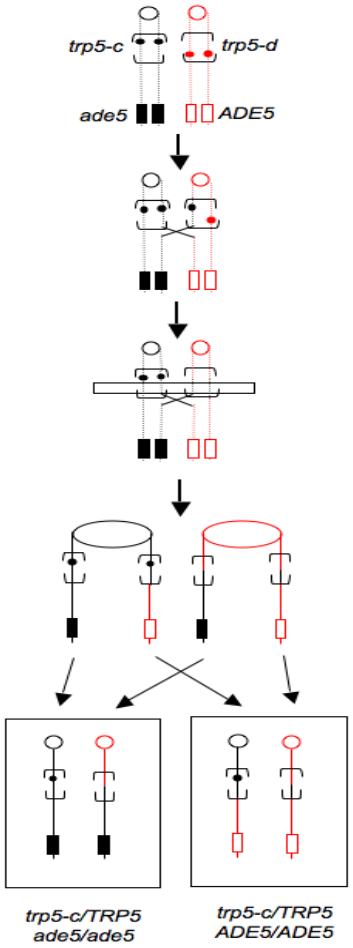


Figure 18 Model proposed by Esposito to explain G1-initiated mitotic recombination

Dotted lines in this figure represent single strands of a DNA duplex. Derived from a strain with *trp5* heteroalleles and a distal heterozygous marker, Esposito observed Trp^+ colonies that had homozygous sectors for the distal marker. To explain such sectors, he suggested that an asymmetric heteroduplex is formed in G1 that includes both of the heteroallelic markers. Repair of both resulting mismatches using wild-type information would result in a wild type allele. The resulting intermediate with an unresolved Holliday junction would be replicated to produce the RCO. Resolution of the Holliday junction in G1 would not produce a reciprocal crossover.

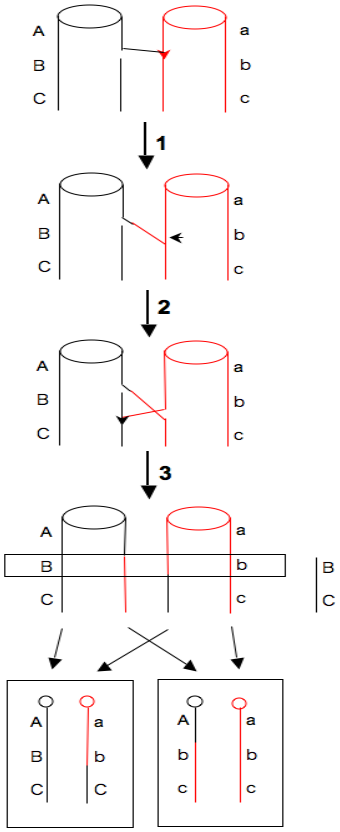


Figure 19 Mitotic conversion tracts with associated crossover generated by a double BIR event

The broken DNA in the black chromatid invades and begins to replicate the red chromatid (step 1). After region B of the chromosome has been replicated, the replication fork breaks (step 2), and the broken end invades the black chromatid (step 3). Completion of DNA synthesis results in a long conversion tract with a flanking RCO (step 4). The acentric chromatid fragment with the B and C regions is lost.

Table 1 Primers used in strain constructions

Primer name	Sequence (5' to 3')
ADE2-KIURA-381	ACTCCAATGACCACGTTAATGGCTCCTTTTCCAATCCTTTGATATCGAAAAACTAGCTGTGGTTATTCGTGGATCTATATC
ADE2-KIURA3 DS	GATTCTTTAGTGTAGGAACATCAACATGCTCAATCTCAATCGTTAGCACATCACATTTTTTTAAACTCTTTTTCGATGAT
ADE2-11	GAACAGTTGGTATATTAGGAGGGGGAC
ADE2-392	TCACTGGCTTGTTCCACAGGAACAC
ADE2 DS	CAAGACGAATGGAAAACCCAAATCTC
CAN1-801	CGAATCAGGGAATCCCTTTTTGC
CAN1-2974	CTGAAGGAGTTTCAAATGCTTC
CAN1-0901	GTTGGATCCAGTTTTTAATCTGTC
CAN1-2800	GTGATCAAAGGTAATAAAACGTC
URA3 US	ACGCATATGTGGTGTTGAAGAAAC
URA3 DS	GTTGTTCTTTGGAGTTCAATGCGTC
MATALPHA NATF	ATATATATATATATTCTACACAGATATATACATATTTGTTTTTCGGGCCGTACGCTGAAGGTCGAC
MATALPHA NATR	TGAACAACATTCAGTACTCGAAAGATAAACAACCTCCGCCACGACCACACTCATCGATGAATTCGAGCTCG
SPO11NATF	ATAACCTCACATATTTGTCTTCACCCTTAAGATTTTACGATTTACTAAGTTCACCTTCTCCGTACGCTGCAGGTCGAC
SPO11NATR	CGTTTTCAATTCTTGAAAAACATTTTTTATAAAGCAACAGCTCCCATTCTTATTCATTTATCGATGAATTCGAGCTCG
nat-R	GTCGACCTGCAGCGTACG
V-63628	GATTGCAGTTGCCCGAAAACTGT

Table 2 Primers used in analysis of polymorphic markers

As described in the text, we identified sequence differences between two yeast strains (W303a and YJM789) that altered restriction sites in the region between *CEN5* and *CAN1*. We examined the segregation of these sites by generating short PCR fragments that included the sites, and treating the resulting fragments with restriction enzymes that cut the DNA derived from one strain, but not the other. The position of the polymorphism is indicated in coordinates based on the Stanford Genome Database. The numbers in parentheses represent the abbreviations of the coordinates used in the figures. This column indicates the enzymes used to diagnose the polymorphism. The enzyme written in boldface has a recognition site at the diagnostic position in YJM789, but not in W303a. The enzyme written in plain face has a recognition site in W303a, but not in YJM789.

polymorphic site	primers	sequences	restriction enzyme used
34,812	V-34290	GTTGCCTGTATCTCCGAATAGGAC	*Taq*I
	V-35030	CAGGTCTTGGAAAATTCTCATCAAT	
40,688	V-40455	TTTAAACCTGGGGCGAAAACGTC	***Hpa*II**
	V-40876	ACTGTGCCGGTACTATCGCTT	
43,075	V-42860	TGTCTGCTTCCTTACTTGGCTG	***Alu*I**
	V-43242	GCTTACTGCTGACCTTTGCCTT	
44,403	V-44164	GTGTTCTACTATGTCGACTC	*Alu*I
	V-44532	AGCTAACGTATCACCATTTAAC	
46,347	V-46267	CGATTGACGTAAACGGTAATAAATGT	*Hha*I
	V-46590	ATTTATAAGACAAAAAGTTAGAATTCT	
48,898	V-48700	TTAGAAGTCAGCATCAGCTTGTG	*Mse*I
	V-49179	GCTTTCTGACGACGGTGGAGA	
51,707	V-51560	TGCACTTGTGGAAAGAATCGCC	*Dra*I
	V-51985	CCTTCAAGGCTACTTTCAGATGC	
54,915	V-54657	TTTCAAAAAGGTTTCTAAGTGGTGAC	*Rsa*I
	V-55143	CTACCAGTTTGCCAAATTCTTCAAC	

Table 2, continued.

56,166	V-55825	CTATCCGCTATGGCTGCTAC	*Hha*I
	V-56394	AAGACAAAGGAACTCGGCAC	
57,448	V-57125	CGATACGGTTCCATTTTTGGCCGCA	***Hpa*II**
	V-57682	GGCTCACCACACAACTGCTTGA	
60,163	V-59896	CCAAGAGATCTGTTTTGATGGTC	*Hpy*188III
	V-60417	TGGTGGCCATAGAAATAGAACG	
63,936	V-63638	ATTGAACTGCTTTGGTTACCTCTC	*Hae*III
	V-64114	CCTTCATTAAATTGGCGTTTGTCTC	
70,336	V-70090	GCCAAGAAGTACTGCGATGTTAC	***Hha*I**
	V-70583	TCTTCAAAGTGGAGACGATGCTG	
76,383	V-76193	GTTGATACTGCATACGATGTAAGGC	*Dra*I
	V-76567	CGGCGGAACATCTTTCGTGAATATA	
80,094	V-79811	TAACCCTTTACCTGACCTGAATGTC	***Bgl*II**
	V-80476	GTAATTGTCTCCCATTTTTGGTATAC	
82,767	V-82592	TAAGCTAACCATTTTTCTATTG	Rsa*I*
	V-82962	GGTCTTTAACTTTTCTTTGAGTG	
86,772	V-86400	GTGTAATTCATTGGGGAGGATGA	***Alu*I**
	V-86943	CTTCCATAATTGACGTTTGTATC	
91,715	V-91473	TTGATTTTCGCTGTTATTGCATCC	*Taq*I
	V-91953	TACTGTTTTTTCTTTCGACAGCCC	
94,329	V-94058	GTTTATATTTTGTTGCTAGCGTTACGG	***Taq*I**
	V-94699	CGGGTCAGAATACGAAGTATATTATG	
99,267	V-98931	ATACTGTTATCGAAACTACGGGC	*Eco*RV
	V-99436	TGTGTGCATGGTTATGTAGATTG	

Table 2, continued.

103,991	V-103659	GAGTTTTGTTCTGGCCACAGTGGC	***Hha*I**
	V- 104202	GAGAGTGGATTTAGAGATTCATTCGT	
107,884	V-107668	TTGAAGGACCCACAGACCGATGC	***Alu*I**
	V-108097	GTGATTTTCGTTCCTTTTTGAGCG	
111,516	V-111339	CACTGCTTGTAAACCATAGAC	***Rsa*I**
	V-111719	GGCTGCAATATGGTTAGTGAATC	
113,600	V-113331	GGGTCGATGAAGCTATTAGAA	***Msp*I**
	V-113830	CAAAAGCTCCAAGGGTGTTA	
115,035	V-114800	CGGAGTACTTGTCCAAATTAA	*Taq*I
	V-115159	CTGTCAATTTCTTGTATTCTA	
117,289	V-117099	AAAGAAAAAGCTTCATGGCC	***Hha*I**
	V-117498	TAGATATATATACGCCAGTAC	
118,783	V-118624	CGCGGTTTATTCTGCCAGGC	***Hha*I**
	V-119048	AACGCGACTATGGGGATTGG	
122,334	V-122161	AGCGTTCATGAACTGCAGCTGATTC	***Hha*I**
	V-122498	CTCTTCGTTTTGTTTGTCCCGTTC	
125,754	V-125473	ACCAATTCTGGCCTATCTTTAAGC	***Xho*I**
	V-126033	TAGATCAAATACTTACTTCAACGGG	
133,080	V-132837	ACCCCTTTTTGCCTATATTAC	***Dde*I**
	V-133228	ACAACCACTTGTCAGCTAGG	
140,703	V-140520	GCACTTTATTCCCCGAAGATCTTC	***Hpy*CH4V**
	V-140946	CAAACGTGGGGGTATAACTACAATC	
144,265	V-143365	CCAATAATAATAGTGCAAGCTCTGC	*Hin*fI
	V-144023-R	GGCAAATGAAGATGAAATTAAAGGCA	

Table 2, continued.

146,855	V-146424	TACAATTAGTAGAAGCCCTTTGC	***Hpa*II**
	V-147181	CGACAGTAATGACATAAACGTG	
151,440	V-151173	CCCGGAATACATCGTGTAGTC	***Hin*fI**
	V-151638	ATTCAATGACAGAAACATTACGAAG	

Table 3 Lengths of mitotic conversion tracts in PSL100/PSL101

The maximum, minimum, and average lengths of mitotic gene conversion tracts were calculated as described in the text. The table is ordered by the average length of the conversion events, beginning with the shortest. In this column, we indicate whether the conversion tract was a 3:1 tract (1), a 4:0 tract (2), or a hybrid 3:1, 4:0 tract (3).

		(bp)		
66	1	3715	1	1858
73	1	4459	1	2230
74	1	6017	1	3009
87	1	5659	1328	3494
121	1	5741	1251	3496
53	1	7525	1	3763
98	1	7525	1	3763
72	2	8264	1	4133
88	1	8617	1	4309
107	2	8617	1	4309
21	1	7299	1494	4397
80	1	10173	1	5087
47	1	8456	2533	5495
55	1	8210	3272	5741
46	3	9592	2387	5990
58	2	8465	3551	6008
94	1	12447	1	6224
125	1	12447	1	6224
39	3	8734	3748	6241
12	3	9405	3519	6462
32	1	8550	4459	6505
128	1	10389	2673	6531
131	1	14949	1	7475
36	3	11536	3715	7626
37	1	9609	5877	7743
135	3	11621	4005	7813
44	1	10899	5773	8336
35	1	11019	5823	8421
136	1	12276	4938	8607

Table 3, continued.

16	3	12229	5248	8739
126	1	14297	3420	8859
70	2	11840	7304	9572
122	2	12154	7299	9727
33	1	14087	5659	9873
22	3	15332	6678	11005
45	3	16220	6400	11310
138	1	14227	8632	11430
43	1	14238	8734	11486
64	1	17870	10818	14344
19	3	20104	11019	15562
54	1	20104	11019	15562
134	1	21112	12276	16694
79	3	23993	15332	19663
14	3	26601	22646	24624
59	1	31425	23067	27246
133	1	64809	59588	62199
127	1	105892	92392	99142

Table 4 Lengths of mitotic conversion tracts in MD457

The maximum, minimum, and average lengths of mitotic gene conversion tracts were calculated as described in the text. The table is ordered by the average length of the conversion events, beginning with the shortest. In this column, we indicate whether the conversion tract was a 3:1 tract (1), a 4:0 tract (2), or a hybrid 3:1, 4:0 tract (3).

Sectored colony # (MD457)	Type of conversion tract	Max. Length (bp)	Min. Length (bp)	Ave. Length (bp)
2-2	1	8210	3272	5741
4-3	1	14949	1	7475
2-9	3	13555	4724	9139.5
2-6	2	16158	6047	11103
2-12	1	18045	9587	13816
2-5	2	24676	15421	20049
4-2	3	23248	17088	20168
2-7	3	31552	22836	27194
2-13	1	33308	25933	29621
2-10	1	35562	27068	31315
2-8	1	41572	29648	35610
2-3	1	107237	98269	102753

Table 5 Lengths of mitotic conversion tracts in PG311

The maximum, minimum, and average lengths of mitotic gene conversion tracts were calculated as described in the text. The table is ordered by the average length of the conversion events, beginning with the shortest. In this column, we indicate whether the conversion tract was a 3:1 tract (1), a 4:0 tract (2), or a hybrid 3:1, 4:0 tract (3).

Sectored colony # (PG311)	Type of conversion tract	Max. Length (bp)	Min. Length (bp)	Ave. Length (bp)
4-3	1	3715	1	1858
4-7	2	6678	1	3339.5
4-9	1	7552	1	3776.5
4-5	2	7557	1	3779
4-10	1	9758	1	4879.5
1-2	2	8568	2809	5688.5
1-5	1	8550	4459	6504.5
4-6	1	12276	4938	8607
4-4	3	18831	9758	14295
4-8	3	27261	19533	23397
1-6	1	30470	25352	27911
4-1	3	68874	30393	49634

Table 6 Lengths of meiotic conversion tracts in PSL101

The maximum, minimum, and average lengths of meiotic gene conversion tracts were calculated as described in the text. The table is ordered by the average length of the conversion events, beginning with the shortest.

PSL101 tetrad #	Max. Length (bp)	Min. Length (bp)	Ave. Length (bp)
31	3715	1	1858
27	3715	1	1858
29	3997	1	1999
9	3997	1	1999
10	9758	1	4880
33	10746	1	5374
28	12447	1	6224
13	12447	1	6224
14	11621	4005	7813
37	14297	3420	8859

2.6.A Supplementary text

2.6.A.1 Materials and methods

Construction of yeast strains

The diploid PSL100 was constructed by crossing the haploids PSL2 and PSL5. PSL2 (*MATa ade2-1 can1-100 ura3-1 trp1-1 V9229::HYG V261553::LEU2*) was derived from sporulation of the diploid MAB6 (Barbera and Petes, 2006) and is isogenic with W303A (Thomas and Rothstein, 1989) except for markers introduced by transformation. The markers *V9229::HYG* and *V261553::LEU2* are insertions of genes encoding hygromycin resistance and *LEU2* into chromosome V at the Stanford Genome Database (SGD) coordinates 9229 and 261553, respectively.

The PSL5 haploid (*MATα ade2-1ura3 can1D::SUP4-o gal2 ho::hisG*) was constructed in several steps. The haploid YJM799 (*MATα ura3 gal2 ho::hisG*) is isogenic to YJM789 (except for changes introduced by transformation or crosses with isogenic strains) and was provided by John McCusker (Department of Molecular Genetics and Microbiology, Duke University School of Medicine); YJM789 was derived from a clinical yeast isolate and its genome has recently been sequenced (Wei *et al*., 2007). The *ADE2* gene in YJM799 was disrupted by transformation with a DNA fragment obtained by PCR amplification of the plasmid pCORE (Storici *et al*., 2001) with the primers ADE2-KlURA-381 and ADE2-KlURA DS; all primer sequences used in strain constructions are in Table 1. The transforming fragment contained the selectable markers *K. lactis URA3* and *KANMX* with 60 bp of flanking homology to the *ADE2* gene. The resulting Ura$^+$ red transformant was PSL3. The *URA3/KANMX* insertion in PSL3 was replaced by the *ade2-1* allele by transformation of PSL3 with a DNA fragment

obtained by PCR amplification of genomic DNA of strain MD235 (Barbera and Petes, 2006) with the primers ADE2-392 and ADE2-11. The replacement of the *URA3/KANMX* insertion with *ade2-1* (an ochre mutation in *ADE2*) was selected using 5-fluoro-orotate (5-FOA)-containing medium (Boeke *et al*., 1984); the resulting haploid was PSL4. The insertion of *ade2-1* was confirmed by sequencing a PCR fragment generated using PSL4 genomic DNA and the primers ADE2-11 and ADE2 DS. The last step in the construction was the replacement of the *CAN1* gene of PSL4 with *SUP4-o* (an ochre-suppressing tRNA). PSL4 was transformed with a DNA fragment containing the *SUP4-o* and flanking sequences derived from the *CAN1* locus, generated by amplification of genomic DNA of MD242-1 (Barbera and Petes, 2006) with primers CAN1-801 and CAN1-2974. The canavanine-resistant transformant that was Ade^+ and formed white colonies (indicating suppression of the *ade2-1* allele) was called PSL5. The construction was confirmed by sequencing a PCR fragment generated using primers CAN1-901 and CAN1-2800 and genomic DNA of PSL5.

The diploid PSL100 was made by crossing PSL2 and PSL5. The closely-related diploid PSL101 was constructed by transforming PSL100 with a fragment generated by amplifying genomic DNA of the strain YJM780 (a *URA3* strain otherwise isogenic with YJM799; provided by John McCusker) with the primers URA3 US and URA3 DS. Subsequent analysis of mitotic recombination events in PSL101 showed that the *URA3* allele was in coupling with *SUP4-o* on chromosome V.

MD457, a *spo11::NAT/spo11::NAT* derivative of PSL101, was constructed by mating the haploids MD454 and MD455. MD454 was constructed by transformation of PSL2 with a DNA fragment obtained by amplifying the *NAT*-containing plasmid pAG25

(Goldstein and McCusker, 2006) with the primers SPO11NATF and SPO11NATR. The resulting nourseothricin-resistant transformant was checked by PCR amplification of genomic DNA with the primers VIII-63628 and nat-R. MD455, the other *spo11::NAT* haploid, was derived from the haploid MD416 by the same procedure. MD416 is a *URA3* derivative of PSL5 resulting from transformation of PSL5 with a *URA3*-containing DNA fragment generated by PCR amplification of genomic DNA of YJM789 with the primers URA3 US and URA3 DS.

PG311 is a *MATa/MATα::NAT* derivative of PSL101. This diploid was constructed by transforming PSL101 with a DNA fragment generated by PCR amplification of the plasmid pAG25 with the primers MATalpha NATF and MATalpha NATR. The resulting nourseothricin-resistant transformant was, as expected, sensitive to the alpha pheromone, capable of mating to a *MATα* haploid, and incapable of sporulation.

Details of physical analysis of markers in sectored colonies

Of the approximately 100 sectored colonies analyzed, 12 were analyzed for all 34 polymorphic sites in both sectors; the PCR primers used in the analysis are in Table 2. For each marker, we usually observed one of four patterns: 1) the marker was heterozygous in both sectors (indicating that the RCO occurred centromere-distal to the marker), 2) the red sector was homozygous for the W303A form of the polymorphism and the white sector was homozygous for the YJM789 form of the polymorphism (indicating that the RCO occurred centromere-proximal to the marker), 3) the red sector was homozygous for the W303A form of the polymorphism and the white sector was heterozygous (indicating a gene conversion event in which the W303A-derived

chromosome was a donor), and 4) the white sector was homozygous for the YJM789 form of the polymorphism and the red sector was heterozygous (indicating a gene conversion event in which the YJM789-derived chromosome was a donor).

For those sectored colonies that were not analyzed for all markers, we mapped the crossover and associated conversion tract (if any) by the following procedure. First, we examined the markers 57, 94, and 122 in both sectors; the numbers of the markers represent their approximate position (in thousands of base pairs) on chromosome V (SGD coordinates). This analysis allowed us to map the crossover within a single quadrant of the 120 kb interval. We subsequently refined the mapping within each quadrant using the additional heterozygous markers. Each crossover and gene conversion event was mapped to the highest degree of resolution possible with the 34 markers. Markers with unusual segregation patterns were re-checked. It should be noted that, in strains heterozygous for the *ura3* mutation, a sectored colony with red Ura^- and white Ura^+ sectors were assumed to reflect a RCO between *CEN5* and *URA3*; the position of the RCO was confirmed by marker analysis.

2.6.A.2 Results and discussion

Mitotic and meiotic conversion tract lengths

The maximum, minimum, and average mitotic conversion tract lengths for strains PSL100/PSL101, MD457, and PG311 are shown in Tables 3-5, respectively. Meiotic conversion tract lengths for PSL101 are shown in Table 6.

Interpretation of recombination events in which the conversion tracts have an unexpected pattern of segregation in the sectored colonies

As discussed in the text, most (42 of 48) of the 3:1 or hybrid 3:1, 4:0 tracts had the pattern of marker segregation shown in Figure 10B. If the W303A-derived chromosome is the donor in the conversion event (the red chromatid in Figure 10B), we expected that the red sector would be homozygous for the converted marker and the white sector would be heterozygous; alternatively, if the YJM789-derived chromosome is the donor, we expected that the white sector would be homozygous for the converted marker and the red sector would be heterozygous. In 3 of 48 sectored colonies, we found a different pattern. One example is shown in the bottom part of Figure 16. In this example, although the W303A-derived chromosome is the donor in the conversion event, the red sector is heterozygous for the converted marker and the white sector is homozygous. This pattern can be explained as a consequence of the repair of two DSBs in which the repair of the first DSB is associated with a crossover with no conversion tract and the repair of the second has an associated conversion, but no associated crossover.

In the second class of exceptional sectored colonies (3 of 48 sectored colonies), the pattern of marker segregation indicated a crossover within the conversion tract (bottom part of Figure 17). This pattern can also be explained as repair of two DSBs resulting from a replicated broken chromosome. The repair of the first DSB is associated with a crossover and conversion of the marker centromere-distal to the DSB. The repair of the second DSB is not associated with a crossover, but results in conversion of a marker centromere-proximal to the DSB. It should be emphasized that there are other patterns of crossovers and conversions in G1 and G2 that could also explain the exceptional classes.

Esposito model of mitotic recombination in G1

As discussed in the main text, Esposito (1978) concluded that a substantial fraction of spontaneous mitotic recombination was initiated in G1. In these experiments, a diploid was constructed that had non-complementing heteroalleles at the *TRP5* locus (*trp5-d* and *trp5-c*) and the heterozygous centromere-distal marker *ADE5*. The two main classes of sectored colonies diagnostic of a G1 event were: Class I (*ADE5/ADE5 TRP5/trp5-d* sector and *ade5/ade5 TRP5/trp5-d* sector) and Class II (*ADE5/ADE5 TRP5/trp5-c* sector and *ade5/ade5 TRP5/trp5-c* sector). For Class I events, since the *trp5-c* allele is not present on either homologue in either sector, this region of the *TRP5* gene has undergone a gene conversion equivalent to a 4:0 event; similarly, the *trp5-d* portion of the gene represents a 4:0 conversion in Class II.

Esposito (1978) suggested that these results could be explained as a consequence of heteroduplexes formed in G1, followed by “patchy” repair of the resulting mismatches (repair of two adjacent mismatches using different strands as templates), and replication of the DNA molecules containing the resulting Holliday junction (Figure 18). Although we cannot exclude the Esposito model, we would expect the Holliday junction to be susceptible to resolvases rather than resolution by DNA replication. In addition, as described in the text, there is a single Holliday junction in the Esposito model, whereas the repair of meiotic DSBs in *S. cerevisiae* is associated with two adjacent Holliday junctions. Resolution of the double junction by replication would produce a double crossover rather than the single crossover required to make the distal marker homozygous. For these reasons, we prefer our model (Figure 14).

3. Mitotic gene conversion events induced in G1-synchronized yeast cells by gamma rays are similar to spontaneous conversion events.

3.1 Introduction

Homologous mitotic recombination is an efficient method of repairing DSBs in the yeast *Saccharomyces cerevisiae* and other organisms (Aguilera and Rothstein, 2007). Although mitotic recombination was described in Drosophila more than 70 years ago (Stern, 1936), many of the basic properties of mitotic recombination are not understood. The chromosomes resulting from a mitotic recombination event are segregated into two daughter cells and one problem is that most analytic methods select for only one of the cells containing the recombinant chromosomes.

A method for selecting both daughter cells with the products expected from reciprocal crossing over (RCO) is shown in Fig. 20. A diploid yeast strain is constructed in which one copy of chromosome V has an ochre mutation in the *CAN1* gene (*can1-100*); in the absence of an ochre suppressor, such strains are resistant to canavanine. On the other homologue, the *CAN1* gene has been replaced by *SUP4-o*, a gene encoding an ochre-suppressing tRNA. The diploid is also homozygous for the *ade2-1* ochre mutation. Yeast strains containing the *ade2-1* allele without a nonsense suppressor are Ade^- and form red colonies as a result of accumulation of red precursor to adenine (Jones an Fink, 1982). In the diploid depicted in Fig. 20, because of the presence of the *SUP4-o* gene, the cells are canavanine-sensitive, Ade^+, and form white colonies.

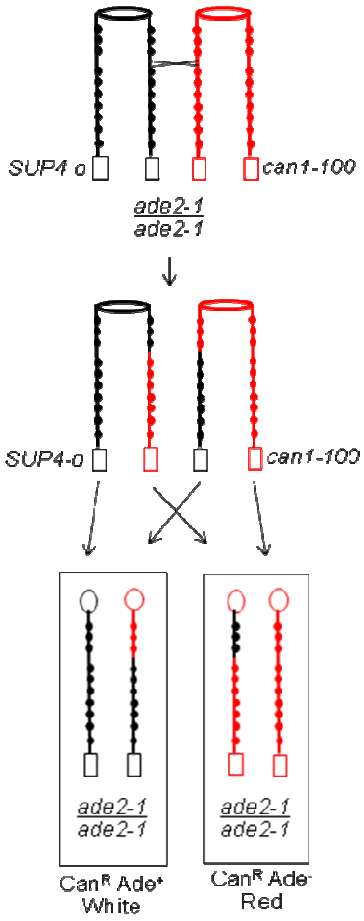


Figure 20 Diploid strain used to select and map RCOs

The diploid PG311 has the *can1-100* allele on one copy of chromosome V and a replacement of *can1* sequences with the ochre-suppressor tRNA gene *SUP4-o* on the other (Barbera and Petes, 2006). This strain is also homozygous for the ochre-suppressible *ade2-1* mutation. The starting diploid is Can^S and Ade^+; strains with an unsuppressed *ade2-1* mutation form red colonies (3). An RCO between the centromere and *can1-100/SUP4-o* can result in two Can^R cells (rectangles at bottom of figure). Subsequent growth of these cells results in a red/white sectored colony. There are many single-nucleotide polymorphisms distinguishing each homologue (indicated by red and black circles) that can be used to map the position of the RCO.

A RCO between the centromere of chromosome V and the *can1-100/SUP4-o* markers will result in two Can^R daughter cells that will subsequently grow to form a

red/white sectored Can^R colony (Barbera and Petes, 2006). As a consequence of the RCO, polymorphisms distal to the crossover point become homozygous in the two sectors, whereas polymorphisms proximal to the exchange retain heterozygosity (Fig. 20). We mapped crossovers to a resolution of 4 kb in this 120 kb interval using a diploid constructed by mating haploids with diverged (0.1%) DNA sequences (Lee *et al*., 2009). Using PCR and restriction enzyme analysis (discussed below), we determined whether individual sectors were heterozygous or homozygous for the markers.

In meiotic tetrads in fungi, although heterozygous markers usually segregate 2:2 into the four spores (for example, 2*A*:2*a*), in some tetrads, there is a net loss of one allele and a net gain of the second (3*A*:1*a* or 1*A*:3*a* segregation). Such events are called “gene conversions”. Meiotic gene conversions reflect DNA mismatch repair in the heteroduplex that initiates the crossover (Petes *et al*., 1991); mitotic gene conversions also occur (Petes *et al*., 1991). In our system, conversion events associated with RCOs are detectable as regions that are homozygous for one or more markers in one sector, but that remain heterozygous for the same markers in the other sector (dotted boxed region in Fig. 21A). In analogy with meiotic conversion events, this type of mitotic conversion is called “3:1”.

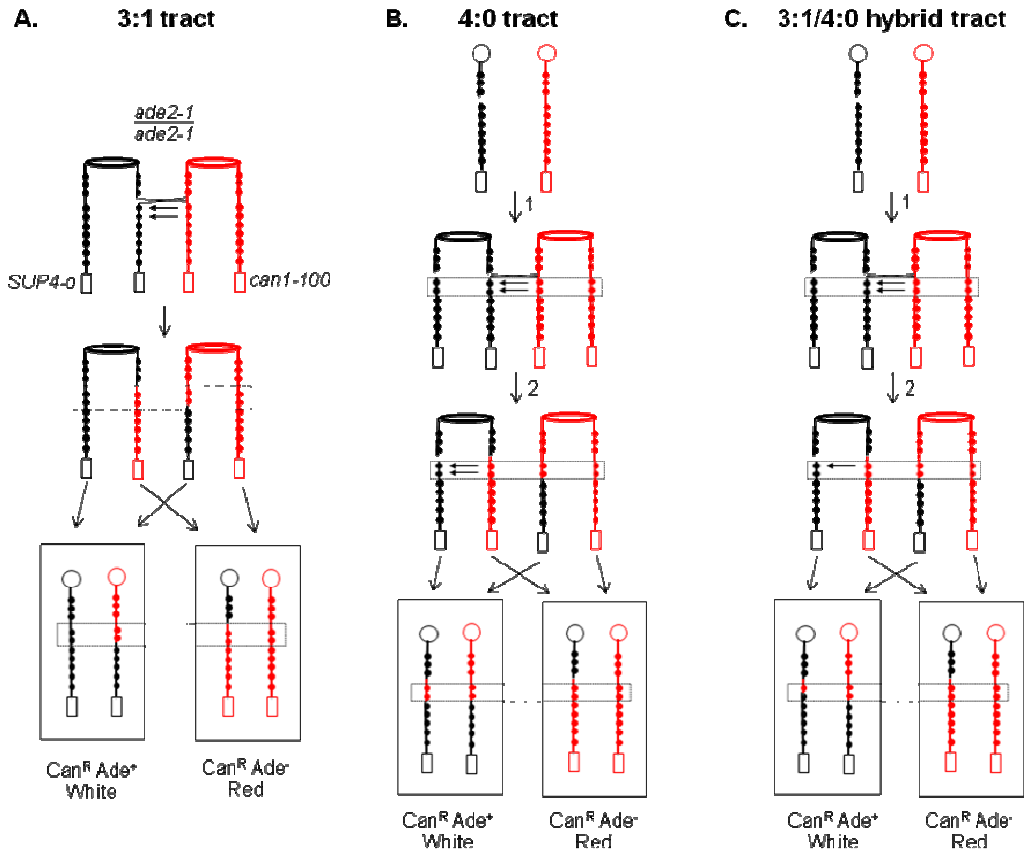


Figure 21 RCOs and gene conversion events associated with G2- and G1-induced DSBs

The homologues containing the *SUP4-o* and *can1-100* alleles are shown in black and red, respectively, with circles indicating polymorphic sites. Dotted boxes mark gene conversion tracts and X's show the position of the RCO. (A). 3:1 conversion tract. Recombination is initiated by a DSB on one of the black chromatids. The associated 3:1 gene conversion involves the broken chromatid receiving information from the red chromatid as observed in many studies of induced DSBs. Two linked markers are transferred as shown by the horizontal arrows. (B). 4:0 conversion tract. Recombination is initiated by a DSB in an unreplicated black chromosome that is then replicated to yield two broken chromatids. The repair of one chromatid is associated with an RCO and conversion of two polymorphisms. The repair of the second DSB is not associated with an RCO but the same two sites are converted, yielding a 4:0 conversion event. (C). 3:1/4:0 hybrid tract. The pattern of DSB formation and conversion is similar to that in Fig. 21B, except that the conversion tract associated with the second repair event is short, generating a hybrid 3:1/4:0 conversion.

We previously showed that mitotic conversion tracts associated with RCOs averaged 12 kb in length (Lee *et al*., 2009), considerably longer than meiotic conversion tracts (Petes *et al*., 1991). In addition, about 40% of the conversion tracts were not of the 3:1 type. For one type of exceptional tract (4:0 events), the same form of the polymorphism was homozygous in both sectors (dotted boxed region of Fig. 21B). We also detected conversion tracts with a 4:0 region immediately adjacent to a 3:1 region (Fig. 21C). Since the 4:0 and hybrid 3:1/4:0 tracts were much too frequent to represent two independent events, we suggested that both 4:0 and 3:1/4:0 hybrid tracts reflected repair of two DSBs, generated by replication of a broken chromosome to form two broken chromatids (Fig. 21B and 21C). Repair of both DSBs associated with conversion tracts of the same size would generate the 4:0 tract and repair of the two DSBs with tracts of different sizes would produce the hybrid tracts.

Several other observations support the model shown in Fig. 21B and 21C. First, X-rays and ultraviolet radiation stimulate mitotic recombination in G1-arrested yeast cells (Wildenberg, 1970; Fabre, 1978). Second, Esposito (1978) showed that some spontaneous heteroallelic conversions in yeast had the properties expected for events initiated in G1. Third, DSBs in G1 cells result in DNA ends that are inefficiently resected (Aylon *et al*., 2004; Ira *et al*., 2004), and these ends fail to recruit Rad52p (Lisby *et al*., 2001). Fourth, cells irradiated in G2 have much more rapid repair than those irradiated in G1 (Brunborg *et al*., 1980). Thus, broken chromosomes generated in G1 are likely to replicate before DNA repair. In addition, G1-irradiated chicken cells have metaphase chromosomes in which both chromatids are broken at the same position, whereas G2-

irradiated cells have metaphase chromosomes with a single broken chromatid (Takata *et al*., 1998).

To provide further evidence for this model, we examined RCOs and their associated conversion events induced by gamma irradiation of yeast cells synchronized in G1 or G2. We find that the conversion events associated with RCOs in cells irradiated in G1 are of the 3:1, 4:0, and 3:1/4:0 hybrid types. The conversions associated with RCOs induced by G2 irradiation are exclusively of the 3:1 type.

3.2 Results

3.2.A Experimental rationale

The diploid used in the study (PG311) has markers allowing the selection of RCOs as canavanine-resistant red/white sectored colonies, and is also heterozygous for the polymorphisms that allow mapping of the RCOs and associated conversion tracts (Fig. 20). Although most diploids have both *MATa* and *MAT*α information, PG311 has a deletion of the *MAT*α locus, allowing its synchronization in G1 using the α pheromone (Bucking-Throm *et al*., 1973). We showed previously that the frequency and distribution of RCOs and conversion tracts were similar in PG311 and an isogenic diploid expressing both mating types (Lee *et al*., 2009). We synchronized PG311 in G2 using nocodazole (Argueso *et al*., 2008).

3.2.B Cell viability and frequency of RCOs in G1- and G2-synchronized cells

PG311 cells synchronized in G1 or G2 were irradiated with 0, 50, or 100 Gy of gamma rays to induce DSBs. The average viabilities for the G1-arrested cells ($\pm$ 95% confidence limits) relative to 100% for unirradiated samples (five independent

experiments) were: 100% (± 10%) for cells treated with 50 Gy and 77% (± 15%) for the samples treated with 100 Gy. The viabilities of the G2-irradiated samples at the 50 and 100 Gy doses were 110% (± 25%) and 89% (± 7%), respectively.

The average rates of sectored colonies per viable cell for the G1 cells were: 1.1 (± 0.4) x 10^{-5}, 0 Gy; 3.2 (± 2.0) x 10^{-4}, 50 Gy; and 2.6 (± 1.1) x 10^{-4}, 100 Gy. For the G2 cells, the comparable rates were: 9.9 (± 7.8) x 10^{-6}, 0 Gy; 2.4 (± 0.9) x 10^{-4}, 50 Gy; and 4.2 (± 2.5) x 10^{-4}, 100 Gy. Thus, in both G1- and G2-irradiated samples, the rates of RCOs were stimulated 20- to 40-fold.

Unexpectedly, the rate of RCOs in the G1-irradiated samples was not elevated by increasing the dose from 50 to 100 Gy. It is possible that cells have a limited capacity to repair G1-induced DSBs by the RCO pathway because of limiting amounts of one of the recombination proteins. Alternatively, because our estimates of the rates of RCOs have large confidence limits, it is possible that the similar rates of RCOs at the two radiation doses simply reflect the large confidence limits on the estimates.

3.2.C Mapping of gamma ray-induced mitotic crossovers and associated gene conversions

Crossovers and associated gene conversion events were mapped as described previously (Lee *et al*., 2009). DNA was isolated from both the red and white sectors of each sectored colony. For this analysis, we used 34 heterozygous polymorphisms in which one allele had a restriction enzyme recognition site that the other allele lacked. By PCR amplification of sequences containing the heterozygous alleles, followed by digestion with the appropriate restriction enzyme, we determined whether the cells of the sector were homozygous for one allele or the other, or were heterozygous.

The mapping data for the G1- and G2-irradiated samples are shown in Fig. 22. We analyzed 29 sectors from the G1-irradiated cells (15 and 14 from the 50 and 100 Gy samples, respectively) and 31 from the G2-irradiated cells (7 and 24 from the 50 and 100 Gy samples, respectively). The patterns of crossovers and conversions were similar for the two doses of radiation and are summed for subsequent analysis. Green Xs depict crossovers unassociated with gene conversion and the horizontal lines show conversion events associated with crossovers. The 3:1 tracts are shown as thin lines and 4:0 tracts as thick lines.

In the G1-irradiated cells, we found 3:1, 4:0, and 3:1/4:0 hybrid tracts whereas in the G2-irradiated sample, we observed only 3:1 tracts. Most (34 of 41) of the conversion events in G1- and G2-irradiated samples had three properties: 1) the conversion event involved a single donor chromosome, 2) the conversion tracts were uninterrupted by markers that did not show conversion, and 3) for 3:1 conversion tracts or the 3:1 portion of 3:1/4:0 hybrid tracts, the sectors that are homozygous or heterozygous for the markers had the patterns of segregation shown in Fig. 21A and 21C in which the conversion event involves the chromatids that crossed over. Thus, if the conversion event involves donating information from the *can1-100*-containing chromosome, the sector that is homozygous for *can1-100* is homozygous for the converted marker with the other sector retaining heterozygosity.

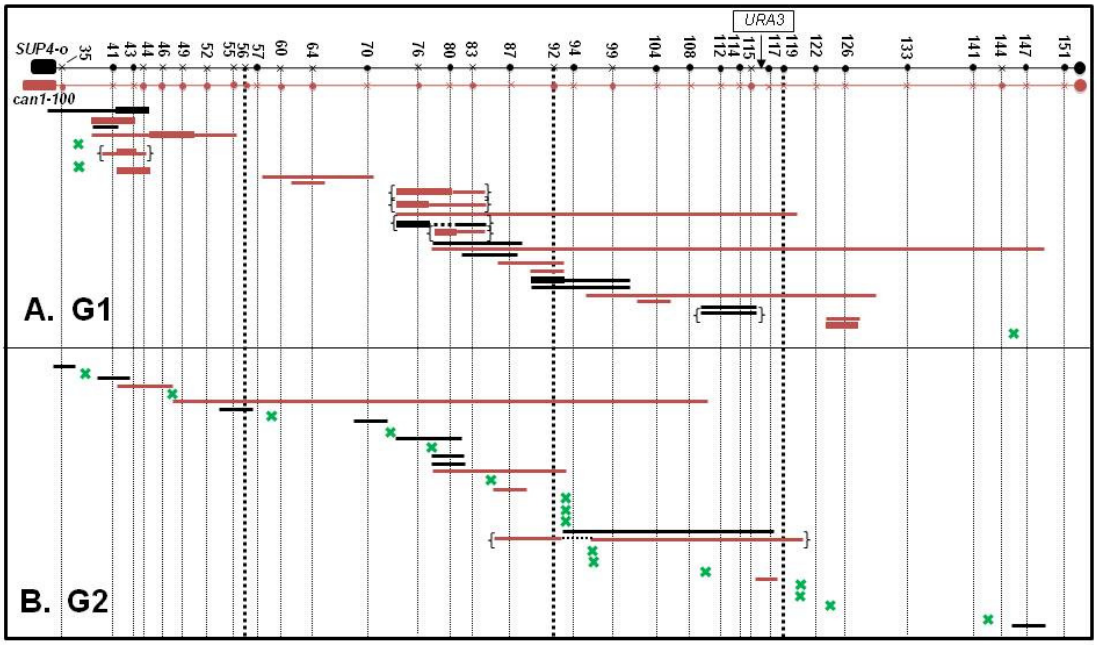


Figure 22 Mapping of RCOs and associated conversions in G1- and G2-irradiated cells

The markers are shown at the top with X's and O's indicating the absence and presence of diagnostic restriction sites, respectively. The thick vertical dotted lines mark the divisions that were used in the analysis of the distribution of recombination events (described in the text). Numbers at the top of the figure are the SGD coordinates of the markers. Conversion events are shown as horizontal lines with 3:1 events, 4:0 events, and 3:1/4:0 hybrid events indicated by thin lines, thick lines, and hybrid thin/thick lines, respectively. The color of the line shows which chromosome was the donor in the conversion event. Brackets indicate complex conversion events and green X's represent RCOs that are not associated with an observable conversion tract. (A). Mapping of 29 RCO events in G1-irradiated cells. (B). Mapping of 31 RCO events in G2-irradiated cells.

We observed two classes of exceptional conversion events (bracketed in Fig. 22). There were five sectored colonies in the G1-irradiated samples involving 3:1 conversion tracts in which the "wrong" sector (as defined in the previous paragraph) was homozygous for the marker (Class 1). There were two sectored colonies in which the gene conversion tract was interrupted by a marker that did not undergo conversion

(Class 2). Mechanisms for these two classes will be presented in the Discussion. Since we have a simple mechanism to explain the Class 1 sectored colonies, we include this class, but not Class 2, in the analyses described below.

Of the simple conversions observed in the G1-irradiated cells, we found fifteen 3:1, three 4:0, and seven 3:1/4:0 hybrid tracts. In the G2-irradiated cells, we observed fourteen 3:1 tracts and no 4:0 or hybrid tracts. These two distributions are significantly different (p=0.02 by Fisher exact test). In our previous study of conversion events associated with spontaneous RCOs in PSL100/101 (Lee *et al*., 2009), we found thirty-three 3:1, six 4:0, and fifteen 3:1/4:0 hybrid tracts. This distribution is not significantly different from that observed in the G1-irradiated cells (p=1 by Fisher exact test), but is significantly different from that observed in the G2-irradiated cells (p=0.01 by Fisher exact test). PSL100/101 are *MATa/MAT*α diploids otherwise isogenic to the *MATa/MAT*$\alpha\Delta$*::NAT* diploid PG311 (Lee *et al*., 2009). We also previously examined a small number of spontaneous events in PG311 and found five 3:1, three 4:0, and four 3:1/4:0 hybrid tracts. This distribution is not significantly different from that observed for the G1-induced events (p=0.40) but is different from that observed for G2-induced events (p=0.001). In summary, the types of gene conversion tracts resulting from G1-irradiated cells are similar to those associated with spontaneous mitotic crossovers and are different from those associated with G2-induced crossovers.

3.2.D Conversion tract sizes in G1- and G2-irradiated cells

Since the lengths of the conversion tracts do not have a symmetrical distribution, we examined the median length of the tracts (95% confidence limits shown in parentheses). We measured the lengths of individual conversion tracts by averaging the

minimum length (the distance between markers included within the tract) and the maximum length (the distance between the closest markers not included in the tract). Since all homologous recombination events are thought to require heteroduplex formation (Aguilera and Rothstein, 2007), it is likely that the RCOs with no detectable associated conversion events reflect short regions of heteroduplex that do not include any markers. Consequently, we estimated the lengths of conversion tracts for the RCO events with no detectable tract by assigning them a length that was the average of the maximum length (the distance between the flanking markers in the interval with the RCO) and the minimum length (1 bp).

The median length of conversion tracts associated with spontaneous RCOs in PSL100/101 was 6.5 kb (95% confidence limits of 5.1-8.6 kb; 5), considerably longer than meiotic conversion tracts (1-5 kb in different studies Lee *et al*., 2009; Judd and Petes, 1988; Borts and Haber, 1989; Mancera *et al*., 2008). The median conversion tract size associated with the G1-irradiated samples was 7.3 kb (5.4-11.4 kb), similar to the size associated with spontaneous crossovers. The median tract size for G2-irradiated cells was 2.7 kb (1.8-3.6 kb), significantly smaller than that associated with spontaneous events or G1-irradiated cells. If mitotic conversion tracts induced in G2 are shorter than those induced in G1, then one would expect that a smaller fraction of RCOs would be associated with conversion in G2-irradiated cells. 26 of the 29 RCOs in G1-irradiated cells were associated with conversion events whereas only 15 of the 31 RCOs in G2-irradiated cells were conversion associated, a significant difference ($p<0.001$ by Fisher exact test).

By the non-parametric Mann-Whitney test, the tracts in the G1 samples were also significantly longer than those of the G2 samples ($p<0.001$), although there was no significant difference in the lengths of the G1 and spontaneous tracts ($p=0.17$). In contrast, by the same test, the G2 tracts were significantly shorter than the spontaneous tracts ($p<0.001$). In summary, the G1-associated conversion tracts were longer than the G2-associated tracts, and G1-associated tracts were similar in size to those associated with spontaneous RCOs. In these comparisons, we included 4:0 and 3:1/4:0 hybrid tracts as single events. We also did the Mann-Whitney test, comparing G1 and G2 conversions, in which each 4:0 and hybrid tracts was counted as two events; the G1 tracts were still significantly longer than the G2 tracts ($p<0.001$).

3.2.E Distribution of gamma-radiation-induced crossovers and conversion events

We previously showed that the distribution of spontaneous conversion events was non-random in the region between *CAN1* and *CEN5* (Lee *et al*., 2009). When this interval was divided into four quandrants (shown as thick dotted lines in Fig. 22), the quadrant near *can1-100/SUP-o* had significantly more events and the quadrant near *CEN5* had significantly fewer events than expected for a random distribution. A similar analysis (chi-square analysis) of the G1- and G2-associated conversion events indicated no significant deviation from a random distribution. This result is not surprising since the distribution of gamma-radiation-induced DNA damage is likely to be random.

3.3 Discussion

Our main conclusion is that DNA damage induced by gamma radiation in G1-arrested yeast cells results in crossovers and gene conversion events that closely mimic the recombination events that occur spontaneously. Recombination events induced by

irradiating G2-arrested cells are significantly different from spontaneous and G1-induced events. These results support our previous conclusion that many spontaneous mitotic recombination events reflect DNA damage occurring in unreplicated chromosomes.

3.3.A Effect of gamma irradiation on cell viability and the frequency of RCOs in diploid cells irradiated in G1 and G2

We found that 50 and 100 Gray (Gy) of gamma radiation had no significant effect on the viability of G2-arrested cells and only a small effect on the viability of G1-arrested cells. In most previous studies, the viability of G2-arrested cells is unaffected by doses of radiation of 100 Gy or less; the viability of G1-arrested cells is usually reduced about two-fold by doses of 100 Gy (20-22). In agreement with these previous studies, gamma rays induced mitotic recombination in both G1- and G2-arrested cells. The level of induction in our study was about 30-fold at the same dose of radiation for both G1- and G2-arrested cells. In analyzing mitotic gene conversion between homologues (heteroallelic recombination), Fabre *et al.* (Fabre *et al*., 1984) and Kadyk and Hartwell (Kadyk and Hartwell, 1992) observed very strong stimulation of conversion in G1-irradiated cells (about 100-fold) and only about 10-fold stimulation of conversion in G2-irradiated cells. These results were interpreted as indicating that sister chromatids were the preferred substrate for repair in G2 cells, reducing the frequency of heteroallelic conversion.

Our conclusion that DSBs generated in G1 and G2 result in RCOs with similar efficiencies is not necessarily in conflict with the previous studies (Fabre *et al*, 1984; Kadyk and Hartwell, 1992). In mitosis, less than 10% of gene conversion events are associated with crossing over (Paques and Haber, 1999). Conversion events

unassociated with crossovers are not detected in our system. One interpretation of our results is that the repair of DSBs leading to RCOs is not preferentially between sister chromatids, unlike the repair of DSBs leading to conversions unassociated with RCOs. Thus, in mitosis, as in meiosis (Allers and Lichten, 2001; Borner *et al.*, 2004), conversions unassociated with crossovers may involve a pathway different from conversions associated with crossovers.

Only a small fraction of DSBs is associated with RCOs. Since a dose of 800 Gy of radiation results in 250 DSBs in a G2-arrested diploid (Argueso *et al.*, 2008), we calculate that about 30% of the G2-arrested diploids treated with 100 Gy would have a DSB in the *can1-CEN5* interval. Because the observed frequency of RCOs in the diploids irradiated in G2 at this dose was only about 4×10^{-4}, we conclude that most (at least 99%) of the DSBs are repaired by a mechanism that does not lead to an RCO (for example, gene conversion without an associated RCO or sister-chromatid crossovers). Although we cannot rule out a low frequency of non-homologous end-joining (NHEJ) repair events, NHEJ is a minor pathway for the repair of radiation-induced DNA damage compared to homologous recombination (Siede *et al.*, 1996).

3.3.B Patterns of gene conversion associated with RCOs

The conversion events in the G1-irradiated cells resemble those previously observed associated with spontaneous RCOs. The 4:0 and 3:1/4:0 events (about 40% of the total) can be simply explained by repair of two DSBs resulting from the replication of a broken chromosome (Fig. 21B and 21C). The 3:1 events in the G1-irradiated cells could be formed in two different ways. First, the 3:1 conversion event could reflect repair of a G1-associated DSB, if the repair of one DSB was associated with conversion of an

adjacent marker, but the repair of the second DSB was not. Alternatively, since gamma rays induce DSBs:single-strand nicks: base damage in a ratio of 1:20:40 (Powell and McMillan, 1990), the 3:1 conversions could reflect a DSB generated by replicating a nicked DNA molecule. Single-stranded nicks are recombinogenic in yeast (Strathern *et al*., 1991). Replication of nicked DNA molecules can give rise to a recombinogenic DSB (Galli and Schiesl, 1999; Cortés-Ledesma and Aguilera, 2006). In addition, nicked or gapped DNA molecules can stimulate conversion directly, without being converted to a DSB (Fabre *et al*., 2002; Lettier *et al*., 2006; Mozlin *et al*., 2008).

All of the simple gene conversions induced by irradiating G2-synchronized cells were 3:1 events. The most straightforward explanation of this result is that irradiation resulted in recombinogenic DSBs in only one of two sister chromatids. Repair of this DSB then resulted in a 3:1 conversion associated with the RCO (Fig. 21A).

3.3.C Complex conversion events

As described in the Section 3.2, we found two classes of complex conversions (bracketed in Fig. 22). The Class 1 events represent conversion in which the “wrong” sector is homozygous for the marker or in which there is a crossover within the conversion tract (Figures 23 and 24). As shown in Fig. 21A, since the conversion event is associated with a crossover, in a 3:1 conversion event in which the “red” alleles are donated, the red sector should be homozygous for the “red” allele and the white sector heterozygous. In the Class 1 event depicted in Fig. 23, for the 3:1 portion of the hybrid tract, the red sector is heterozygous and the white sector is homozygous. This pattern of segregation is explicable as a consequence of the independent repair of two DSBs

(Fig. 23). All of the Class 1 sectored colonies can be explained by similar mechanisms (Lee *et al*., 2009).

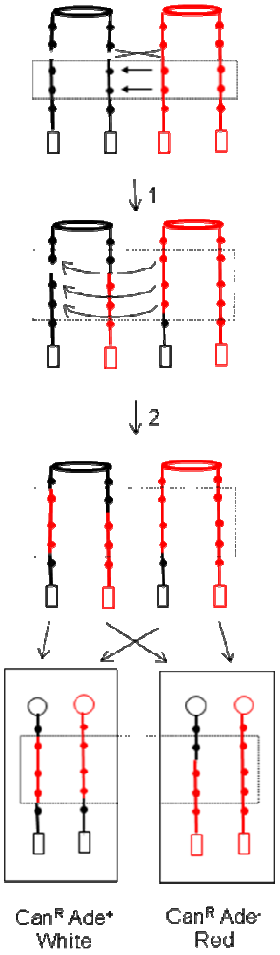


Figure 23 Mechanism for generating a Class 1 complex conversion event

As shown in Fig. 21B, if the event initiates on the *SUP4-o*-containing chromosome, the sector that is homozygous for *SUP4-o* will be heterozygous for the marker and the sector that is homozygous for *can1-100* will be homozygous. In Class 1 complex events, this expectation is violated, since the sector that is homozygous for *SUP4-o* is homozygous for the marker in the 3:1 portion of the hybrid tract. This pattern is readily explained by a double repair event of a G1-associated DSB. Repair of the first DSB is associated with the RCO and conversion of two markers. Repair of the second DSB is unassociated with a crossover but involves conversion of three markers, two on one side of the DSB and one on the other.

In Class 2 events (Fig. 24), the continuity of the gene conversion tract was interrupted by one marker that was not converted. Such a conversion tract could result from a heteroduplex including multiple markers (and, therefore, multiple mismatches) in which one mismatch was repaired in the opposite direction of the other mismatches. Such "patchy" events have been observed previously in studies of mitotic (Nickoloff *et al*., 1999) and meiotic gene conversion (Symington and Petes, 1988).

3.3.D Conversion tract sizes associated with RCOs in synchronized G1 and G2 cells

The conversion tracts in G1-irradiated cells were approximately two times longer than those in G2-irradiated cells. One explanation of this difference is that it reflects the kinetics of processing and repair of DSBs induced in G1 and G2. More specifically, since mitotic recombination in G1 cells is inefficient, the broken ends generated in G1 are likely to undergo extensive processing as cells enter S before recombination is initiated. In contrast, in G2 cells, the time interval between producing the DSB and initiating the recombination event would likely be short, leading to shorter gene conversion tracts.

From previous studies, it is unclear whether the length of conversion tracts has a simple relationship with the amount of resection. In a study of transformed linearized plasmids, mutations resulting in less resection had smaller conversion tracts (Symington *et al*., 2000), whereas, in experiments examining HO-induced recombination, mutations that increased resection did not result in longer conversion tracts (Krishna *et al*., 2007). It should also be noted that 5' to 3' resection of a DNA molecule broken in G1, followed by replication, would result in two chromatids with asymmetric double-stranded gaps. By

this model, the conversion of a G1-associated DSB (gap repair?) might be mechanistically different from conversion events of a G2-associated DSB (mismatch repair in a heteroduplex).

3.3.E Mechanism of spontaneous mitotic reciprocal crossovers

Our analysis supports the model for spontaneous mitotic RCOs shown in Fig. 21B and 21C. We suggest that about half of spontaneous mitotic RCOs initiate by formation of a DSB on an unreplicated chromosome. This estimate is based on the observation that 40% of conversion tracts are 4:0 or 3:1/4:0 hybrid tracts. Since 3:1 conversion tracts can also be generated by a DSB formed in G1, this estimate is a minimum. The DSB could be formed in G1 (as shown) or in an unreplicated portion of a chromosome that has already initiated DNA synthesis. This broken chromosome is then replicated to yield two chromatids broken at the same position.

We suggest that the resulting broken ends are processed independently by either degradation of both DNA strands, producing a gap, or by processing of one strand 5' to 3'. Although, in studies of HO-induced DSBs in yeast, nuclease degradation of only one of the two strands is observed (Paques and Haber, 1999; Mimitou and Symington, 2009), degradation of both strands producing a gap has been recently observed (Zierhut and Diffley, 2008). Depending on the extent of processing the four broken ends, the subsequent repair events can result in 3:1, 4:0 or 3:1/4:0 hybrid conversion tracts associated with the RCO. Alternatively, some fraction of spontaneous RCOs can be generated by a DSB formed in S (possibly as a consequence of a stalled DNA replication fork) and repaired in G2. The conversion events associated with this type of DSB would be expected to be exclusively of the 3:1 type (Fig. 21A). As argued above,

the DNA ends of a G2-associated DSB would be quickly engaged in recombination, subjected to limited nuclease processing, and thus be associated with shorter conversion tracts than for G1-associated events.

There are several points to be mentioned. First, our conclusions are based on only a sub-set of mitotic recombination events, those that give rise to RCOs. Gene conversion events that are not associated with RCOs, sister chromatid exchanges, and non-homologous end-joining events are not detected by our system. Second, we do not know the source of the G1-associated DSBs. These DSBs could reflect unrepaired DNA lesions generated by the action of topoisomerases, excision of closely-spaced damaged bases located on different strands, or other types of DNA damage repair gone awry. Alternatively, a G1-initiated DSB could be mimicked by a chromosome broken in G2 that escaped repair and was segregated into a daughter cell. Since no 4:0 or hybrid conversion tracts are observed in G2-irradiated cells, however, this pathway is probably a minor (or non-existent) one. Finally, since the experiments of Stern in 1936, most geneticists have assumed that mitotic crossovers occur in G2. The model shown in Fig. 21B and 21C is completely consistent with Stern's observations, although not Stern's interpretation of his observations.

3.4 Material and Methods

3.4.A Description of the diploid yeast strain PG311

The construction of the diploid PG311 has been described previously (Lee *et al.*, 2009). The genotype of PG311 is: *MAT*a/*MATα*Δ::*NAT ade2-1/ade2-1 trp1-1/TRP1 ura3-1/URA3 can1-100/can1-*Δ*::SUP4-o gal2/GAL2 ho/ho::hisG*. In addition, PG311 is heterozygous for an insertion of the gene encoding hygromycin resistance centromere-

distal to the *can1-100* allele (*V9229::HYG*) and heterozygous for an insertion of *LEU2* on the right arm of chromosome V (*V261553::LEU2*).

3.4.B Media

Rich growth medium (YPD [yeast extract, peptone, dextrose]) and omission media were made according to standard recipes (Guthrie and Fink, 1991), except that the omission medium contained only 10 μg/ml of adenine. The solid medium used to select for mitotic crossovers lacked arginine (SD-arg) and contained 120 μg/ml canavanine. The solid medium used as the control non-selective medium (SD-arg) contained no canavanine.

3.4.C Synchronization and irradiation of cells in G1 and G2

The PG311 strain was synchronized in G1 using alpha factor and in G2 using nocodazole. Cells were irradiated with a Shepherd Mark 1 Cesium-137 irradiator. The details of the synchronization and irradiation procedures are in the Supporting Information.

3.4.D Genetic and physical analysis of irradiated synchronized cells

We monitored the rate of RCOs by measuring the frequency of Can^R red/white sectored colonies (Lee *et al*., 2009). We purified cells from the red and white sectors and determined whether they were homozygous or heterozygous for the 34 restriction fragment length polymorphisms used to map crossovers and conversions. This analysis involved generating a PCR fragment containing the polymorphism and treating the fragment with the relevant restriction enzyme. Details of the procedure are in Section 3.5 and Lee *et al.* (2009).

3.4.E Statistical analyses

Depending on the nature of the comparison, we used the Fisher exact test, the "goodness" of fit chi-square test, or the Mann-Whitney test on the VassarStats Web Site (http://faculty.vassar.edu/lowry/VassarStats.html). Calculations of confidence intervals of the median were made using Table B11 of Altman (Altman, 1990).

3.5 Supporting Information

3.5.1 Materials and Methods

Synchronization and irradiation of cells in G1

The strain PG311 was grown at 30° in 10 ml liquid YPD medium to OD550 of 0.2 to 0.5. At this density, at least 90% of the cells were budded. To synchronize cells in G1, we harvested cells by centrifugation and washed these cells twice with water. The resulting pellet was resuspended in 10 ml of YPD medium (pH reduced to 3.5 by addition of hydrochloric acid) containing 60 µl of a stock solution of (5mg/ml α factor [Sigma-Aldrich] in dimethyl sulfoxide [DMSO]). The cells were incubated at 30° with vigorous shaking for two hours. The cells were briefly sonicated and examined for their morphology; more than 90% were unbudded. The G1-arrested cells were then collected and resuspended in 10 ml ice-cold water with the same concentration of α factor as used in the initial treatment.

Gamma irradiation of the synchronized cells was done using the Shepherd Mark 1 Cesium-137 irradiator. For most experiments, we used 5 or 10 krads of radiation. For each experiment, 2 ml were removed from the synchronized cells before radiation and 2 ml were removed for each subsequent irradiated sample. The irradiator produced about

0.5 krads of gamma rays per minute. After treatment, the cell suspensions were kept on ice until plating.

Synchronization and irradiation of cells in G2

Exponentially-growing PG311 cells were grown to OD_{660} of 0.2 to 0.5 in 10 ml of YPD. We added 20 μl of a stock solution containing 20mg/ml nocodazole (Sigma-Aldrich) in DMSO. The cells were then incubated with constant shaking for two hours at 30^{o}. The cells were then sonicated and their morphology examined. More than 95% of cells were arrested as doublets, with the mother cell and the bud of approximately the same size. The arrested cells were then resuspended in 10 ml of ice-cold water containing nocodazole at the same concentration as that used in the initial arrest. We irradiated the G2 cells by the same procedure described above for the G1 cells.

Genetic and physical analysis of irradiated synchronized cells

The procedures to measure the frequency of reciprocal crossovers in irradiated samples and to analyze the products of reciprocal crossing over (RCO) were identical to those used previously for unirradiated samples (Lee *et al*., 2009). As described elsewhere, the frequency of RCO is monitored by the frequency of canavanine-resistant red/white sectored colonies. Thus, we plated approximately 10^5-10^6 cells of each sample on SD-arg plates containing canavanine. The plates were incubated at room temperature for four days, and then incubated at 4^0 for one day. This procedure minimizes the background growth of Can^S cells and maximizes the red color of the sectored colonies. We then counted sectored Can^R colonies in which the smallest sector was at least one-eighth of the total colony size. To monitor the total number of viable cells in the samples, we also plated dilutions of the irradiated cells on SD-arg

plates lacking canavanine. The resulting colonies were also counted after three days of growth at 30^0.

The red and white sectors of each sectored Can^R were re-streaked on rich growth medium and single colonies were isolated for further analysis. As described previously (Lee *et al*., 2009), DNA was purified from the isolates and each heterozygous restriction site polymorphism was examined by using PCR to amplify a DNA fragment of about 500 bp containing the site. Subsequent treatment of this fragment with a diagnostic restriction enzyme, followed by gel electrophoresis, allowed us to determine whether the cells of each sector were homozygous for the allele lacking the restriction site, homozygous for the allele containing the restriction site, or heterozygous for the two alleles. The PCR primers and restriction enzymes used for this analysis are described by Lee *et al.* (2009).

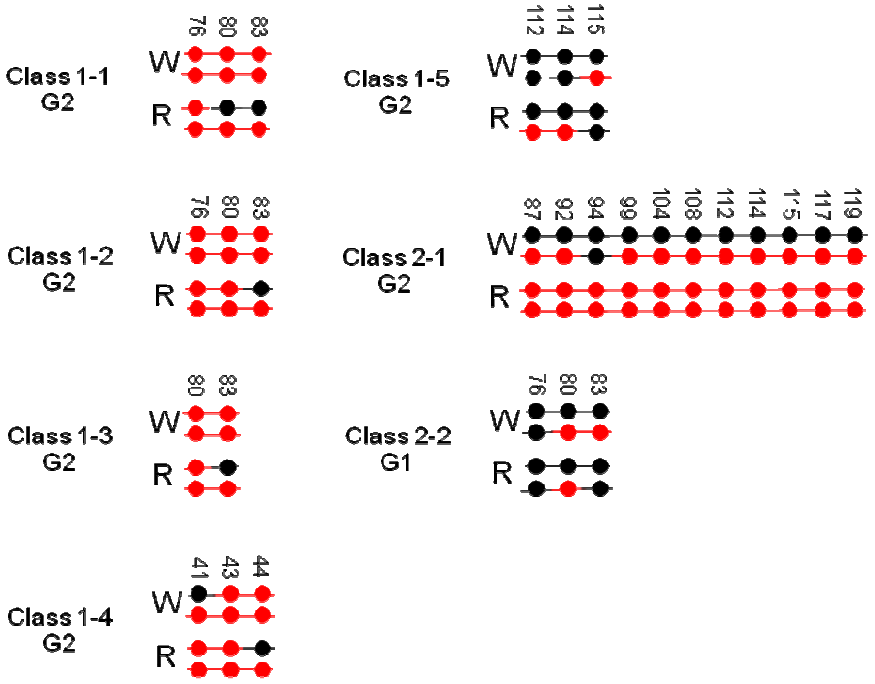


Figure 24 Patterns of marker segregation in sectored colonies with complex gene conversion events

In this figure, we show the segregation of markers for complex gene conversion events. The red and black circles indicate markers originally derived from the *can1-100*-containing homologue and the *SUP4-o*-containing homologue, respectively. Each line of circles represents the markers on one homologue in the sectored colony. The pairs of circles reflect the homologues in the white (W) and red (R) sectors. The numbers above the circles show the approximate SGD coordinates in thousands. Only those markers exhibiting gene conversion are shown in this figure. Markers that are centromere-proximal to those shown in the figure are heterozygous in both sectors, and the markers that are centromere-distal are homozygous. For Class 1 3:1 conversion events, the “wrong” sector is homozygous for the marker (discussed in detail in the main text) or there is a crossover within the conversion tract. For Class 2 events, the conversion tract is interrupted by a marker that segregates 2:2.

4. Discussion

The initial focus of this thesis was to generate the first fine-resolution map of spontaneous reciprocal crossovers (RCOs) in *S. cerevisiae*. In the process of generating this map, I made several important observations: 1) mitotic gene conversion tracts were considerably longer than those observed in meiotic cells, 2) about half of the spontaneous mitotic gene conversion tracts had properties expected for the repair of a DSB in unreplicated chromosomal DNA, 3) gamma irradiation of G1-arrested cells resulted in conversion events that mimicked spontaneous events, whereas irradiation of G2-arrested cells resulted in conversion tracts different than spontaneous events, 4) most DSBs are repaired by mechanisms that do not produce an RCO, and 5) spontaneous mitotic recombination events are distributed non-randomly in the 120 kb interval between *CEN5* and *can1-100*/*SUP4-o*. I will divide the discussion of these findings and possible extensions of my results in the following order: 4.1 Spontaneous and induced gene conversions, 4.2 Relationship between mitotic gene conversion and mitotic crossovers, 4.3 Hotspots and coldspots of mitotic recombination, and 4.4 Summary of findings.

4.1 Spontaneous and induced gene conversions

In this section of the Discussion, I will describe the nature of the DNA lesion that initiates gene conversion and when this lesion is produced during the cell cycle. I will also discuss mechanisms for the production of the long continuous gene conversion tracts associated with spontaneous and G1-induced RCOs.

As discussed in Chapter 1, mitotic recombination can be induced by DSBs or by single-strand DNA nicks, although the lesion that initiates spontaneous mitotic

recombination events is not clear. My analysis of spontaneous events and gamma ray-induced events in synchronized cells strongly argues that DSBs are one of the lesions responsible for spontaneous reciprocal crossovers. In Chapter 2, I describe the evidence that about 40% of the gene conversion events associated with RCOs are 4:0 events or hybrid 3:1/4:0 events. The simplest explanation of these conversion events is that they represent the repair of two broken chromatids resulting from a DSB in an unreplicated chromosome. The frequency of the 4:0 and 3:1/4:0 hybrid conversion events was much too high to represent two independent events, and two independent events would not be expected to involve conversion of the same region of the 120 kb *CEN5-can1-100*/*SUP4-o* interval.

About 60% of the spontaneous RCOs are associated with 3:1 tracts (Chapter 2). These tracts could reflect a DSB formed in S or G2, or could also be a consequence of a G1-initiated event. One argument in favor of the second possibility is that about half of the conversion events associated with RCOs induced by gamma rays in G1-synchronized cells were 3:1 events (Chapter 3). Assuming that the recombinogenic lesion resulting from gamma rays is a DSB, this observation argues that 3:1 conversion events can be a consequence of a DSB in unreplicated DNA. A simple explanation for this result is that the two broken chromatids resulting from replication of the broken chromosome are repaired somewhat differently. One broken chromatid is repaired associated with a conversion tract that does not include a heterozygous marker, whereas the repair of the second broken chromatid is repaired associated with a longer conversion tract that includes a marker. The net result of these events would be a 3:1 conversion.

I found that the gene conversion tracts associated with spontaneous RCOs and G1-induced RCOs were considerably longer than meiotic conversion tracts. In addition, the conversion tracts associated with spontaneous RCOs and G1-induced RCOs were longer than those associated with G2-induced RCOs. Below, I discuss some explanations for these observations and additional experiments that could be performed to investigate these issues.

One explanation for the long conversion tracts associated with spontaneous RCOs is that these conversion events are mechanistically different from those observed in meiosis. It is clear that most meiotic recombination events are a consequence of heteroduplex formation followed by repair of mismatches within the heteroduplex (Petes *et al*., 1991; Paques and Haber, 1999). In a relatively small number of studies involving HO-induced DSBs (Clikeman *et al*., 2001) or plasmid-chromosome recombination (S. Jinks-Robertson, personal communication), evidence that some mitotic recombination events involve mismatch repair in heteroduplexes has been obtained. The conversion events examined in these studies, however, had relatively short tracts. One alternative pathway of gene conversion is gap repair. In this pathway (Orr-Weaver and Szostak, 1983; Szostak *et al*., 1983), gene conversion is a consequence of the repair of a double-stranded DNA gap. The main distinguishing feature of these two types of conversion is the effect of mutations in the mismatch repair (MMR) pathway on the events. If conversion is a consequence of gap repair, then the lack of MMR will have little effect on the events. If conversion is a consequence of the repair of mismatches within heteroduplexes, in an MMR mutant, for the markers included within the heteroduplex,

one would expect to find two different classes of cells within a sector as shown in Figure 25.

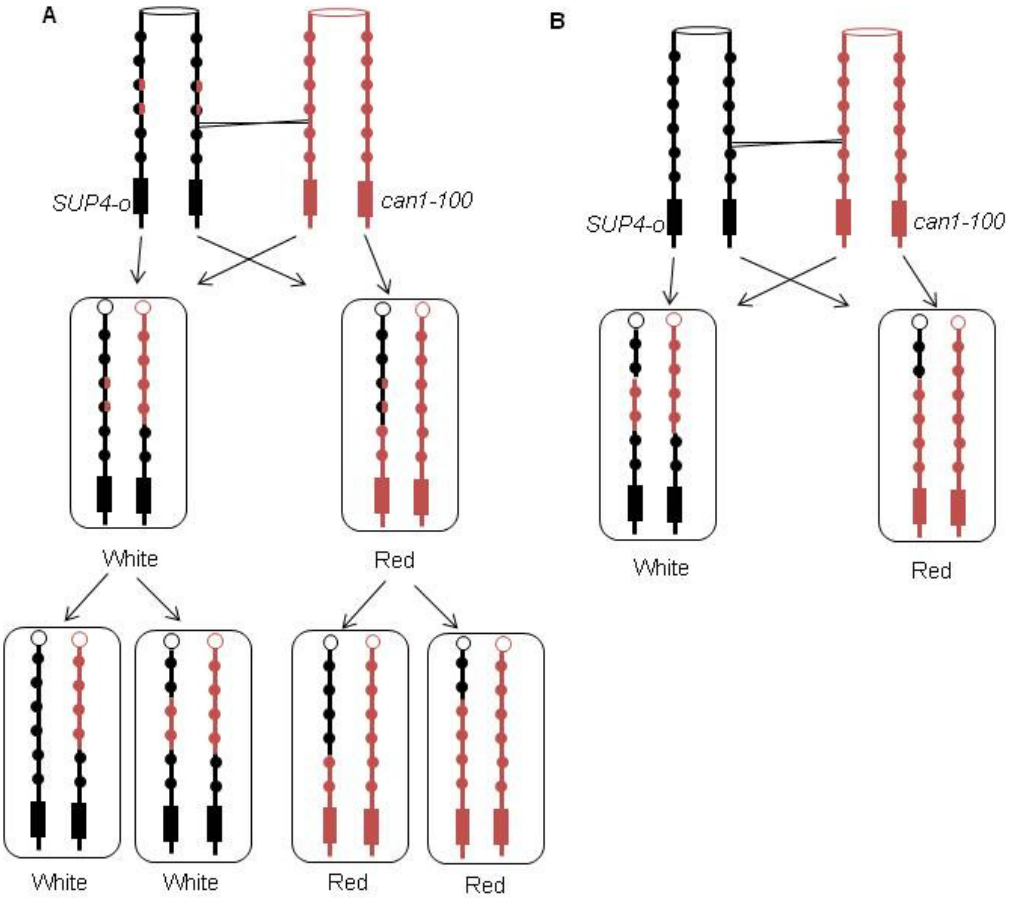


Figure 25 Expected pattern of marker segregation associated with gene conversion in a MMR-deficient strain.

In this figure, I show that patterns expected as a consequence of DSB in G1 on the *SUP4-o* containing chromosome. (A) Pattern of marker segregation if conversion occurs by mismatch repair in a heteroduplex. The mismatch is shown by circles that are half red and half black. In the absence of MMR, both daughter cells containing the products of the RCO will have a chromatid with an unrepaired mismatch. At the first mitotic division of these daughter cells, DNA replication will produce cells of two different genotypes within each sector. (B) Pattern of marker segregation expected from gap repair. If conversion occurs by gap repair, then each sector will have a single genotype.

To analyze the effects of MMR on mitotic conversion, I will first construct a diploid with polymorphisms (W303 x YJM789 cross) that is homozygous for the *msh2* mutation. I will then select for RCOs on plates containing canavanine as done previously (Lee *et al*., 2009). In these previous experiments, I purify and analyze a single colony from the red and white sectors. For the experiments in the *msh2* strain, I will isolate 10 colonies from the two types of sectors. I will initially map the position of the RCO using a single colony from each sector. Once I have identified the region of recombination, I will examine the markers in this region in all 10 colonies from each sector by the standard procedure (PCR followed by restriction digest). If the RCOs are associated with heteroduplexes, I should frequently observe two different types of cells within one or both sectors; these events are the mitotic equivalent of post-meiotic segregation events (Fig. 25). If 4:0 conversion events reflect the repair of mismatches in two heteroduplexes, I expect to find two different cell types in both sectors. If all 10 colonies in the white sectors are identical and all 10 colonies in the red sectors are identical, it would argue that the gene conversion events do not involve the repair of mismatches within a heteroduplex, and probably are a consequence of gap repair. One possibility is that the 4:0 events reflect gap repair whereas the 3:1 events reflect mismatch repair in a heteroduplex. The proposed experiment should clarify this issue.

If the experiments described above indicate that all of the mitotic gene conversion events are a consequence of repair of mismatches in a heteroduplex, the next question that could be addressed is why the spontaneous and G1-induced conversions are longer than those associated with G2-induced crossovers. In Chapter 3, I suggested that broken ends resulting from a G1-induced DSB might have longer

single-stranded “tails” than those resulting from a G2-induced DSB as a consequence of a longer exposure to cellular exonucleases. I propose to test this possibility by inducing DSBs with gamma rays in cells synchronized in three different positions in the cell cycle: Start (cells arrested with alpha factor), the beginning of the S-period (cells arrested at the *cdc7* block), and G2/M (cells arrested with nocodazole). Following irradiation, some of the cells will be immediately transferred to medium containing canavanine, whereas others will be maintained at the block for various times up to 6 hours before being transferred to the canavanine plates. The resulting Can^R sectored colonies will be analyzed by the usual procedures.

Ira *et al*. (2004) found that the resection of broken ends was very slow or absent in cells arrested at Start, but active at the Cdc7-dependent block. Thus, I expect that incubation of gamma-irradiated cells arrested for various times at Start will not alter the lengths of conversion tracts. However, cells arrested at the Cdc7 block or in nocodazole following gamma irradiation may have longer conversion tracts. For these experiments, I will have to construct a diploid with a temperature-sensitive or an analogue-sensitive allele of *CDC7* (Ira *et al*., 2004).

I will also examine the regulation of conversion tract length using another approach. The Rad54p influences the formation and extension of heteroduplexes during mitotic recombination (Bugreev *et al*., 2006); therefore, I propose to study the length of gene conversions associated with G1 and G2 gamma irradiation–induced crossovers in a *rad54-Δ* yeast strain. If G1-induced events involve gap repair and G2-induced events involve extensive heteroduplex formation, I expect that the *rad54* mutation might have

little effect on the length of G1-induced events, but reduce the length of G2-induced gene conversion tracts.

4.2 Relationship between mitotic gene conversion and mitotic crossovers

The system that I have used in my thesis involved selection of RCOs and we then mapped the position of RCOs and associated conversions. This system, however, was not designed to detect other types of mitotic recombination events such as conversions that were unassociated with crossovers, BIR events, or sister-chromatid recombination. From calculations of the number of DSBs induced by gamma rays and the number of induced RCOs (Chapter 3), I estimated that only about 1% of DSBs result in a crossover. In this section of Chapter 4, I discuss an alternative method of analyzing genetic events associated with the repair of a DSB. I then discuss the genetic regulation of the fraction of conversions that are associated with a crossover.

4.2.A System to examine gene conversion and associated crossovers

The site-specific endonuclease HO has been extensively used by Haber and Nickoloff labs (reviewed by Paques and Haber, 1999) to examine various aspects of DNA repair and recombination in yeast. The HO cut site can be inserted into an appropriate target locus and the expression of the HO endonuclease can be regulated by the galactose-inducible *GAL1,10* promoter (Clikeman *et al.*, 2001). I will propose an experiment to determine what fraction of DSBs induced in G1 are associated with gene conversions and crossovers.

The starting strain for the construction is the diploid PG311 that has the *can1-100*/*SUP4-o* system, the single-nucleotide polymorphisms allowing mapping of events, and the *MATa*/*MATα*::*NAT* genotype (allowing synchronization of the diploid using the

alpha pheromone). In addition, the strain will be heterozygous for an insertion of the HO-endonuclease cut site within the *URA3* gene (*ura3*::*HO* site/*URA3*), and HO-cut sites at other positions in the genome will be removed. The *URA3* gene is located between *CEN5* and the *can1-100*/*SUP4-o* markers. Finally, the strain will contain a copy of *HO* gene fused to the *GAL1*,*10* promoter (Fig. 26).

For the experiment, I will arrest the cells in G1 using the alpha pheromone and then incubate the cells in medium containing the pheromone and high levels of galactose in order to stimulate DSB formation at the *ura3*::*HO* site allele. I will monitor the efficiency of DSB formation by Southern analysis. Assuming that DSB formation is efficient, I will then transfer the cells onto YPD plates and allow them to form colonies. The colonies will be replica-plated to medium lacking uracil, to medium containing 5-fluoro-orotate, and to canavanine-containing medium. If DSB formation occurs on the chromosome with the *ura3*::HO site allele, and the broken chromosome replicates, I expect to detect either conversion events associated with an RCO or conversion events unassociated with an RCO (Figure 26). If the conversion event is associated with an RCO, the colony will be Ura^+ and $5\text{-}FOA^S$ with no $5\text{-}FOA^R$ papillae; in strains that are homozygous for a wild-type *URA3* gene, no $5\text{-}FOA^R$ papillae will be observed. In addition, on the plates containing canavanine, the colony will be sectored (red/white) and Can^R. If the conversion event is unassociated with an RCO, I will also find a colony that is Ura^+ and $5\text{-}FOA^S$ without $5\text{-}FOA^R$ papillae, but the colony will be sensitive to canavanine. If the cell did not undergo a DSB, I would expect to find a canavanine-sensitive, Ura^+, and $5\text{-}FOA^S$ colony with $5\text{-}FOA^R$ papillae. In diploids with the *ura3*/*URA3* genotype, $5\text{-}FOA^R$ papillae arise as a consequence of loss of the wild-type *URA3* allele

by chromosome loss, mutation, or secondary recombination events. In summary, this experiment will reveal the fraction of G1-induced gene conversion events that are associated with RCOs.

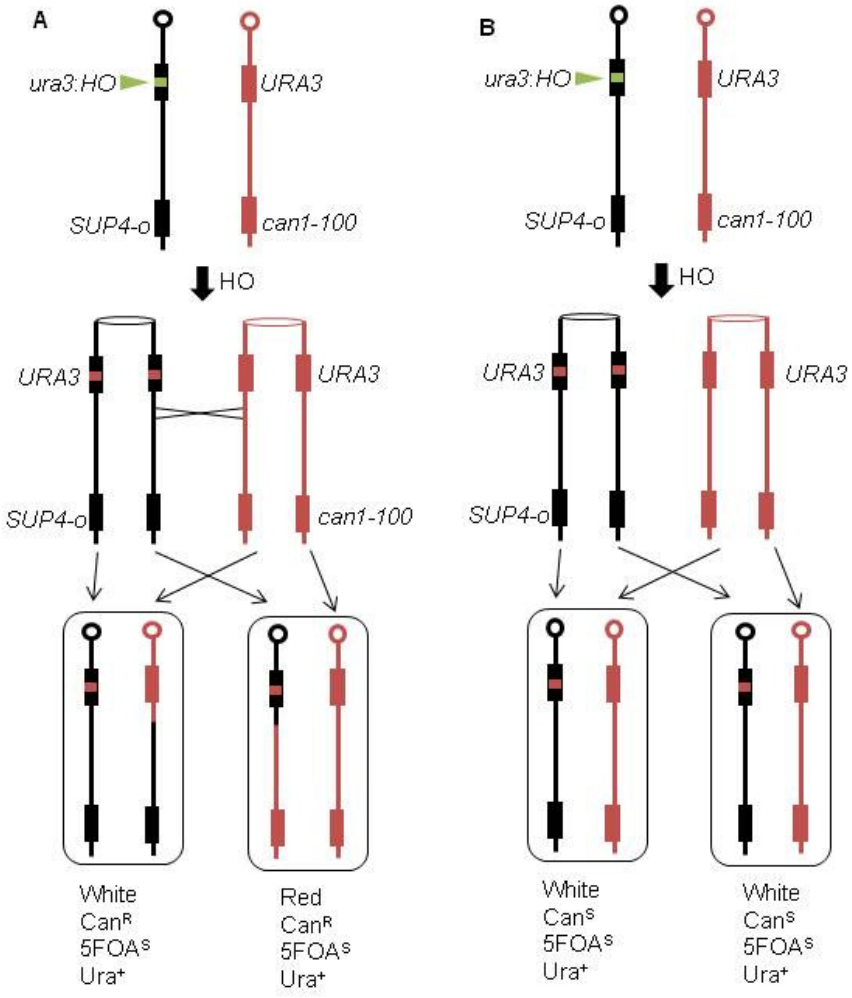


Figure 26 Diploid designed to detect both conversion events associated with RCOs and conversion events that are unassociated with RCOs

The diploid has one wild-type *URA3* gene and one mutant gene resulting from the insertion of a cut site for the HO endonuclease into the middle of the gene. The strain also has the previously described *can1-100/SUP4-o* sequences. The diploid will be synchronized in G1 and the HO endonuclease will be expressed. When the broken chromosome is replicated, both broken *ura3* genes will be repaired using the wild-type *URA3* gene as a template. If there is an RCO associated with this conversion, a canavanine-resistant red/white sectored colony will be observed (A). If there is no associated RCO, the colony will be canavanine-sensitive (B). Colonies that have undergone a conversion without an associated crossover will be 5-FOA^S and will not have 5-FOA^R papillae. Colonies that are identical to the starting strain will also be FOA^S, but will have 5-FOA^R papillae.

4.2 B Genetic regulation of the fraction of gene conversion events associated with crossovers

Three proteins that are known to affect the frequency of crossovers are the anti-recombination proteins Srs2, Sgs1, and Mph1. The Srs2p was identified as a DNA helicase with 3'–5' polarity with mutant strains exhibiting elevated levels of gene conversion in response to DNA damage (Aboussekhra *et al*., 1989; Rong *et al*., 1991, Rong *et al*., 1993). Genetic and biochemical studies show that Srs2 negatively modulates recombination by disrupting Rad51p–DNA filament formation; this disruption directs all DSB-repair intermediates into the SDSA pathway, resulting in gene conversion rather than crossover repair products (Aguilera and Klein, 1988; Chan *et al*., 1995; Ira *et al*., 2003; Kaytor *et al*., 1995; Krejci *et al*., 2003; Milne *et al*., 1995; Schild 1995; Veaute *et al*., 2003). Sgs1p, acting with the Top3 and Rmi1 proteins, disrupts double Holliday junctions, and suppresses crossovers (Tay *et al*., 2010). *MPH1* encodes an ATP-dependent 3'–5' DNA helicase (Prakash *et al*., 2005) that participates in the homologous recombination/repair of DSBs. The Mph1p is recruited to the DSB site, and its helicase activity promotes gene conversion by unwinding the D-loop intermediate independently of Srs2p, leading to suppression of crossovers (Prakash *et al*., 2009). In addition, the Mph1p is involved in a second pathway involving MMR enzymes that stimulates gene conversion over crossovers (Tay *et al*., 2010).

I will construct strains homozygous for mutations in *SRS2*, *SGS1*, or *MPH1* in the background described in Section 4.2.A. I will then examine the frequency of gene conversion and crossovers for the mutant strains. I expect to find that all three strains

will have relatively more crossovers associated with conversions compared to the wild-type strain. Since these proteins act in somewhat different pathways, one could also examine conversion and crossing over in various double mutants and the triple mutant.

4.3 Hotspots and coldspots of mitotic recombination

In my analysis of spontaneous RCOs and associated conversion tracts, I observed a non-random distribution, although there were no very striking hotspots or coldspots. The 30 kb region near the centromere had fewer conversion events than expected for a random distribution and the interval between SGD coordinates 43075 and 44403 was significantly "hot". We were unable to identify a unique feature of this region likely to account for the elevated frequency of recombination. First, we examined whether the region included or was next to genes with high transcription rates, as high rates of transcription stimulate mitotic recombination (Bratty *et al.*, 1996; Nevo-Caspi and Kupiec, 1994). However, neither *SOM1* nor *PCM1*, the two genes closest to the mitotic recombination hotspots, are highly transcribed. The region was also devoid of simple repetitive tracts, replication origins, or other obvious structural elements likely to stimulate DSB formation. Below, I briefly discuss two approaches to further characterize sequences or structures that affect mitotic recombination.

One obvious approach is to map crossovers in a larger chromosomal region. The 120 kb interval that I examined represents only about 1% of the yeast genome. One could move the *can1-100*/*SUP4-o* system to examine RCOs on the right arms of chromosome IV and/or chromosome XII. Each of these arms represents about 10% of the yeast genome. Examining the frequencies of recombination in a much larger interval might allow detection of common elements for hotspots and coldspots. One technical

issue is how to map crossovers and conversion events in very large intervals to high resolution. For the 120 kb interval, I mapped each of the 34 heterozygous markers individually using PCR and restriction digests. To map recombination events in an interval that is ten times larger by this method would be extremely time-consuming and expensive. Consequently, a better method of mapping would be to design an oligonucleotide-based microarray that can distinguish polymorphisms of the two parental haploids, YJM789 and W303a. This method was used by Mancera *et al*. (2009) to map meiotic recombination events.

Once hotspots and coldspots have been mapped in large chromosome intervals, one interesting comparison would be to correlate recombination levels with cohesin binding. Cohesin binding has been mapped throughout the yeast genome by Glynn *et al*. (2004). If any interesting correlation, positive or negative, is observed, it would be important to examine the effects of mutants that decrease cohesin binding on the frequency and pattern of reciprocal crossovers.

A second approach is to introduce putative recombinogenic sequences into the *CEN5-can1-100*/*SUP4-o* interval. I would then determine whether the insertion stimulates the frequency of sectored colonies. If increased levels of RCOs were observed, I could map them to determine if the crossovers and conversion events were adjacent to the insertion. Some sequences that could be tested are certain trinucleotide repeats (for example, CAG/CTG and CCG/CGG), “hairpin” structures, and genes with inducible levels of transcription. For the last class of experiment, I could use a gene with the *GAL1*,*10* promoter and examine the effects of the insertion in high and low levels of galactose, resulting in high and low levels of transcription, respectively.

4.4. Summary

At the beginning of this thesis research, I set out to study the properties of spontaneous mitotic reciprocal crossovers in yeast, including the location of crossovers and the lengths of gene conversion tracts associated with crossovers. From my analysis, I showed that mitotic recombination events were distributed fairly evenly in the 120 kb interval studied, although I detected one hotspot and showed that the centromeric region was cold for exchange. I also showed that mitotic gene conversion tracts were considerably larger than meiotic conversion tracts and found classes of gene conversion events (4:0 and hybrid 3:1/4:0) that are likely to reflect a DSB in an unreplicated chromosome. I confirmed this conclusion by examining crossovers and gene conversions induced by gamma radiation in G1- and G2-arrested cells. The work in this thesis changes our understanding about the mechanism of spontaneous mitotic recombination in yeast.

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Biography

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