SELECTION AND ANALYSIS OF MITOTIC CROSSOVERS IN *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

MARIA ANTONIA BARBERA: Selection and Analysis of Mitotic Crossovers in *Saccharomyces cerevisiae*
(Under the direction of Thomas D. Petes)

Mitotic recombination is an important mechanism of DNA repair in eukaryotic cells. I have developed a novel system that allows the selection of the reciprocal products resulting from spontaneous mitotic crossovers in the yeast *Saccharomyces cerevisiae*. A number of other types of genetic events, including chromosome loss, can be monitored with this system. For a 120 kb chromosome interval on chromosome V (*CEN5* - *CAN1*), the rate of mitotic crossovers was 4×10^{-5} per division, a rate approximately 25,000-fold lower than the meiotic rate of crossovers. We found no suppression of mitotic crossovers near the centromere of chromosome V, unlike the suppression observed for meiotic exchanges. A tract of trinucleotide repeats, $(CTG)_{115}$, did not stimulate mitotic recombination or chromosome loss. The rate of reciprocal crossovers was substantially (38-fold) elevated by treatment of cells with hydroxyurea, a drug that reduces nucleotide pools and slows DNA replication. When cells were irradiated with 20 J/m^2 UV-light, a dose that results in 90% survival of the cells, mitotic crossovers were stimulated 175-fold. The effect of the mating-type alleles on spontaneous and induced recombination was also examined. Although the analysis with this system was limited to one genetic interval on chromosome V, the same approach can be extended to any region of the yeast genome.

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CHAPTER I

GENERAL INTRODUCTION

In this chapter I will describe the introductory information in the following four sections: A) overview of the mitotic and meiotic cell cycle, B) review of meiotic recombination, C) review of mitotic recombination, and D) mechanisms of loss of heterozygosity and cancer.

A. Overview of the mitotic and meiotic cell cycle.

Cells divide and give rise to new cells. The different stages a cell passes through from one cell division to the next constitute the cell cycle. The cell cycle can be divided into two major phases: interphase, which consists of three stages (G_1, S, and G_2), and mitotic phase (M). During G_1, the cell grows and prepares for DNA replication. In the S stage, the DNA of each chromosome is replicated and the product of chromosome duplication is two exact copies, called sister chromatids, which are held together by the replicated but unseparated centromere. In G_2, the cell prepares for cell division or M phase. The mitotic phase includes (1) the process of mitosis, comprised of four cytologically distinguishable stages called *prophase, metaphase, anaphase,*and *telophase,* during which duplicated chromosomes are separated into two nuclei, and (2) cytokinesis, where the entire cell divides into two daughter cells. The result of the mitotic cell cycle is the production of daughter cells

that contain identical chromosome number and that are genetically identical to the mother cell from which they arose.

Meiosis is a specialized cell cycle that enables diploid organisms to reproduce sexually through one round of DNA synthesis followed by two successive divisions. At meiosis I, the reductional division, homologous chromosomes disjoin from each other, and at meiosis II, the equational division, sister chromatids separate and move to opposite poles. The net result of this process is the formation of four haploid products.

Once meiosis is initiated, DNA replication occurs. Premeiotic DNA synthesis is followed by a lengthy prophase where homologous chromosomes pair, synapse and recombine. Prophase I is divided into five stages: *leptotene, zygotene, pachytene, diplotene and diakinesis.* The first stage of prophase I is *leptotene*, during which pairing (loose alignment of homologous chromosomes along their length) occurs. DNA double-strand break (DSB) formation, the initiating lesion of meiotic recombination, occurs at this step (Padmore *et al.*, 1991; Goyon and Lichten, 1993). A key event in *zygotene* is synapsis, defined as the intimate association of homologous chromosomes in the context of the synaptonemal complex (SC) (von Wettstein *et al.*, 1984). The next stage of prophase I, called *pachytene,* is characterized by a fully formed SC. It is during this stage that high levels of recombination occur. In the *diplotene* stage, the chromosomes begin to move apart and the result of crossing-over becomes evident as "chiasmata". During the final stage of meiotic prophase I, *diakinesis,* shortening and thickening of paired chromosomes occurs and the meiotic spindle is formed. In metaphase I, the bivalents become aligned on the equatorial plane of the cell. In anaphase I, the homologues disjoin and migrate toward opposite poles,

and in telophase I, the homologues complete their migration to opposite poles of the cell. The end of meiosis I results in two daughter cells with replicated chromosomes. The second meiotic division, meiosis II, is very similar to a mitotic division.

Two significant events distinguish mitosis from meiosis: chromosome pairing and high levels of recombination. These two processes play an essential role ensuring the proper segregation of the chromosomes during meiosis I. It has been shown in several organisms, including *Drosophila melanogaster* (Parry, 1973; Carpenter and Sandler, 1974) and *Saccharomyces cerevisiae* (Engebrecht and Roeder, 1990; Rockmill and Roeder, 1988) that mutations that affect pairing and recombination in meiosis lead to missegregation of the chromosomes. In addition, meiotic recombination has been interpreted as a way of generating genetic variability from one generation to the next.

B. Review of meiotic recombination.

1. Genetic recombination models. Several models have been proposed to explain the molecular mechanisms of recombination with two of them being the most consistent with the available genetic data. One of the models is the double-strand break repair model (DSBR) proposed by Szostak in 1983 (Szostak *et al.*, 1983) and revised in 1991 (Sun *et al.*, 1991). In this model, meiotic recombination initiates with the formation of a DNA double-strand break, catalyzed by the enzyme Spo11 (Keeney *et al.*, 1997). The 5' ends of the break are resected by an unknown 5'-3' exonuclease activity to create long 3'-overhanging single-stranded tails. One of these tails invades a homologous duplex, displacing a region of single-stranded DNA referred to as a D-loop. The displaced sequence is used as a template for the

repair of the DSB. Ligation of the newly synthesized products creates a joint molecule connected by double Holliday junctions. Resolution of the Holliday junctions can generate crossover or non-crossover configuration of flanking markers (Figure 1.1). Another resolution mechanism (also shown in Figure 1.1) has been proposed in which the extended single-strand end disassociates and reanneals with its original partner followed by ligation, yielding non-crossover products (synthesis-dependent strand annealing model, SDSA) (Allers and Lichten, 2001; Hunter and Kleckner, 2001).

2. Measures of meiotic recombination in *Saccharomyces cerevisiae*. In the yeast *Saccharomyces cerevisiae*, the four meiotic products (spores) are packaged together in a single tetrad allowing for all the products of a single meiosis to be examined. There are two commonly used genetic measures of meiotic recombination in yeast. One of them is to compare the level of reciprocal recombination (crossovers) between two heterozygous markers for a region of known physical length. Alternatively, the level of non-reciprocal recombination (gene conversion) for heterozygous single markers can be monitored. In a diploid heterozygous for alleles *A* and *a*, Mendelian segregation results in a pattern of 2*A*:2*a* spores. Single gene conversions result in tetrads with 3*A*:1*a* or 1*A*:3*a* spores. Another measure of the frequency of meiotic recombination is the level of meiosis-specific DSBs. DSBs are the initiating lesion of meiotic recombination (Szostak *et al.*, 1983). All known recombination hotspots in yeast are associated with DSBs (Lichten and Goldman, 1995), and the levels of local DSBs are well correlated with gene conversion frequencies (Fan *et al.*, 1995; Sun *et al.*, 1989; de Massy and Nicolas, 1993).

3. Meiotic recombination hotspots and coldspots. Meiotic recombination events are distributed nonrandomly throughout the genome with some regions having higher levels of recombination than others. Hotspots are genomic regions with unusually high levels of recombination (Lichten and Goldman, 1995; Petes, 2001), and coldspots are regions that display a lower than average frequency of recombination. Global mapping of meiotic recombination hotspots and coldspots in the yeast *Saccharomyces cerevisiae*, done using DNA microarrays to measure DSB frequencies, showed that coldspots were nonrandomly associated with the centromere and telomeres (Gerton *et al.*, 2000). This observation was consistent with genetic and physical studies indicating suppression of meiotic recombination near the centromere of yeast chromosome III (Baudat and Nicolas, 1997; Lambie and Roeder, 1988).

There is no unique DNA sequence, structure or specific characteristic that can be associated with all recombination hotspots in yeast. Some hotspot loci require the binding of specific transcription factors for hotspot activity (α hotspots). Bas1, Bas2, Gcn1 and Rap1 are transcription factors that bind to the upstream region of *HIS4*. White *et al.* (White *et al.*, 1993) showed, by deleting the transcription factor binding sites or the genes encoding the transcription factors, that Bas1, Bas2, and Rap1 were required for hotspot activity. Other hotspots seem to be created by nucleosome-excluding sequences (β hotspots) (Kirkpatrick *et al.*, 1999). Lastly, some hotspot loci are associated with local regions of high G + C base composition (γ hotspots) (Gerton *et al.*, 2000). Although it is clear that hotspot activity is related to chromatin structure, the mechanistic details of this relationship are unclear at present.

C. Review of mitotic recombination.

Recombination is usually thought of as meiotic phenomena, but these processes also take place in vegetative growth although at much lower frequency (Smith and Nicolas, 1998). Below I summarize some of the previous observations of mitotic recombination, including the first description of mitotic crossing-over.

1. First description of mitotic crossing-over. In the 1930s, Calvin Bridges was studying *Drosophila* females that were genotypically M^+/M (M is a dominant allele that causes slender bristles) and noticed that some of the females had a patch of wild-type bristles on a body with an M phenotype. His conclusion was that this was probably the result of mitotic nondisjunction of the chromosomes.

Around the same time, in 1936, Curt Stern described the first case of mitotic crossing-over (Stern, 1936). He was working with the *Drosophila* X-linked genes *y* (yellow body) and *sn* (singed which causes short, curly bristles), and made the following cross:

$$y^+ sn / y^+ sn \quad \text{X} \quad y\, sn^+ / Y$$

The females recovered were wild type in appearance, as expected from their $y^+ sn/ y\, sn^+$ genotypes. However, he noticed that some of the females had patches of yellow tissue and others had patches of singed tissue. These could be explained as the result of nondisjunction or chromosome loss. But, in addition, he also observed that some of the progeny showed "twin spots" with a patch of yellow tissue adjacent to a patch of singed tissue. Stern noticed that the twin spots were too common to be chance juxtaposition of single spots, and he reasoned that they were probably reciprocal products of the same event.

2. Mitotic recombination pathways. In most organisms, homologous recombination and nonhomologous recombination are the two major pathways for the repair of DSBs. Homologous recombination, unlike nonhomologous recombination, requires the presence of a homologous sequence with hundreds of nearly perfectly matched base pairs. The focus of this dissertation is on homologous recombination.

Different types of homologous mitotic recombination have been observed in yeast cells based on the mechanism of the event and on the location of the sequence homology. Below, I will describe recombination events occurring between two homologous chromosomes (allelic recombination), between dispersed repeated genes (ectopic recombination) and between sister chromatids.

1) Allelic recombination refers to events occurring at allelic positions between homologous chromosomes and can occur through a number of different pathways. In a *reciprocal crossover* there is a reciprocal exchange of information between the two homologous. In this type of event, all heterozygous markers distal to the crossover become homozygous. *Gene conversion* is an event in which DNA sequence information is transferred non-reciprocally from one homologous chromosome to another. This event usually occurs between two alleles of a gene, but it can also comprise many contiguous genes. In a *Break-induced replication (BIR)* event, one broken end invades a homologous region, setting up a replication fork that duplicates the entire chromosome from the point of invasion to the telomere. This event can be described as a very large conversion event.

2) Ectopic recombination refers to events that occur between any homologous pair of sequences at non-allelic positions, either within the same chromosome or between different chromosomes. Such recombination events can generate deletions, duplications, inversions,

and translocations (Petes and Hill, 1988). In addition, ectopic recombination is a source of telomere propagation in yeast cells that lack the enzyme telomerase.

3) Sister chromatid exchange (SCE) refers to events occurring between the sister chromatids. The study of SCE has been difficult due to the difficulty of genetically detecting recombination between two identical DNA molecules. Most of the work done on SCE comes from studies of unequal sister chromatid exchange (USCE) events between repeated sequences. Repeats located in sister chromatids may misalign, leading to deletions or inversions giving rise to genetically detectable products (Szostak and Wu, 1980).

3. Proteins involved in mitotic recombination. Genes important for recombination were identified by their requirement for the repair of X-ray induced DNA damage in *Saccharomyces cerevisiae* (Paques and Haber, 1999). Ten genes were classified as the *RAD52* epistasis group: *RAD50, RAD51, RAD52, RAD53, RAD54, RAD55, RAD56, RAD57, MRE11,* and *XRS2*. These genes can be classified into at least four subgroups. *RAD52* is the only gene required for all homologous recombination events. *RAD51, RAD54, RAD55,* and *RAD57* are required for some homologous recombination events but are dispensable or less necessary for others. *RAD50, MRE11,* and *XRS2* form another group of interacting proteins. *RAD53* is an essential gene that is not directly involved in the repair of DNA but is part of the DNA damage checkpoint function of the cell.

Biochemical characterization of these recombination proteins indicates that Mre11p, Rad50p, and Xrs2p are involved in nuclease activity and Rad51p, Rad52p, Rad54p, Rad55p, and Rad57p participate in the strand transfer reaction (Symington, 2002). A model for strand invasion of Rad51p and the mediator proteins Rad52p, Rad54p, and Rad55/Rad57 include

the following steps. After 3'ssDNA tails are produced by the MRX complex and/or a 5'-to-3' resection exonuclease, the ssDNA tails are coated by RPA to eliminate secondary structures. Then, Rad52p recruits Rad51p to the RPA-ssDNA complex. The Rad51p extends on the ssDNA mediated by Rad55/Rad57 and RPA is displaced. The Rad51 filament then locates a homologous sequence. Rad54 interacts with Rad51 facilitating the interaction between the donor DNA and the Rad51 filament.

4. Classical methods of studying mitotic recombination. In this section, I will describe some of the systems that have been commonly used in the study of mitotic recombination in diploid strains of yeast.

4.1. Non-selective method (Roman, 1973). The diploid strain used by Roman is heterozygous for the recessive tryptophan (*trp5*), leucine (*leu1*), and adenine (*ade6*) mutations, and for the co-dominant markers *SUC1* and *MAL1*, involved in sucrose and maltose fermentation (Figure 1.2.A). These genes are located on chromosome VII. The cell is also homozygous for the *ade2* gene (located on chromosome XV); cells harboring this mutation accumulate a red pigment and the colony derived from those cells is red. The Ade6 and Ade2 proteins catalyze steps four and six of the adenine biosynthesis pathway, respectively. If the *ade6* allele becomes homozygous, the production of the red pigment is blocked and a white colony is formed.

Two of the events that could achieve this homozygosity are 1) a reciprocal crossover between the centromere of chromosome VII and the *ADE6* locus (a distance of ~115 kb) (Figure 1.2.B) or 2) a local conversion event where one of the wild type alleles is converted to the *ade6* mutation (Figure 1.2.C). A reciprocal crossover between *CEN7* and the *ADE6*

locus will result in a red/white sectored colony. The red cells will be homozygous for the *ADE6* wild type allele and the *SUC* marker, and the white cells will be homozygous for the *ade6* mutation and the *MAL* marker. A local conversion event where one of the *ADE6* wild type alleles is converted to the *ade6* mutation will also produce a red/white sectored colony. In this case, however, the red cells will remain heterozygous at the *ADE6* locus and for the fermentation markers while the white cells will become homozygous for the *ade6* mutation and will remain heterozygous for the fermentation markers.

Because mitotic events are infrequent (Roman determined the frequency of red/white sectored colonies under spontaneous conditions to be approximately in the order of 1 in 10,000 cells plated), he studied mitotic recombination using chemicals and radiation in order to enhance the frequency of recombinational events. From those studies, he concluded that 1) mitotic gene conversion events were less often associated with crossovers than meiotic gene conversion events, 2) ultraviolet light mainly induced crossing over and 3) chemical mutagens (ethyl methane-sulfonate [EMS] and nitrosoguanidine [NG]) mainly produced conversion events.

Although this system allows the recovery of both products result of a reciprocal mitotic crossover and a conversion event, it has several limitations. First, since mitotic events are usually rare, it would be very inefficient to study them using non-selective techniques. Second, in order for a conversion event to be detected it should occur in the direction from *ADE6* to *ade6*, and not in the reverse direction.

4.2. Selective methods. Spontaneous mitotic recombination between homologous chromosomes occurs at rates several orders of magnitude lower than

spontaneous meiotic recombination for the same genetic intervals (Fogel and Mortimer, 1971). Because these mitotic events are too infrequent to be analyzed by nonselective techniques, a number of selective methods have been developed for their detection.

4.2.1. Heteroalleles system. One method, also developed by Roman (Roman, 1957), allows selection of spontaneous mitotic gene conversion (Figure 1.3). It consists of a diploid strain carrying two noncomplementing mutant alleles of the *ADE5* gene. Mitotic gene conversion events involving the heteroalleles can result in prototrophic derivatives. This method has been applied to a wide range of genes in the yeast genome. Schmuckli-Maurer *et al* (Schmuckli-Maurer *et al.*, 2003) examined intergenic recombination in cells carrying heteroalleles in the *HIS7* (*his7-2, his7-1*), *TYR1* (*tyr1-1, tyr1-2*), *URA3* (*ura3-13, ura3-1*), *TRP5* (*trp5-d, trp5-c*) and *LEU1*(*leu1-12, leu1-c*) genes, and reported rates of 8×10^{-7}, 1×10^{-6}, 2.5×10^{-6}, 8×10^{-6} and 8×10^{-6}, respectively. In another study (Freeman and Hoffmann, 2006) where the *TRP5* (*trp5-12, trp5-27*) gene was analyzed, the frequency of convertants was 6.1×10^{-6}. This system has several limitations: 1) only a very specific type of event can be studied, 2) only one of the two products of the mitotic event can be selected, and 3) recombination is assayed in a very limited region of the genome.

4.2.2. Canavanine assay. Another selective system that is commonly used in *Saccharomyces cerevisiae* (Hartwell and Smith, 1985) is shown in Figure 1.4. A diploid is constructed that is heterozygous for two recessive mutations on chromosome V, *can1* and *hom3*. The resulting diploid is sensitive to the drug canavanine (Can^S) and is a methionine prototroph (Met^+). A mitotic crossover followed by disjunction of the recombined chromatids into different daughter cells results in one cell that is homozygous for *can1* and, therefore, canavanine resistant (Can^R), and a second that is homozygous for the

wild type allele and Can^S. The *hom3* marker is used to screen for Can^R derivatives that reflect loss of the homologue containing the wild type *CAN1*, since such derivatives should be Can^R Met^-. In the study by Hartwell and Smith, the rate of mitotic recombination between the centromere and *can1* was determined to be $1.2x10^{-5}$/division and the rate of chromosome loss $8.3x10^{-6}$ losses/division.

The system shown in Figure 1.4 detects only one of the two expected products of the reciprocal crossover. The failure to detect both products is a problem because two types of non-reciprocal recombination can also generate a strain homozygous for the *can1* mutant allele. In *Saccharomyces cerevisiae*, it is likely that most recombination events are initiated by a DSB that can then be repaired in several different ways (Paques and Haber, 1999). In two-ended repair, the broken ends form heteroduplexes with the unbroken homologue. If there are mismatches within these heteroduplex regions, repair of these mismatches can result in a gene conversion event. Since the length of the heteroduplexes is usually less than a few kb, the amount of DNA transferred non-reciprocally between the homologues is usually 100 bp to several kb. We will refer to this type of conversion as "local" gene conversion. In a second type of DSB repair, one broken end invades a homologous region, setting up a replication fork that duplicates the entire chromosome from the point of invasion to the telomere; this event has been termed "break-induced replication" (BIR). Both local conversion events and BIR events can produce Can^R cells. The distinction between these events and the reciprocal crossover is that the Can^S cell resulting from local conversion or BIR events is *can1/CAN1*, whereas the Can^S cell resulting from the reciprocal crossover is *CAN1/CAN1*. Since these two types of Can^S cells are non-selectable, the system shown in Figure 1.4 cannot distinguish reciprocal crossovers from various classes of non-reciprocal

events. Another limitation of this system is that only conversion events where *CAN1* is converted to *can1* can be detected.

As part of my dissertation research, I developed a novel method in which mitotic events occurring on either of the two homologues can be recovered, eliminating some of the limitations of the previous methods. The distinctive feature of this system is that it is the first system developed that allows the selection of both products result of a spontaneous reciprocal mitotic crossover, providing a fast and accurate assessment of this type of mitotic event.

D. Mechanisms of loss of heterozygosity and cancer.

A diploid wild type cell usually contains two copies of a functionally normal gene. Except for dominant mutations, cells carrying functionally normal alleles on both homologous chromosomes will require two genetic events, where both alleles are modified, to obtain a phenotypic change. For example, in the case of a tumor suppressor gene, when a mutation occurs in one of the two copies of the gene, the cell becomes heterozygous but since the mutation is recessive the cell still behaves as wild type. Loss of heterozygosity (LOH) refers to the loss of the remaining normal allele predisposing a human cell to develop into a tumor (Knudson, Jr., 1971; Knudson, 1993; Knudson, 2005). LOH may arise by any of several possible mechanisms. (*i*) The second allele can be inactivated by a second point mutation. (*ii*) Alternatively, the chromosome on which the normal allele resides can be lost. (*iii*) A gross rearrangement such as a deletion could cause loss of the remaining normal

allele. (*iv*) Finally, it could be the result of mitotic recombination between alleles (Cavenee et al., 1983).

Loss of heterozygosity is the most common mechanism by which the remaining functional copy at the retinoblastoma tumor suppressor locus (*RB1*) is lost in heterozygous retinal cells. A retinoblastoma can arise in cells homozygous or hemizygous for the mutant allele. In a study where a set of matched retinoblastomas and leukocyte DNA samples from 158 patients were analyzed, approximately half of the tumors presented homozygosity at all informative marker loci (including the marker closest to the centromere), indicating that the homozygosity was result of chromosomal nondisjunction or a crossover that occurred very close to the centromere. The rest of the tumors retained heterozygosity for one or more proximal markers suggesting that homozygosity at the *RB1* locus had occurred through somatic recombination distal to the most centromeric marker (Hagstrom and Dryja, 1999). Many other human cancers arise after loss of heterozygosity of other tumor suppressor genes (Lasko *et al.*, 1991).

The aim of my work has been to further our understanding of mitotic recombination and chromosome loss using the model organism *Saccharomyces cerevisiae*. For this purpose, I have developed a novel and powerful system that allows the selection of spontaneous reciprocal mitotic crossovers and other types of mitotic events and used this system to address several important questions: 1) what fraction of mitotic recombination events produces reciprocal products?, 2) are there hotspots and coldspots for mitotic reciprocal crossovers?, 3) are there DNA sequences that predispose to increases in mitotic events?, 4) what is the effect of DNA-damaging agents on these types of events?, and 5) what is the role of the mating-type alleles on reciprocal mitotic recombination and chromosome loss?

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Figure 1.1. Models of recombination. In the DSB repair model, the ends of a DSB are resected 5'-to-3' producing 3'-ended single-stranded DNA. The ssDNA invades a homologous molecule and primes new DNA synthesis. The invasion of both ends produces two Holliday junctions whose cleavage will yield a crossover or non-crossover product (DSBR branch). In the SDSA pathway, after strand invasion and initiation of new DNA synthesis, the newly synthesized DNA disassociates from the template and anneals back with the original partner.

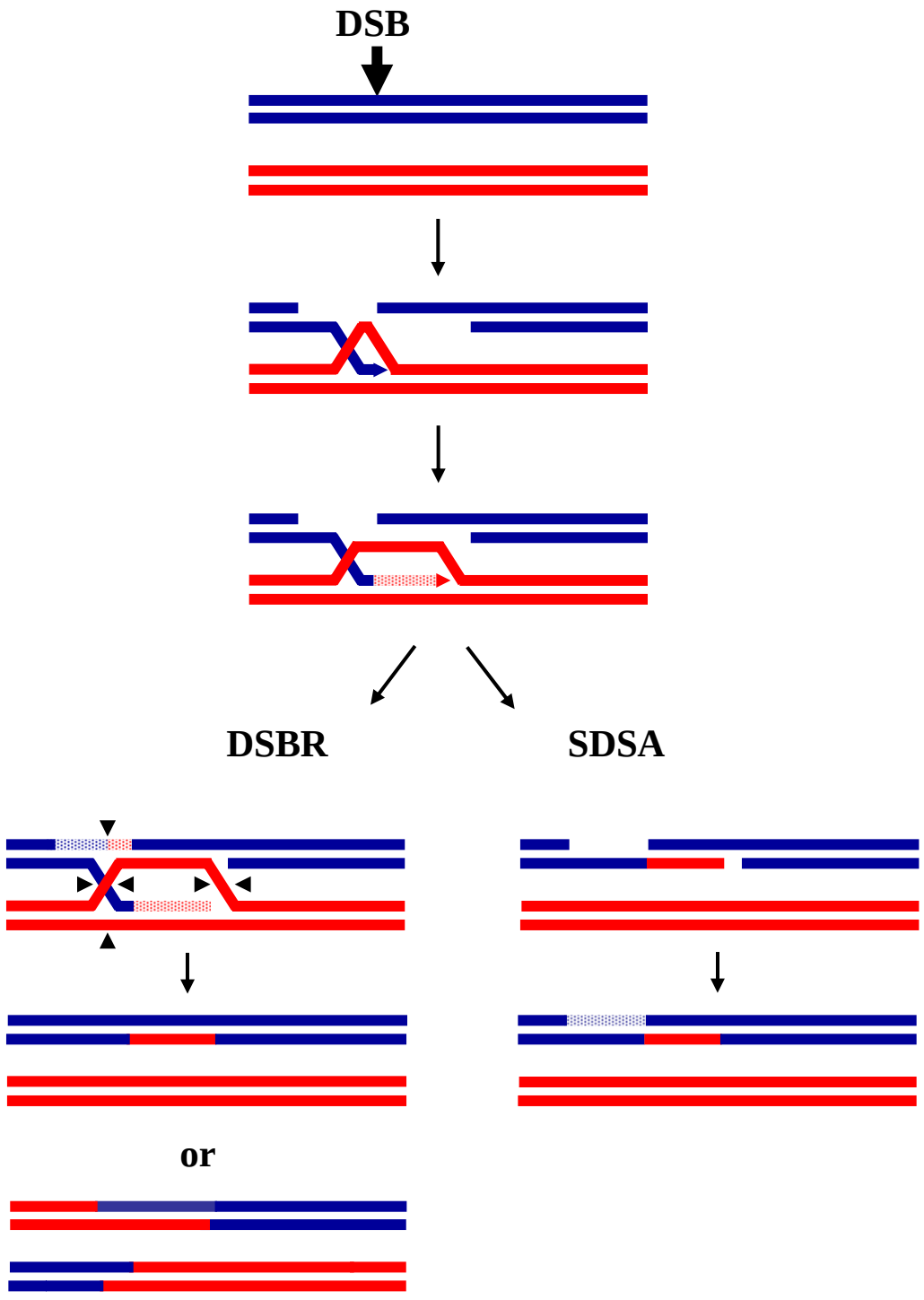
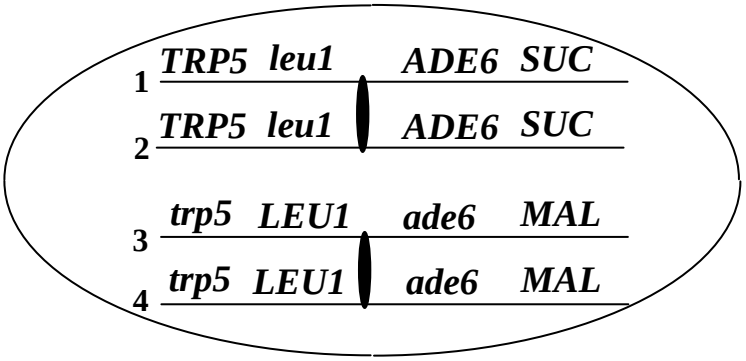

DSB
DSBR
SDSA
or

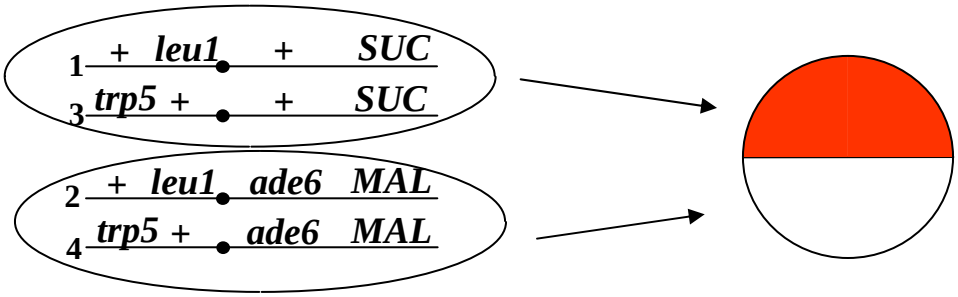
Figure 1.2. Detection of mitotic reciprocal crossovers and conversion events. The system developed by Roman allows the detection (although not selection) of mitotic reciprocal crossovers and conversion events. (A) Cells in this system harbor an *ade2* mutation which makes the colonies red. Several markers are located in chromosome VII. The cells are heterozygous for *trp5*, *leu1*, *ade6* and the co-dominant markers *SUC1* and *MAL1*. (B) A reciprocal crossover between *CEN7* and the *ADE6* locus will result in a red/white sectored colony. The red cells will be homozygous for *ADE6* and *SUC1*, and the white cells will be homozygous for *ade6* and *MAL1*. (C) A local conversion event where *ADE6* in converted to *ade6* will also produce a red/white sector. The red cell will remain heterozygous at the *ADE6* locus and the white cell will become homozygous for the *ade6*.

A.

DIPLOID CELL



B. **DAUGHTER CELLS AFTER CROSSING-OVER**



C. **DAUGHTER CELLS AFTER CONVERSION**

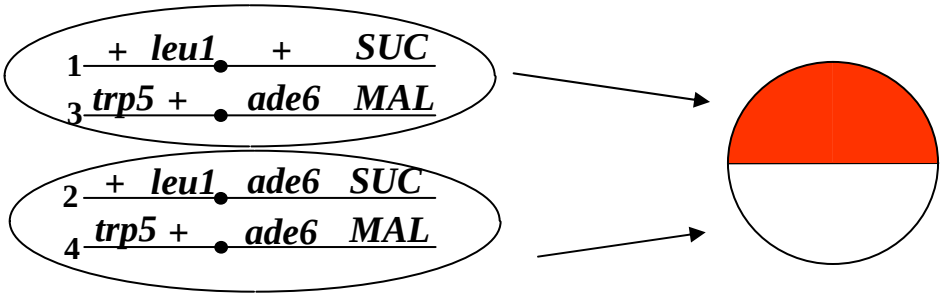


Figure 1.3. Heteroalleles system for the detection of mitotic recombination in diploid yeast cells. This system allows the selection of spontaneous mitotic gene conversion events. It consists of a diploid strain carrying two noncomplementing mutant alleles of the *ADE5* gene. Mitotic gene conversion events involving the heteroalleles can result in prototrophic derivatives.

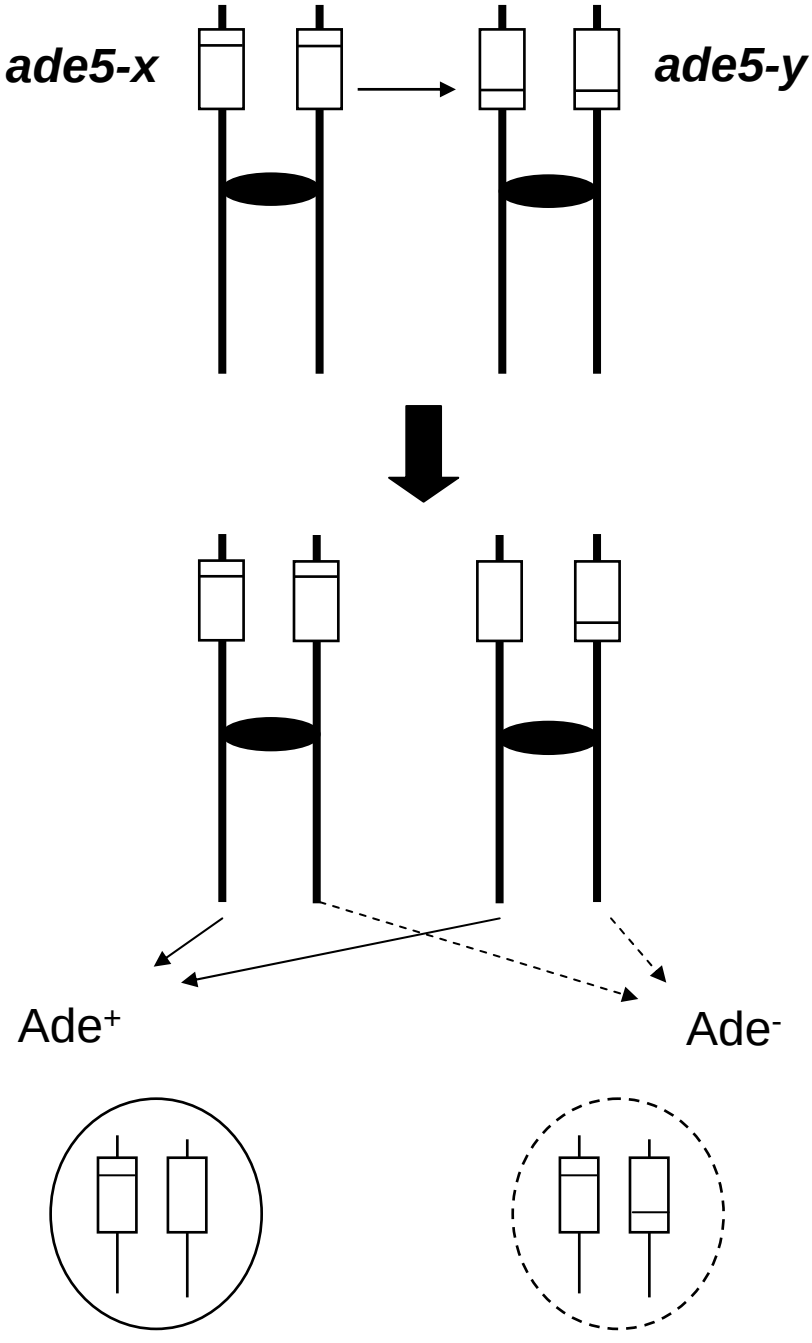
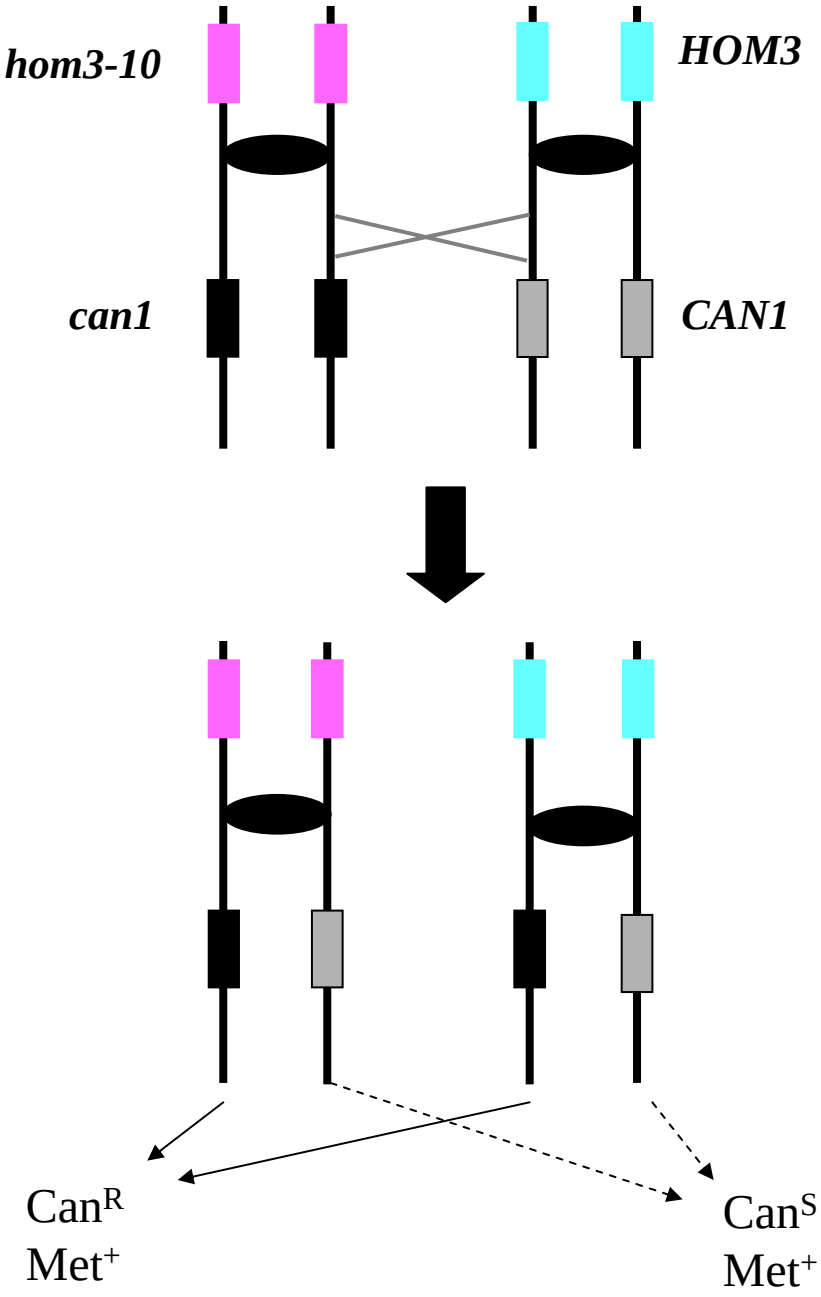

ade5-x
ade5-y
Ade+
Ade-

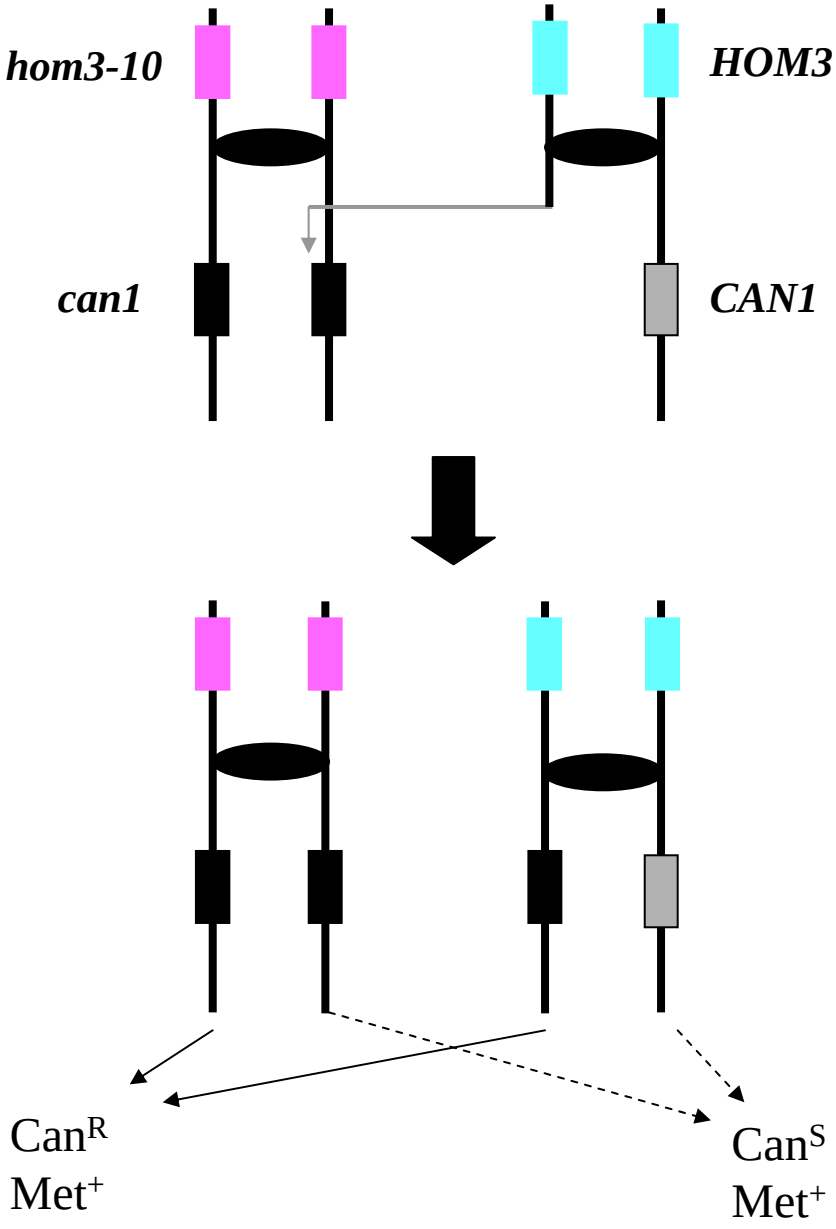
Figure 1.4. Canavanine system for the detection of mitotic recombination and chromosome loss in diploid yeast cells.

One commonly used system for detection of mitotic recombination events employs a diploid that is heterozygous for mutations in the *can1* and *hom3* loci. The starting diploid strain is Can^S and Met^+. Cells are transferred to plates containing canavanine, and any Can^R derivatives are tested for their ability to grow in the absence of methionine. (A) A reciprocal crossover will result in a Can^R cell (*can1/can1*) and a Can^S cell (*CAN1/CAN1*); both cells will be Met^+. (B) A Break-Induced Replication event initiated on the CAN1-containing homologue will result in duplication of all sequences distal to the DSB. One of the expected products will be Can^R (*can1/can1*) and the other will be Can^S (*can1/CAN1*). Both cells will be Met^+. (C) A local gene conversion event will produce the same products as a BIR event by this assay. (D) Loss of the *CAN1*-containing homologue will result in a Can^R colony that is also Met^-.

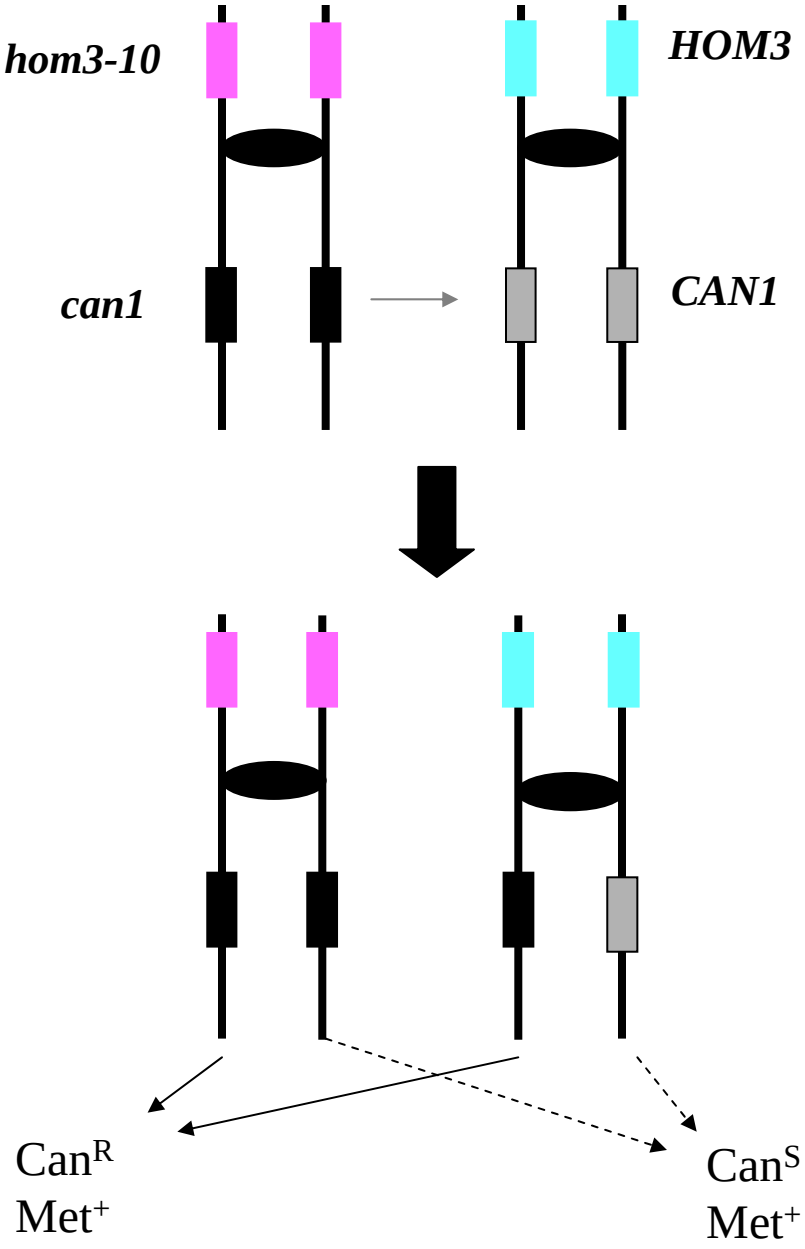
A. Mitotic Crossover



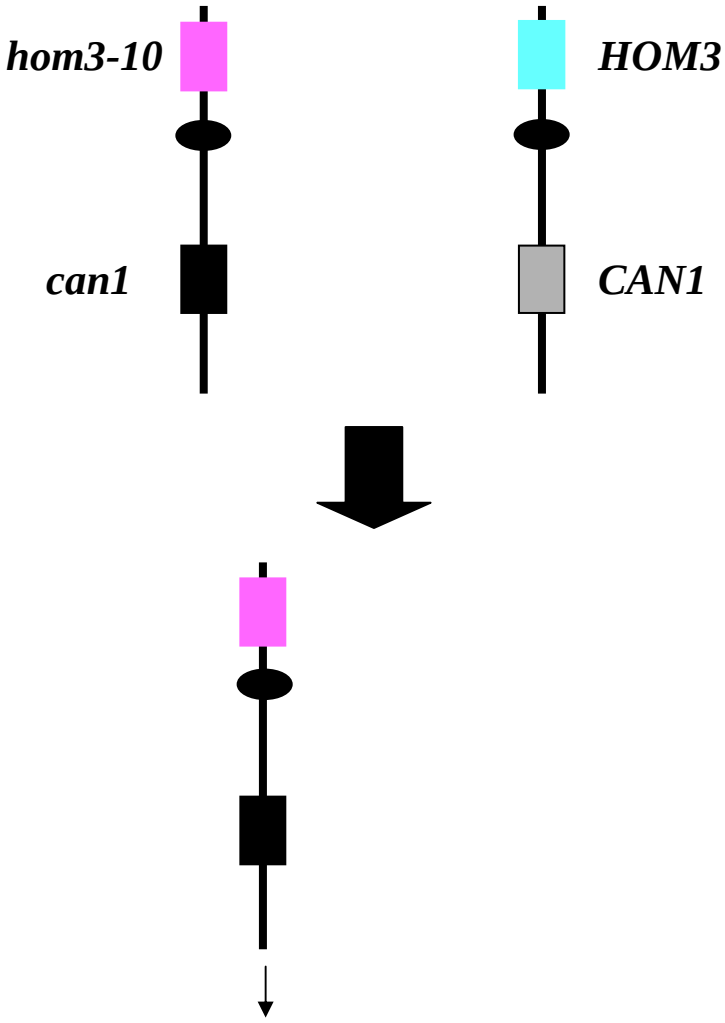
B. Break-Induced Replication



C. Local Gene Conversion



D. Chromosome loss



Can^{R}
Met^{-}

CHAPTER II

ANALYSIS OF SPONTANEOUS AND INDUCED MITOTIC CROSSOVERS USING A NOVEL METHOD DEVELOPED IN THE YEAST *SACCHAROMYCES CEREVISIAE* [1]

A. Introduction

Because of the way in which genetic maps are usually constructed, most geneticists are more concerned with meiotic recombination than mitotic recombination. Mitotic recombination, however, has a number of important roles in eukaryotes including 1) repairing of DNA lesions such as double-strand DNA breaks (DSBs), 2) re-starting of stalled replication forks, 3) providing an alternative pathway of telomere replication in cells lacking telomerase, and 4) contributing to the evolution of the genome by generating novel chromosome rearrangements (Symington, 2002;Helleday, 2003). In addition, human cells that are heterozygous for a mutation in a tumor suppressor gene are at risk for developing into a tumor cell as a consequence of loss of the protective wild type gene (LOH) (Knudson, 1993). Although LOH has a variety of causes, about half of the LOH events in one large study of retinoblastomas reflected mitotic recombination (Hagstrom and Dryja, 1999).

Mitotic recombination events are rare and a number of selective methods have been developed for their detection. One common method used in *Saccharomyces cerevisiae* is described in chapter I. Briefly, a diploid is constructed that is heterozygous for two recessive

[1]This chapter is an adapted version of Barbera & Petes 2006 PNAS **103**: 12819-12824, supplemented with unpublished results.

mutations on chromosome V, *can1* and *hom3*. The resulting diploid is Can^S and Met^+. A mitotic crossover between *CEN5* and *CAN1* will produce one cell homozygous for the mutation (*can1/can1*) and therefore, canavanine-resistant (Can^R), and one cell homozygous for the wild type allele (*CAN1/CAN1*) and Can^S. This system detects only one of the two expected products of the reciprocal crossover. The failure to detect both products is a problem because two types of nonreciprocal recombination (conversion and BIR) can also generate a strain homozygous for the *can1* mutant allele.

Here we describe a genetic system that allows selection of both products of a reciprocal crossover. Although our analysis was limited to one genetic interval on chromosome V, the same approach can be extended to any region of the yeast genome.

B. Results

1. Description of the system. As discussed in chapter I, previous systems for the analysis of mitotic recombination in yeast allow for the selection of only one of the two expected products of a given event, and also mitotic crossovers could not be distinguished from other types of non-reciprocal exchange. We have developed a system that allows the selection of both products result of spontaneous reciprocal mitotic crossovers in *Saccharomyces cerevisiae*. The system consists of a diploid strain, MAB6, with the *can1-100* allele (an ochre-suppressible nonsense mutation) on one copy of chromosome V, and the *SUP4-o* (an ochre suppressor) gene replacing the *CAN1* locus on the other copy of chromosome V. The *CAN1* gene encodes an arginine permease, and canavanine is a toxic arginine analog. Thus, cells with a wild type *CAN1* gene are sensitive to canavanine. The *can1-100* mutation, in the absence of the suppressor, results in a strain that is resistant to the

drug canavanine. The MAB6 diploid is sensitive to canavanine because the *can1-100* mutation is suppressed by *SUP4-o*. In addition, drug resistance markers (*KAN*: kanamycin, *HYG*: hygromycin) were introduced at allelic positions centromere-distal to *CAN1*. Yeast strains with the *KAN* and *HYG* genes are resistant to geneticin (Gen^R) and to hygromycin (Hyg^R), respectively. On the opposite arm of chromosome V, we inserted the *LEU2* and *HIS3* markers at allelic positions. The MAB6 diploid is also homozygous for the *ade2-1* allele, an ochre-suppressible mutation. In the absence of the suppressor, strains carrying this mutation form red colonies and are adenine dependent; when the suppressor is present, *ade2-1*-containing strains form white colonies. Thus, the starting diploid strain has the following phenotypes: sensitive to canavanine, forms white colonies, resistant to geneticin and hygromycin, Leu^+ and His^+.

A reciprocal crossover between the centromere and the *CAN1* locus will produce two canavanine-resistant cells, one cell homozygous for the *can1-100* allele and lacking the suppressor and one homozygous for the suppressor and lacking *can1-100*. In addition, each of the canavanine-resistant cells will now be resistant to one of the drugs (geneticin or hygromycin) but sensitive to the other one. Because the strain is homozygous for the *ade2-1* mutation, and the cell with two copies of the *can1-100* allele lacks the suppressor, a reciprocal crossover that occurs as the cell is plated onto the canavanine-containing plate will result in a colony with one red and one white sector (Figure 2.1).

In addition to the red/white sectored Can^R colonies resulting from reciprocal crossovers, six different phenotypic classes of unsectored Can^R colonies were observed (Figure 2.2). Class 1 colonies are likely to reflect two types of genetic events: *(i)* a BIR event in which the initiating lesion was on the chromosome with the *SUP4-o* gene, and *(ii)* one of

the two types of cells produced by a reciprocal crossover before the plating of the cells on canavanine-containing medium. Class 2 white colonies represent the comparable classes: a BIR event initiated on the other homologue, and the other product of the reciprocal exchange. Class 3 colonies represent local gene conversion events (unassociated with a crossover) in which the *can1-100* gene replaces the *SUP4-o* gene. Alternatively, class 3 colonies could be a consequence of additional mutations in the *SUP4-o* gene. Class 4 colonies represent conversion events in which the *SUP4-o* gene replaces the *can1-100* gene. Class 5 colonies reflect loss of the *SUP4-o*-containing homologue, and class 6 events reflect loss of the *can1-100*-containing homologue. Table 2.1 summarizes the phenotypes of Can^R colonies expected from these various classes of events.

2. Rates of spontaneous mitotic recombination and chromosome loss. To measure the rates of the various mitotic events, we plated cells from multiple (~40) independent cultures on canavanine-containing medium (to measure the frequencies of the various canavanine-resistant phenotypes) and on nonselective medium (to measure the number of cells in the culture). Colonies formed on the canavanine-containing plate were then replica-plated to five different types of diagnostic media: those lacking histidine, leucine, or adenine, and those containing geneticin or hygromycin. Photographs of colonies formed on a canavanine-containing plate and replica-plated to the various diagnostic plates are shown in Figure 2.3. A red/white-sectored colony and all six classes of unsectored colonies are shown.

The data for the different classes of events for this experiment are shown in Table 2.2 and Figure 2.4.A. Rates were calculated in two different ways. Because a sectored colony

requires the event to occur at the time the cell is plated on canavanine-containing medium, the frequency of such colonies is the same as the rate. The average rate of sectored colonies (40 cultures in two experiments) was 2.0 x 10^{-5} per division. Since in cells that have undergone a reciprocal crossover only half of the segregation events will produce Can^R cells, we conclude that the actual rate of reciprocal exchange between *CEN5* and *CAN1* is 4.0 x 10^{-5} per division.

The rates for all classes of unsectored colonies were determined by fluctuation analysis (Lea and Coulson, 1949). The rates of class 1 and 2 events (2.0 x 10^{-5} per division and 2.2 x 10^{-5} per division, respectively) were about the same as observed for the rate of reciprocal crossovers. The simplest interpretation of this result is that probably most of the class 1 and 2 events represent reciprocal crossovers that occurred before plating, rather than BIR events.

The rate of local gene conversions where *SUP4-o* is converted to *can1-100* (class 3) was 2.9 x 10^{-6} per division, 10-fold less frequent than reciprocal crossovers. Class 3 could also be generated by additional mutations in the *SUP4-o* gene. A class 3 event resulting from conversion would be homozygous for the *can1-100* gene, whereas a class 3 event resulting from mutation in *SUP4-o* would retain *SUP4-o* sequences. These two types of class 3 events can be readily distinguished by PCR analysis. Of 128 class 3 events analyzed, 90 were local conversion events and 38 were additional mutations within *SUP4-o*. The rates of class 3 events shown in Table 2.2 and Figure 2.4.A were adjusted to exclude those derivatives with additional mutations in *SUP4-o*. The rate of mutation in the *SUP4-o* gene was ~7.5 x 10^{-7}, similar to the rate previously reported by Pierce *et al.* (Pierce *et al.*, 1987).

The rate of local gene conversions where *can1-100* is converted to *SUP4-o* (class 4) was 3.9 x 10^{-6} per division. As mentioned before, class 4 cells have the phenotype: Can^R Gen^R Hyg^R His^+ Leu^+ Ade^+ white. Additional mutations in the *can1-100* allele of MAB6 that would render the cell unresponsive to the suppressor will produce cells with the phenotype: Can^R Gen^R Hyg^R His^+ Leu^+ $Ade^{+/-}$ white. The $Ade^{+/-}$ phenotype (diploid cell with one copy of *SUP4-o*) is easily distinguishable from the Ade^+ phenotype (diploid cell with two copies of *SUP4-o*) in SD-Adenine medium.

The rates of chromosome loss (class 5 and 6) were ~0.8 x 10^{-5} per cell division. To confirm that these classes represent chromosome loss rather than a BIR event that covers all three heterozygous markers, we sporulated and dissected two independent class 5 and six independent class 6 strains. In all eight strains, the majority of the tetrads had two viable spores or less, as expected for chromosome V monosomic strains. We also analyzed two class 5 and two class 6 strains by using microarrays containing all of the yeast genes. All four strains had one copy of chromosome V and two copies of all other chromosomes (Figure 2.5). Chromosome losses can reflect either nondisjunction events or failure to replicate one of the homologues. Because a chromosome V trisomic strain (the other expected product of a nondisjunction event) will probably produce a Can^S cell, we cannot distinguish these possibilities with our system.

In addition to the classes described above, there are several other types of genetic events that could produce Can^R colonies. A local conversion event associated with crossover could contribute to classes 1 and 2. Because less than half of mitotic gene conversion events are associated with crossovers (Chua and Jinks-Robertson, 1991) and local gene conversion events unassociated with crossovers are10-fold less frequent than the reciprocal crossovers

class, this type of event is unlikely to contribute significantly to these classes. Two further points should be made. First, our data do not allow accurate comparison of the relative rates of local gene conversion and crossovers because the rate of conversion is determined at a single site, whereas the crossover rate is assayed in a 120-kb interval. In addition, the conversion event involves a sequence heterology that could reduce rates. Second, because local conversions associated with crossovers produce the same colony phenotypes as BIR events and reciprocal crossover events that occur before the plating of the cells on canavanine-containing medium, we cannot determine the fraction of conversion events that are associated with crossovers.

Another type of mitotic event that would be expected to be infrequent is a two-strand double reciprocal crossover event, with one crossover between *CEN5* and *can1-100/SUP4-o* and a second crossover between *can1-100/SUP4-o* and the drug resistance markers. This type of exchange would produce a sectored colony with the sector phenotypes: Can^R Gen^R Hyg^R His^+ Leu^+ Ade^- red and Can^R Gen^R Hyg^R His^+ Leu^+ Ade^+ white. Of 135 red/white-sectored colonies examined, only two had these phenotypes.

One final class of Can^R colony had the same phenotype for all markers on chromosome V as the parental MAB6 strain except that the colonies were reddish instead of white. These colonies, which appeared at a rate of ~0.7×10^{-5} per cell division, grew slowly, similar to the growth rates of the monosomic class 5 and 6 strains. We sporulated and dissected four of these strains, and three of these strains segregated two live to two dead spores. This pattern of spore viability is expected for a recessive lethal or a monosomic strain. Microarrays analysis on the four strains showed that these strains were monosomic for chromosome XVI. This result suggests that there is a gene (or genes) on XVI that

positively regulates the expression or function of the Can1p and, because of the low expression of Can1p in MAB6, loss of one of the copies of these genes in the diploid results in a Can^R colony. Because this class is not relevant to the recombination and chromosome loss events involving chromosome V, it will not be discussed further.

In the experiment described above, the MAB6 cells were grown on rich medium (YPD) for 2 days and then plated on canavanine-containing medium. It has been reported that in yeast cells that have undergone >20 divisions, the rate of nonreciprocal events is increased compared to younger cells (McMurray and Gottschling, 2003). We decided to analyze if there was any change in any of the classes when the cells were allowed to divide for a few more generations. MAB6 cells were grown on YPD for 3 days and then plated on canavanine-containing medium. The data for this experiment are shown in Figure 2.4.B. The same number (~40) of independent colonies were plated on both experiments (2 days vs. 3 days), and no significant difference was observed for any of the rates.

3. Mitotic crossing-over in an interval near the centromere of chromosome V. In many organisms, including *Saccharomyces cerevisiae*, meiotic recombination is reduced near the centromere (Lambie and Roeder, 1988). According to the genetic and physical maps in the *Saccharomyces* Genome Database (www.yeastgenome.org), the 36-kb *CEN5-URA3* interval is 8 cM, whereas the 84-kb *URA3-CAN1* interval is 42 cM. Thus, there are 0.22 cM/kb in the first interval and 0.5 cM/kb in the second, indicating substantial suppression of meiotic recombination near *CEN5*. We measured meiotic recombination in these same two intervals in MAB54, an isogenic derivative of MAB6 that was heterozygous for mutations at

the *URA3* and *CAN1* loci, in addition to being heterozygous at the centromere-linked *TRP1* locus. From analysis of 360 tetrads, we measured the *CEN5-URA3* distance to be 7 cM and the *URA3-CAN1* distance to be 41 cM.

To investigate whether this suppression near the centromere was also seen in mitotic recombination, we constructed a strain (MAB13) that was isogenic with MAB6 except that it was also heterozygous at the *URA3* locus. In MAB13, the wild type *URA3* allele was on the homologue with the *can1-100* and *HYG* markers, and the mutant allele was on the homologue with the *SUP4-o* and *KAN* markers. Strains with a wild type *URA3* allele are sensitive to 5-fluoroorotic acid, whereas cells harboring only the mutant allele are resistant (Boeke *et al.*, 1984). A reciprocal crossover in MAB13 between *CEN5* and the *URA3* locus would be expected to produce a red Ura^+/white Ura^--sectored colony (Figure 2.6.A). A reciprocal crossover between *URA3* and *CAN1* would yield a red/white Can^R colony in which both sectors are Ura^+ (Figure 2.6.B).

The average rate (60 independent cultures) of Can^R red/white sectored colonies in MAB13 was 1.3×10^{-5} per cell division, similar to the rate observed in MAB6. The rate of reciprocal crossovers between *CEN5* and *URA3* was 0.4×10^{-5}, and the rate of reciprocal crossovers between *URA3* and *CAN1* was 0.9×10^{-5}. Thus, the ratio of crossovers in these two intervals is 0.44, similar to the ratio of the physical distances of the intervals (0.42). Thus, mitotic crossovers, unlike meiotic crossovers, are not significantly suppressed near *CEN5* (Figure 2.7). Of 176 red/white-sectored colonies observed, 171 had the phenotypes expected for single crossover events, and five had the phenotypes expected for double crossover events (one between *URA3* and *can1-100/SUP4-o*, and one between *can1-100/ SUP4-o* and the drug resistant markers).

4. Effect of trinucleotide repeat array on mitotic recombination and chromosome loss. Long CTG tracts stimulate spontaneous ectopic mitotic exchange in yeast and are associated with DSB formation (Freudenreich *et al.*, 1998). Freudenreich *et al.* found that ectopic recombination in haploid cells was ~5-fold, ~9-fold, ~27-fold and ~53-fold greater for strains carrying a tract of $(CTG)_{130}$, $(CTG)_{180}$, $(CTG)_{200}$ and $(CTG)_{250}$, respectively (compared to the control strain). It has also been reported that long trinucleotide repeats stimulate unequal sister-chromatid exchange (USCE) during vegetative growth in yeast (Nag *et al.*, 2004). Nag *et al.* studied the effect of a $(CAG)_{71}$ tract on unequal SCE in a haploid strain and reported a subtle (2-fold) increase in the strain carrying the tract.

Based on the idea that long CTG tracts act as fragile sites in yeast resulting in stimulation of ectopic exchange, we wished to test the hypothesis that a $(CTG)_{115}$ tract would also stimulate reciprocal mitotic crossovers and/or conversion events between the homologous chromosomes. To test this possibility, we inserted a $(CTG)_{115}$ tract in each of the parent strains of MAB6 at the *ura3-1* locus on chromosome V. We then constructed a diploid strain, MAB33, homozygous for the insertion. MAB33 cells were grown on rich medium and then plated on canavanine-containing medium. Colonies were allowed to form on the canavanine plates and then replica-plated to the different diagnostic media. The different phenotypes were scored and the data for the different events for this experiment are in Table 2.2 and Figure 2.8. Contrary to the results obtained by Freudenreich *et al.*, we did not see any effect on mitotic recombination between the homologous chromosomes (reciprocal or nonreciprocal) or chromosome loss.

5. Stimulation of reciprocal crossovers and other mitotic recombination events by hydroxyurea (HU). Ribonucleotide reductase (RNR) is a heterodimeric allosteric enzyme that catalyzes the reduction of ribonucleotides to deoxyribonucleotides (Thelander and Reichard, 1979;Jordan and Reichard, 1998), an essential step in DNA synthesis and repair. RNR accomplishes the reduction via a complex radical transfer mechanism that has been extensively studied (Fontecave, 1998). In budding yeast, deoxyribonucleotide synthesis is entirely dependent on this enzyme because yeast cells posses no deoxyribonucleoside kinase activities. Hydroxyurea, a radical scavenger, is a highly specific inhibitor of ribonucleotide reductase (Atkin *et al.*, 1973;Yarbro, 1992;Hendricks and Mathews, 1998); this inhibition leads to a decline in the level of dNTPs (Koc *et al.*, 2004) affecting DNA synthesis and repair (Slater, 1973). Hydroxyurea does not inhibit initiation of DNA synthesis, although it greatly slows the progression of replication forks after initiation (Vassilev and Russev, 1984;Santocanale and Diffley, 1998) which can lead to stalled replication forks and double-strand breaks formation.

The metabolism of hydroxyurea acts as a source of reactive oxygen species (ROS) (King, 2004). ROS-initiated DNA damage includes oxidized bases, abasic sites, DNA-DNA intrastrand adducts, DNA strand breaks and DNA-protein cross-links (Cadet *et al.*, 1999). Thus, in addition to the DNA breaks that can occur as a result of stalled forks, hydroxyurea can also induce site-specific DNA-damage by forming hydrogen peroxide and nitric oxide (Sakano *et al.*, 2001).

Yeast cells exposed to hydroxyurea have elevated levels of gene conversion, deletions, and chromosome loss (Mayer *et al.*, 1986;Galli and Schiestl, 1996). Using a

nonselective assay for crossovers, Mayer *et al.* also reported that hydroxyurea induced reciprocal exchange, although the level of induction was not statistically significant.

To examine the effect of hydroxyurea in more detail, we grew MAB6 cells in medium containing 100 mM HU and then plated the treated cells on canavanine-containing medium. As shown in Table 2.3 and Figure 2.9.A, hydroxyurea treatment stimulated reciprocal mitotic crossovers ~40-fold and classes 1 – 6 ~10- to 20-fold. These results argue that hydroxyurea treatment leads to recombinogenic DNA lesions that are often repaired to generate a reciprocal crossover. The elevated chromosome loss in hydroxyurea-treated cells is likely to reflect either chromosome loss associated with unrepaired DNA lesions or incomplete chromosome replication.

Although MAB6 and related strains allow us to determine unambiguously the rate of reciprocal crossovers, we cannot directly determine the rate of spontaneous BIR events, because class 1 and 2 events represent either BIR events or reciprocal crossovers that occurred before the plating of cells on canavanine-containing medium. Because of the large increase in mitotic recombination events in hydroxyurea-treated cells, we were able to use nonselective methods in these cells to detect BIR events. Colonies grown on hydroxyurea-containing plates for 3 days were suspended in water, diluted and plated on nonselective medium (SD-Arginine + 10 μg/ml adenine). We screened the resulting colonies for those that had red/white sectors, and then checked the phenotypes of the sectors to determine whether they represented reciprocal crossovers or BIR events. Class 1 BIR events would produce a red/white-sectored colony. In examining 66,464 colonies, we found 67 sectors, 26 with the phenotype expected for a reciprocal crossover (3.9×10^{-4} per division) and 41 with the phenotype expected for a BIR event (6.1×10^{-4} per division).

For the reciprocal crossover, both red and white sectors have phenotypes different from the parental MAB6 strain. For the BIR event, however, one of the expected sectors has exactly the same phenotype as the parental strain. Consequently, a sectored colony indicative of a class 1 BIR event could be a false sector resulting from a cell of the Can^R Gen^S Hyg^R His^+ Leu^+ Ade^- red phenotype (reflecting a previous reciprocal crossover) being adjacent to a wild type MAB6 cell at the time of plating. Experiments in which we mixed cells of the Ade^- red phenotype with cells of the Ade^+ white phenotype indicate that most of the sectors indicative of a class 1 event are not false sectors (data not shown). In summary, we conclude that hydroxyurea treatment stimulates both reciprocal crossovers and BIR events.

6. Stimulation of reciprocal crossovers and other mitotic recombination events by ultraviolet light (UV). Although the lesion(s) that triggers spontaneous mitotic recombination still remains elusive, it is known that a variety of agents causing different types of lesions stimulate mitotic recombination. One of those lesions is a DSB, the same lesion that initiates meiotic recombination. Examples of DSBs that stimulates mitotic recombination are those induced by the HO-endonuclease (Haber, 1992) or γ-irradiation (Symington, 2002). A variety of compounds such as hydroxyurea, where the initiating lesion has not been defined, also stimulate mitotic recombination. Although in these cases the lesion is unknown, it is assumed that they are probably nicks or ssDNA gaps that then may result in a DSB. UV-irradiation has also been reported to stimulate mitotic recombination; this is an interesting finding because the main lesions produced by UV are cyclobutane

pyrimidine dimers and 6-4 photoproducts that are repaired by the nucleotide excision repair (NER) system (Prakash and Prakash, 2000).

Most of the studies done in the past to examine the effect of UV on recombination used haploid yeast cells (Ira *et al.*, 2003;Lopes *et al.*, 2006) or diploids hemizygous at the mating type locus (Kadyk and Hartwell, 1993) where the nucleotide excision repair and/or other DNA repair systems were compromised. We decided to use our system to analyze the effect of UV-light on reciprocal mitotic crossovers between the homologues chromosomes in diploid cells with proficient DNA repair systems. MAB6 cells were grown on YPD for 2 days and plated on canavanine-containing medium. Immediately after plating them, the cells were irradiated with 20 J/m^2 UV and allowed to form colonies for 4 days in the dark. The data for this experiment are in Table 2.3 and Figure 2.9.B. The rate of reciprocal crossovers was 3.5×10^{-3}, a 175-fold increase compared to unirradiated cells. The rates for classes 1 and 2 were ~22-fold higher. Since the cells were treated with UV after plating them to canavanine, the increases in classes 1 and 2 are probably the result of BIR events that occurred soon after treatment. Conversion events (classes 3 and 4) were elevated ~40- to 50-fold. Interestingly, the rates for classes 1 – 4 were very similar suggesting that stimulation of conversion events is higher in UV- treated than in hydroxyurea-treated cells. Chromosome loss events (classes 5 and 6) were stimulated ~15-fold. The rate of survival of the irradiated cells in this experiment was 90% when compared to unirradiated cells.

As in the experiment with hydroxyurea, because of the increase in mitotic recombination events when the cells were treated with UV, we decided to analyze these events using nonselective methods. We grew the colonies on rich medium for 2 days, plated them to nonselective medium (SD-Arginine + 10 µg/ml adenine) and then irradiated them

with 20 J/m^2 UV. The cells were allowed to form colonies for 3 days in the dark and then screened for red/white sectors. This is an ongoing experiment, but of the 1718 colonies analyzed so far, 11 had the phenotype expected for a reciprocal crossover (6.4 x 10^{-3}), 6 had the phenotype expected for a BIR (3.4 x 10^{-3}), and 3 the phenotype expected for a conversion event (1.7 x 10^{-3}).

7. Effect of mating-type heterozygosity on the rate of mitotic recombination and chromosome loss. Haploid yeast cells carry either the *MATa* or *MATα* allele which defines their mating type. Two haploid cells, one of each type, fuse and produce a *MATa*/*MATα* diploid cell. *MATa*/*MATα* is the normal genotype but diploid cells can also be homozygous at the *MAT* locus (*MATa*/*MATa* or *MATα*/*MATα*). From the analysis of such strains, it has been demonstrated that in diploid cells the mating-type alleles are involved in the expression of mating ability, sporulation, and resistance to ionizing radiation (Baker *et al.*, 1976; Crandall *et al.*, 1977).

It has also been reported that the mating-type alleles are involved in mitotic recombination; this effect was first observed by Friis and Roman (Friis and Roman, 1968) who determined that UV-induced intragenic recombination was 3- to 6-fold lower in *MATa*/*MATa* and *MATα*/*MATα* diploids than in closely related *MATa*/*MATα* hybrids. In contrast to those results, when Kadyk and Hartwell (Kadyk and Hartwell, 1992) examined the effect of the mating-type alleles on X-rays induced mitotic recombination, they did not see any difference on intragenic recombination or on unequal sister chromatid recombination in *MATa*/*MATa* compared to *MATa*/*MATα* diploid cells. Another study (Klein, 2001)

reported a 4.6-fold decrease in spontaneous mitotic recombination at the *CAN1* locus and a 1.6-fold increase in chromosome loss in a diploid strain hemizygous at the *MAT* locus (-/*MATα*) compared to the heterozygous diploid. Since those studies were done using different assays and strain backgrounds, we decided to examine the effect of the mating-type alleles on spontaneous and induced mitotic recombination and chromosome loss using our system.

We examined the effect of mating type on spontaneous mitotic recombination by constructing two diploids that were isogenic with MAB6, except that one of them was deleted for the *MATα* locus (MAB35) and one was deleted for the *MATa* locus (MAB38). As shown in Figure 2.10.A and Table 2.2, MAB35 and MAB38 had about the same rates of reciprocal crossovers and class 1 – 4 events as we observed in MAB6, demonstrating that heterozygosity at the mating type locus does not affect spontaneous mitotic recombination. Chromosome loss, however, was elevated 3- to 5-fold in MAB35 and MAB38.

We then examined the effect of the mating type alleles on recombination and chromosome loss induced by UV-light and hydroxyurea. We found different effects depending on the DNA-damaging agent used. In the case of UV, our results show that reciprocal crossovers and classes 1 – 4 were reduced 2- to 5-fold in MAB35 compared to MAB6 and we did not see any effect on chromosome loss (Figure 2.10.B and Table 2.3). In contrast, the results for the hydroxyurea experiment were similar to those under spontaneous conditions; there was no difference on recombination between MAB35 and MAB6 and chromosome loss was elevated 4-fold in the hemizygous strain (Figure 2.10.C and Table 2.3).

C. Discussion

The system that we have described allows an accurate measurement of the rate of spontaneous mitotic reciprocal crossovers. For the 120-kb *CEN5*-*CAN1* interval, this rate is 4×10^{-5} per cell division. Assuming this rate is the average for the genome, we calculate that the chance of a reciprocal crossover within the 14-Mb yeast genome is ~0.5% per cell division. Thus, one would expect that genetic variants that arise in diploid cells to become homozygous fairly quickly. The meiotic genetic distance between *CEN5* and *CAN1* is 51 cM, indicating approximately one crossover per meiotic cell. For the same interval, therefore, meiotic crossovers are 25,000-fold more frequent per division than mitotic crossovers.

Our data do not provide unambiguous evidence for spontaneous BIR events, but we cannot rule out the possibility that some of the class 1 and 2 events reflect BIR. McMurray and Gottschling (McMurray and Gottschling, 2003) found that most mitotic recombination events observed in nonselected "young" diploid cells were reciprocal, and our data are consistent with this conclusion. McMurray and Gottschling also found that the frequency of nonreciprocal recombination events increased in old cells (cells that had undergone >20 divisions). Because our experiments involve exponentially growing cultures, almost all of the cells in our experiments are young.

Meiotic recombination events are distributed nonrandomly along the chromosomes (Petes, 2001). Recombination rates are controlled both regionally (suppression of recombination at the telomeres and centromeres) and locally (for example, by local G-C content and transcription factor binding). Although no detailed mitotic recombination maps have been constructed in yeast or any other eukaryote, a number of factors have been

associated with elevated levels of mitotic recombination, including high levels of transcription, stalled DNA replication forks, and inverted repeated DNA sequences capable of forming secondary structures (cruciforms and "hairpins") (Freudenreich *et al.*, 1998; Aguilera, 2002;Lobachev *et al.*, 2002).

Our method can be used for any chromosome and any interval by constructing strains in which the *can1-100, SUP4-o*, and the drug resistance markers are inserted into the appropriate positions. By using two parental haploids that have sufficient sequence divergence to provide polymorphisms at ~1-kb intervals, a fine-structure mitotic crossover map could be constructed. Such maps are likely to be informative about the mechanisms of mitotic crossovers. For example, one could determine whether "hotspots" for mitotic crossovers correlated with highly expressed genes, inverted repeats, or regions with stalled replication forks.

It has been reported that long CTG tracts stimulate spontaneous ectopic exchange (Freudenreich *et al.*, 1998) and unequal SCE (Nag *et al.*, 2004) during vegetative growth in haploid yeast cells. We tested if a $(CTG)_{115}$ tract would also stimulate exchange between the homologous chromosomes and found that the rates for mitotic recombination between the homologs in the strain carrying the $(CTG)_{115}$ tract (MAB33) were very similar to the rates in the strain without the tract (MAB6). In addition, we did not see any effect on chromosome loss. There are several possible explanations for this result. One possibility is the type of recombination being examined. Freundereich *et al.* were looking at ectopic mitotic exchange (recombination between two direct repeats) in a haploid strain, and we analyzed recombination between homologous chromosomes in a diploid strain. Alternatively, the lack of effect could be due to the length of the tract. Freundereich *et al.* detected the most

pronounced increase in recombination with tracts that contained over 200 repeats (> 27-fold), with a more subtle (5-fold) stimulation for 130 repeats. Lastly, the chromosome may repair the damage using the sister chromatid as template rather than the homologous chromosome which is something we cannot detect with our system.

Hydroxyurea-treated cells had elevated rates of mitotic crossovers, local gene conversion, BIR events and chromosome loss. Because hydroxyurea treatment leads to slow progression of DNA replication forks (Vassilev and Russev, 1984;Santocanale and Diffley, 1998) and hydroxyurea-stimulated increases in recombination are observed in cycling but not arrested yeast cells (Galli and Schiestl, 1996), it is likely that hydroxyurea treatment leads to stalled replication forks that are susceptible to DSBs. Some of the resulting DSBs are repaired by using both broken ends to generate a local gene conversion event or a reciprocal crossover, whereas others are repaired by using only a single end, resulting in a BIR event. Although it is clear that DSBs stimulate recombination (Paques and Haber, 1999), it should be emphasized that the DNA lesions responsible for spontaneous mitotic events and hydroxyurea-induced events have not been demonstrated to be DSBs.

Treatment of diploid cells with 20 J/m^2 UV-light, a dose that results in 90% survival of the cells, stimulated mainly reciprocal crossovers but also the rest of mitotic events. A dose of 100 J/m^2 produces 1 photoproduct (PP) every 1 kb; 85% of them are pyrimidine dimers and 15% are 6-4 photoproducts (Jiang and Sancar, 2006). Based on that data, a dose of 20 J/m^2 will create 1 PP every ~5 kb (~24 PP will be formed in the 120 kb interval between *CEN5* and *CAN1*) indicating that PPs are not rare and form pretty frequently. The observed rate of UV-stimulated reciprocal crossovers of 3.5 x 10^{-3} indicates that 1 out of 285 cells had a reciprocal crossover in the interval being examined. Since the NER system

discriminates between the two photoproducts created by UV-light and tends to repair more efficiently 6-4 photoproducts than cyclobutane pyrimidine dimers (Reardon and Sancar, 2003), one interpretation of these observations is that some of the pyrimidine dimers probably remain unrepaired and are likely to become a barrier or obstacle for the replication fork during replication causing them to stall or break. This may trigger a lesion that can then be repaired by the recombination machinery.

As demonstrated by the hydroxyurea and UV-light experiments, recombination is an important mechanism for the repair of induced DNA damage present in the cells. Reciprocal mitotic crossovers were stimulated by both DNA-damaging agents, although UV stimulated mitotic crossovers more efficiently. Although we do not know the recombinogenic lesions generated in hydroxyurea- and UV-treated cells, the observation that these two agents had different effects on the various classes of mitotic events argues that these two treatments produce different types of recombinogenic lesions. In a genetic screen looking for diploid yeast cells that would present an increase in LOH at the 3 different loci that were being examined, D.E. Gottschling found mutants that mimicked the results observed in our experiments (http://videocast.nih.gov/PastEventDetail.asp?13215). Mutants that are critical in DNA replication (*top1, rrm3, dph3, pol32*) had an increase mainly in reciprocal crossovers. These results taken together, argue that hydroxyurea- and UV-induced lesions perturb DNA replication and that most of the damage is being repaired by the recombination machinery generating reciprocal crossovers.

We found that heterozygosity at the mating-type locus had no effect on spontaneous or hydroxyurea-induced mitotic recombination events; hemizygosity at the mating type locus, however, resulted in an increase in chromosome loss in those two experiments. In

contrast to those results, and consistent with Roman data (Friis and Roman, 1968), we found that UV-induced mitotic recombination (reciprocal and nonreciprocal) was 2- to 5-fold lower in the hemizygous diploid strain (MAB35) than in the heterozygous diploid (MAB6). The results of the hydroxyurea experiment are similar to Hartwell's experiments with X-rays (Kadyk and Hartwell, 1992), suggesting that the lesion produced by those two damaging agents and the cell's response to the damage is similar and does not involve the *MAT* locus. Although our results on spontaneous chromosome loss are consistent with Klein's findings (Klein, 2001), it is unclear at present why the results on spontaneous mitotic recombination are different. In conclusion, it is clear that mating type heterozygosity affects the rate of some types of mitotic recombination under certain conditions, although these effects in general are subtle.

The observed increase in chromosome loss in *MATa/matαΔ* and *mataΔ/MATα* diploids is consistent with the observation that the stability of centromere-containing plasmids is higher in diploids that are heterozygous at the mating-type locus than in homozygous diploids (Steinberg-Neifach and Eshel, 2002). Because the heterozygous diploids are less sensitive to microtubules-depolymerizing drugs than the homozygous diploids, Steinberg-Neifach and Eshel argue that heterozygous diploids have more stable microtubules, leading to lower rates of chromosome loss. Our results are consistent with this possibility.

In summary, the system that we have developed should be a useful tool for investigating the mechanisms involved in mitotic recombination and the repair of DSBs and other lesions.

D. Materials and Methods

Genetic Analysis and Media. The rich growth medium (yeast extract/ peptone/ dextrose, YPD), sporulation medium, and various types of omission media were standard (Guthrie and Fink, 1991). Strains were grown at 30C unless otherwise noted. Mating, transformation, and tetrad dissection procedures were also standard.

Strain Construction. All strains in this study were isogenic with W303a (*a leu2-3,112 his3-11,15 ura3-1 ade2-1 trp1-1 can1-100 rad5-535*; (Thomas and Rothstein, 1989)) except for changes introduced by transformation or crosses with isogenic strains. All strains were *RAD5*. The sequences of oligonucleotides used in the constructions, the genotypes of the haploid strains, and the genotypes of the diploid strains are in Tables 2.4, 2.5, and 2.6, respectively.

The haploid strain MAB1 was constructed in two steps. First, RCY317-9a (a *MAT*α *RAD5* derivative of W303a) was a spore derived from the diploid RCY317. To introduce the *HYG*^{R} within chromosome V, centromere-distal to *can1-100* on the left arm, we transformed RCY317-9a with a PCR fragment generated by amplifying the *HYG*^{R}-containing plasmid pAG32 (Goldstein and McCusker, 1999) with the primers CHRVF and CHRVR. The resulting strain (PG128.1) has an insertion of the *HYG*^{R} gene between bases 9229 and 9230 of chromosome V; this allele is *V9229::HYG*. Strain PG128.1 was transformed with a PCR fragment generated by amplifying yeast genomic DNA with primers PG/HIS3F and PG/HIS3R. The resulting strain (MAB1) has an insertion of *HIS3* between bases 261553 and 261554 on the right arm of chromosome V; this allele is *V261553::HIS3.*

The haploid strain MAB4 was constructed in three steps. First, RCY317-16c (a *MATa RAD5* derivative of W303a [spore of RCY317]) was transformed with a PCR fragment generated by amplifying the plasmid p.FAG-KANMX, which contains the *KANMX* gene (Wach *et al.*, 1994), with the primers CHRVF and CHRVR. The resulting strain (MD235) has an insertion of the *KANMX* gene between bases 9229 and 9230 of chromosome V; this allele is *V9229::KANMX*. This insertion is at the same position on chromosome V as the HYG^R gene in MAB1. The strain MD235 was transformed with a PCR fragment generated by amplifying the plasmid YCpMP2 (which contains the *SUP4-o* gene; (Pierce *et al.*, 1987)) with the primers SUP/CANF and SUP/CANR. In the resulting strain (MD242-2), the *can1-100* allele is replaced by the *SUP4-o* gene. Lastly, the strain MAB4 was created by transforming MD242-2 with a PCR fragment generated by amplifying yeast genomic DNA with primers MD/LEU2F and MD/LEU2R. The resulting strain (MAB4) has an insertion of *LEU2* between bases 261553 and 261554 of chromosome V; this allele is *V261553::LEU2* and is at the same position as *HIS3* in MAB1.

Two other haploid strains related to MAB1 were also constructed. MAB10 was made by transforming MAB1 with a PCR fragment generated by amplifying yeast genomic DNA with primers wtURA3F and wtURA3R. In this strain, the wild type *URA3* gene replaces *ura3-1* at its native locus in chromosome V. The strain MAB50 was formed by transforming MAB10 with a PCR fragment generated by amplifying yeast genomic DNA with primers TRP1F and TRP1R. The new strain (MAB50) has the wild type *TRP1* gene replacing the *trp1-1* allele at its native locus on chromosome IV.

MAB29 and MAB31 are haploids containing a $(CAG)_{115}$ tract inserted in the *ura3-1* locus in chromosome V. To construct MAB29, the pMAB1 plasmid was digested with the restriction enzyme *NcoI*, which cuts solely within the *URA3* gene, and transformed into MAB1. MAB31 was made by transforming the digested plasmid into MAB4.

The plasmid pMAB1 was derived from pWJH8 (obtained from P. Detloff, University of Alabama-Birmingham). pWJH8 is pUC19 with an insertion of a tract of ~115 CAG between the *AatII* and *BamH1* restriction sites of the plasmid. To construct pMAB1, a PCR product of a wild type *URA3* gene was created by amplifying genomic DNA with the primers URA3 upstream HIII F and URA3 upstream HIII R. The wild type *URA3* gene was inserted between the *BamH1* and *HindIII* restriction sites of the pWJH8 plasmid. pMAB1 consists of a pUC19 plasmid with a tract of ~115 CAG and a wild type *URA3* gene.

The diploids MAB6, MAB13, MAB54 and MAB33 were made by crossing MAB1 and MAB4, MAB10 and MAB4, MAB50 and MAB4, and MAB29 and MAB31, respectively (Table 2.6). The diploid strains MAB35 and MAB38 were derived from the diploid MAB6. The strains are hemizygous for mating type information, unlike the heterozygous *MATa/MATα* MAB6 strain. The MAB35 strain (*MATa/matαΔ::URA3*) was constructed by transforming MAB6 with a PCR fragment generated by amplifying yeast genomic DNA with primers Malpha2/URA3F and Malpha1/URA3R, and MAB38 (*mataΔ::URA3/MATα*) was made by transforming MAB6 with a PCR fragment generated by amplifying yeast genomic DNA with primers MATA2/URA3F and MATA1/URA3R.

Detection of Mitotic Recombination Events in MAB6 and Related Strains.

Diploid cells were streaked for single colonies on rich growth medium (YPD) and incubated at 30C. After 2 days, independent colonies were picked, resuspended in water, and plated on solid medium lacking arginine (SD-Arg), or SD-Arg with 120 μg/ml of canavanine. Four days after plating, the Can^R colonies were replica-plated to SD-Arg media containing canavanine and lacking histidine or leucine, SD-Arg media containing canavanine and, in addition, containing hygromycin (300 μg/ml) or geneticin (200 μg/ml), and to SD-adenine media. All omission media (except medium lacking adenine completely) had 10 μg/ml adenine (which is 2-fold less than the standard omission media). After replica-plating, cells were incubated at 30C for 2 days and then transferred to 5C for 1 day. The incubation in the cold results in a clearer distinction between the red and white color. The MAB6 strain and related strains that have one copy of *SUP4-o* grow partially on plates lacking adenine ($Ade^{+/-}$ phenotype), but derivatives with two copies of *SUP4-o* (for example, classes 2 and 4) grow completely on plates lacking adenine (Ade^+ phenotype).

In experiments involving HU, cells were grown for 3 days at 30C on rich growth medium containing 100 mM HU. Subsequent analysis of these colonies was the same as for the cells grown in the absence of HU. For the UV experiments, cells were grown and plated the same as the cells grown on YPD and then irradiated with 20 Joules/m^2 using a shortwave ultraviolet UVP CL-1000 crosslinker. After irradiation, the plates were kept in the dark at 30C for 4 days. Subsequent analysis of these colonies was the same as for the rest of experiments.

On most of the plates containing canavanine, the Can^R colonies were easily distinguishable from Can^S background. A small fraction of the plates (< 5 %), however, had an increased background of small colonies. For the MAB6 strain to have the Can^S phenotype, the strain must have efficient suppression of the *can1-100* mutation. This suppression requires both the *SUP4-o*-encoded tRNA and the prion Psi (ψ) factor. ψ is a misfolded version of a translation termination factor that increases the efficiency of nonsense suppression (Wickner et al., 2001). Although the ψ factor is reasonably stable in the W303a genetic background, it is likely that the background growth observed in a small fraction of colonies reflects progenitor cells with low level of ψ.

Two types of events gave rise to class 3 colonies, local gene conversion events (replacement of *SUP4-o* with *can1-100*) and additional mutations within the *SUP4-o* gene. These possibilities were distinguished by PCR analysis with a mixture of three primers (primer sequences in Table 2.4): VL33517R, VL32915F, and SUP4R. Class 3 colonies that represent a gene conversion event result in a single 600-bp PCR fragment, whereas class 3 colonies that represent a *de novo* mutation result in two fragments of 600 and 275 bp.

Microarray Analysis. The use of microarray analysis to determine gene dosage was done as described previously (Lemoine et al., 2005). In brief, DNA was isolated from each Can^R strain that was examined and labeled *in vitro* with Cy3-dUTP. DNA samples from the control MAB6 strain were labeled with Cy5-dUTP. The labeled samples were mixed and hybridized to glass slides that contained all yeast ORFs and intergenic regions. The slides

were subsequently scanned using a GenePix 4000B (Axon Instruments, Union City, CA) scanner and patterns were analyzed using Gene Spring 5.1 (Silicon Genetics, Palo Alto, CA).

Statistical Analysis. Rate calculations for classes 1-6 were done using the method of the median (Lea and Coulson, 1949), and 95% confidence limits for these rates were calculated as described (Wierdl et al., 1996). Calculations of 95% confidence limits on proportions were done by using VassarStats (http://faculty.vassar.edu/lowry/VassarStats.html), and calculations of 95% confidence limits on the rates of red/white sectors were done with an Excel spreadsheet.

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Figure 2.1. Selection of both products result of a Reciprocal Mitotic Crossover in the diploid MAB6.

The starting diploid, MAB6, is phenotypically His^{+} Leu^{+} $Ade^{+/-}$ Hyg^{R} Gen^{R} Can^{S} and forms white colonies.

A reciprocal crossover between the centromere and the *CAN1* locus will result in a Can^{R} colony with one red and one white sector, resulting from the growth of two Can^{R} cells, one with the genotype *can-100/can1-100*, and one with the genotype *SUP4-o/SUP4-o*. The red sector will be His^{+} Leu^{+} Ade^{-} Hyg^{R} Gen^{S} Can^{R} (*can1-100/can1-100*), and the white sector will be His^{+} Leu^{+} Ade^{+} Hyg^{S} Gen^{R} Can^{R} (*SUP4-o/SUP4-o*).

Mitotic Crossover

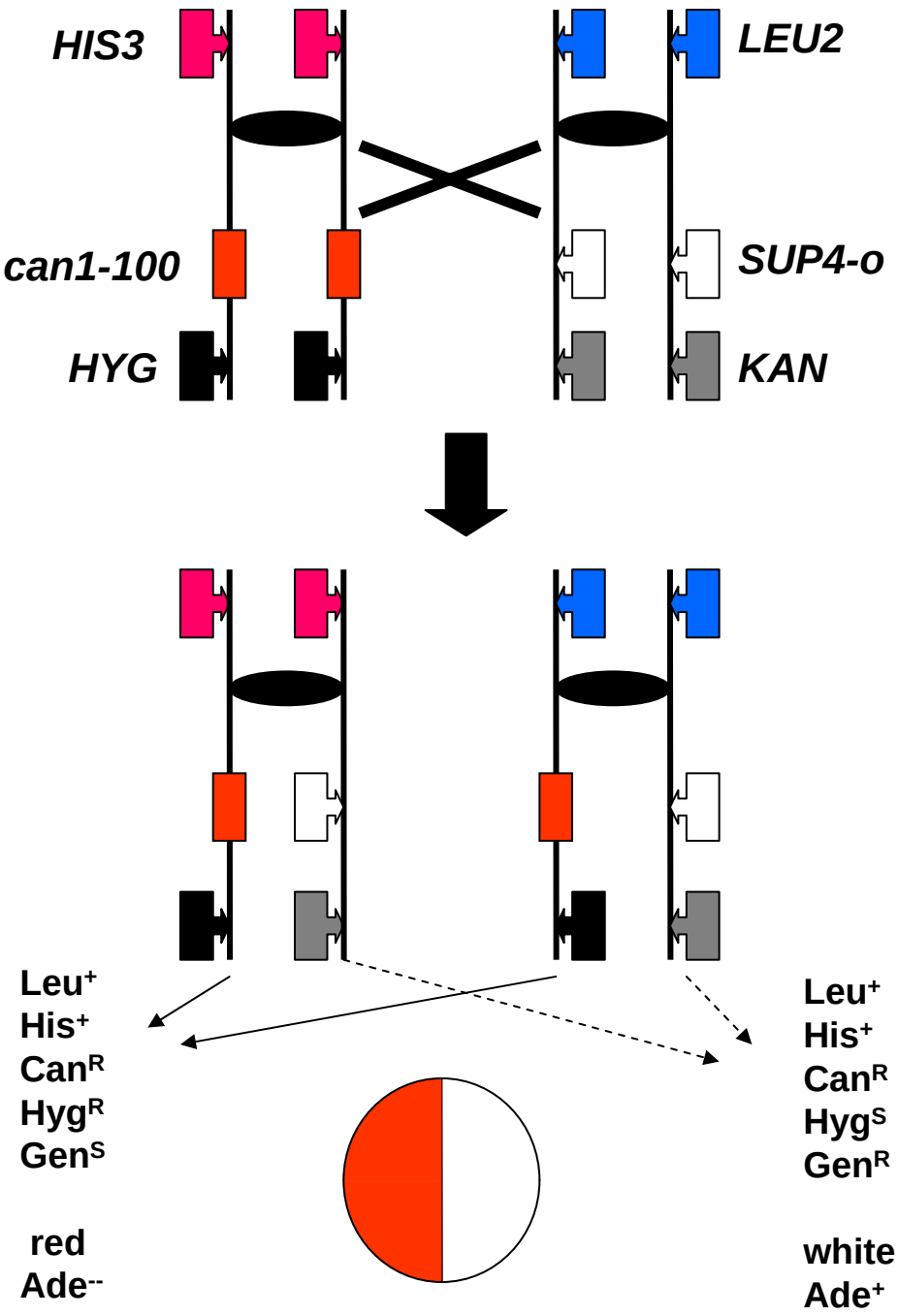
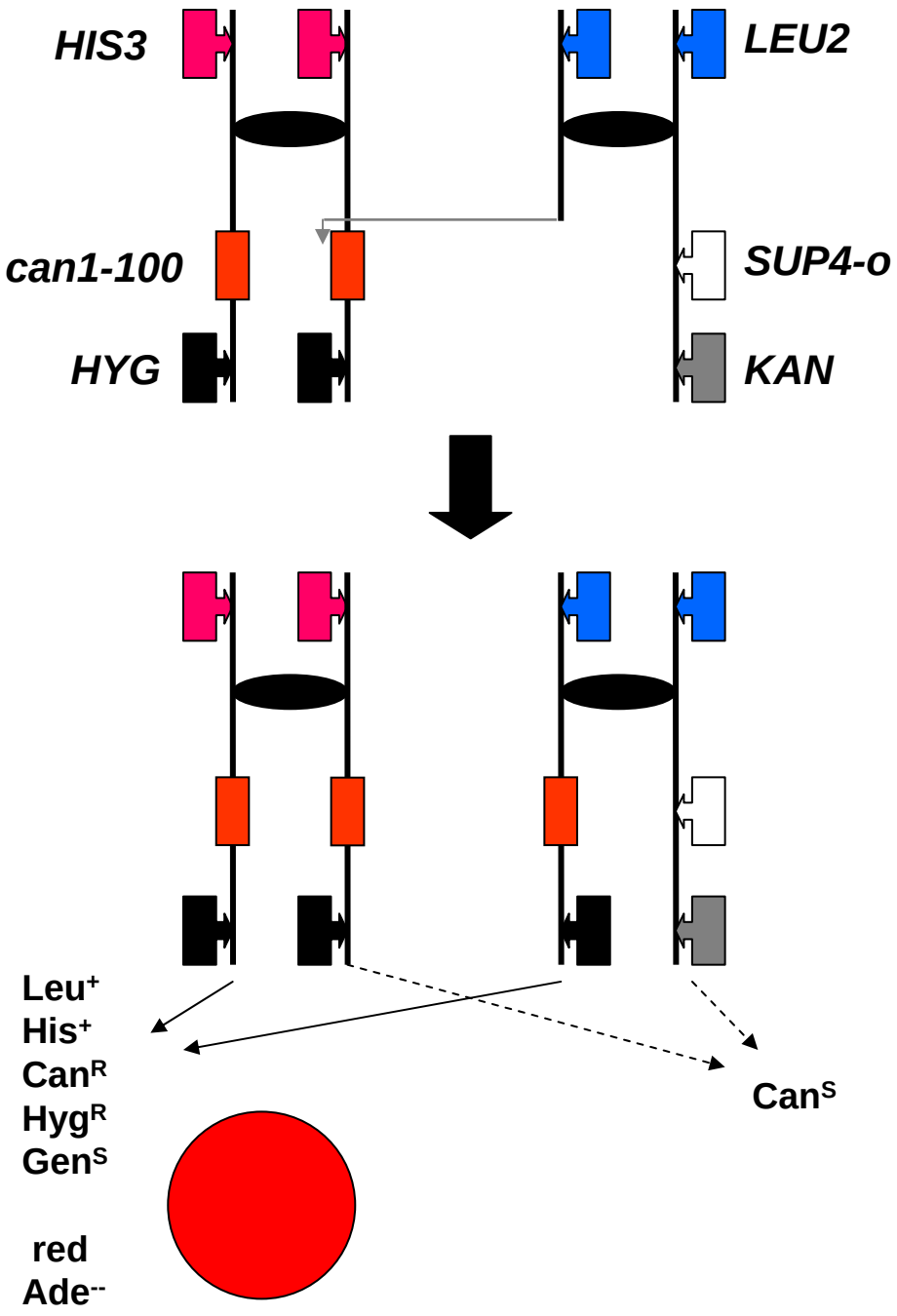
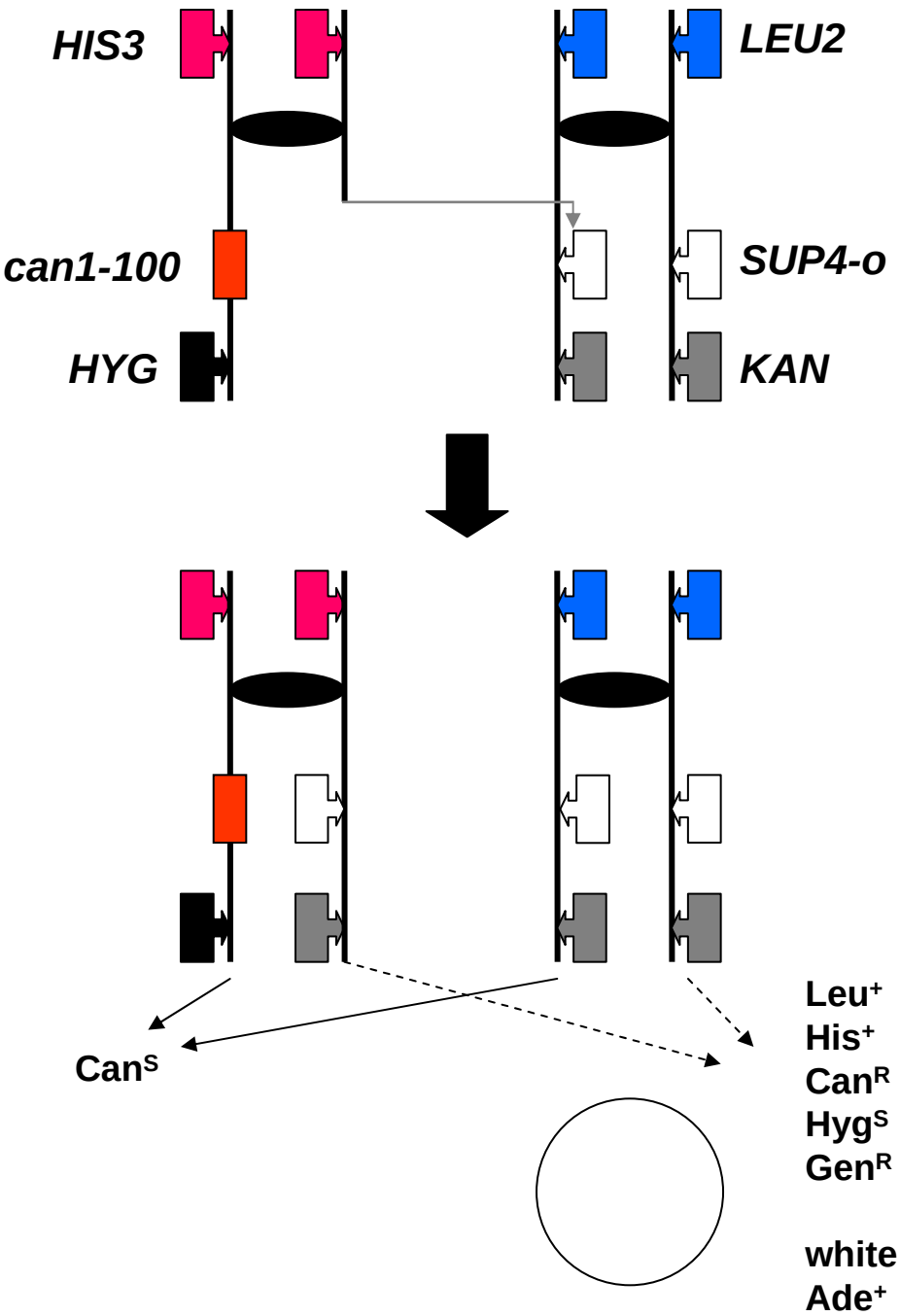


Figure 2.2. Phenotypic classes of unsectored Can^R colonies (derived from MAB6) resulting from nonreciprocal mitotic recombination or chromosome loss events. (A) Class 1: A Break-Induced Replication (BIR) event initiated in the *SUP4-o*-containing chromosome will give rise to one Can^R cell and one Can^S cell. Class 1 events also reflect a reciprocal crossover that ocurred in the culture, before the plating of the cells on medium containing canavanine. (B) Class 2: These BIR events are comparable to Class 1, except that the event initiates by breakage of the *can1-100*-containing homologue. (C) Class 3: A local gene conversion (unassociated with a crossover) in which *SUP4-o* is converted to the *can1-100* allele will produce a His$^+$ Leu$^+$ Ade$^-$ Hyg^R Gen^R Can^R red colony. The same phenotype can be produce by a new mutation within *SUP4-o*. These two possibilities can be distinguished by PCR. (D) Class 4: This class is similar to class 3 except *can1-100* is converted to *SUP4-o*. (E) Class 5: This class results from loss of the chromosome containing *SUP4-o*. (F) Class 6: This class results from loss of the chromosome containing *can1-100*.

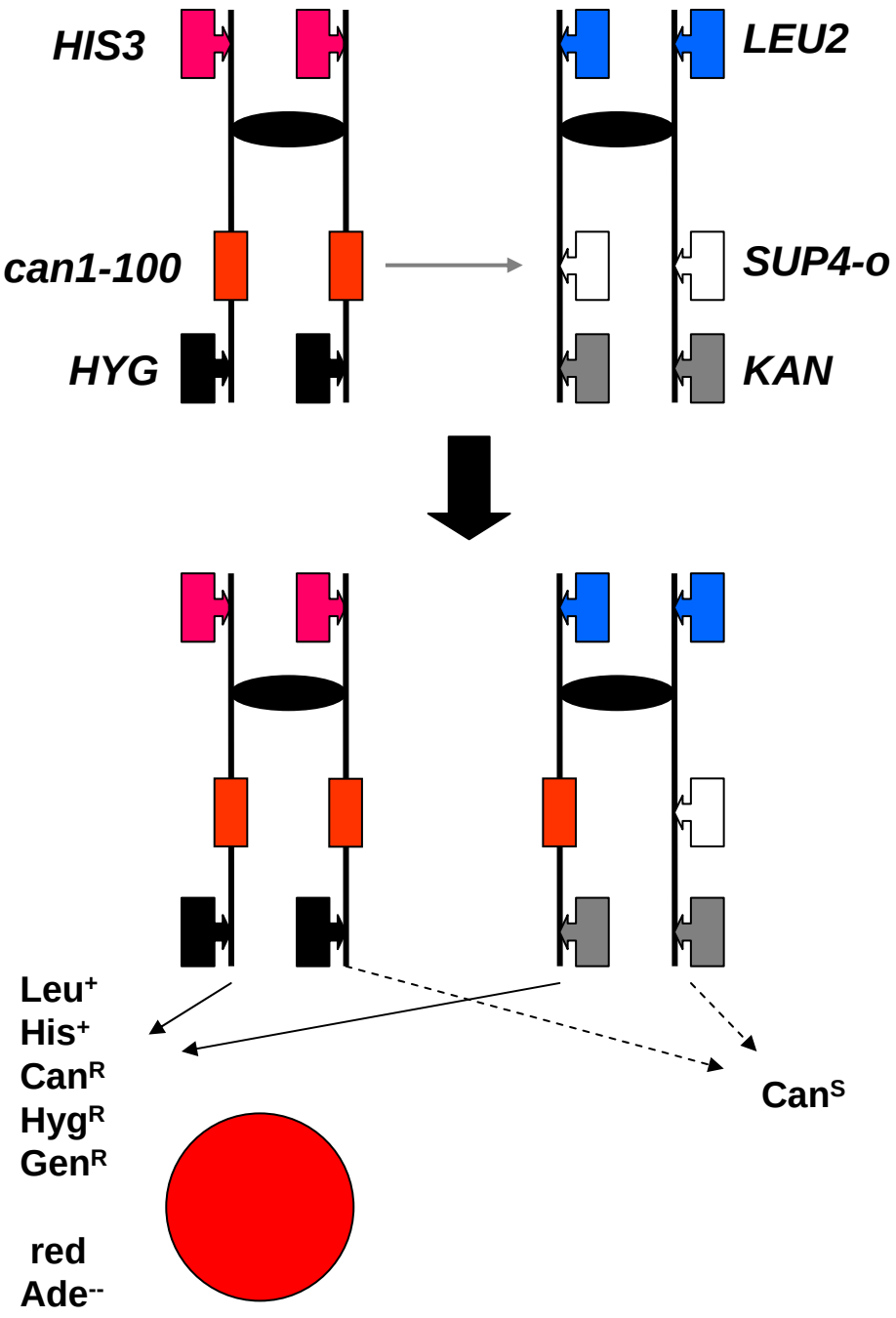
A. Break-Induced Replication (Class 1)



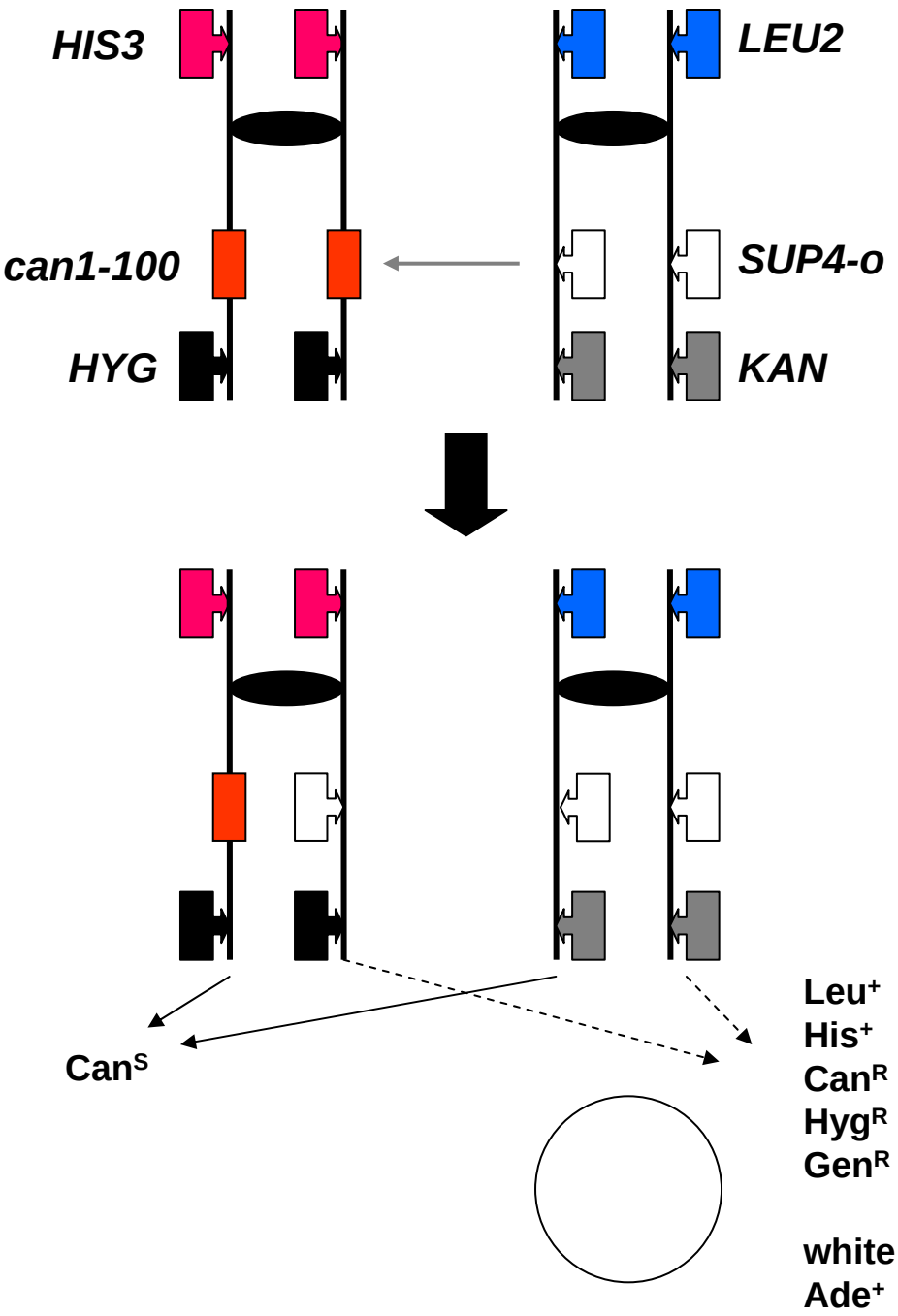
B. Break-Induced Replication (Class 2)



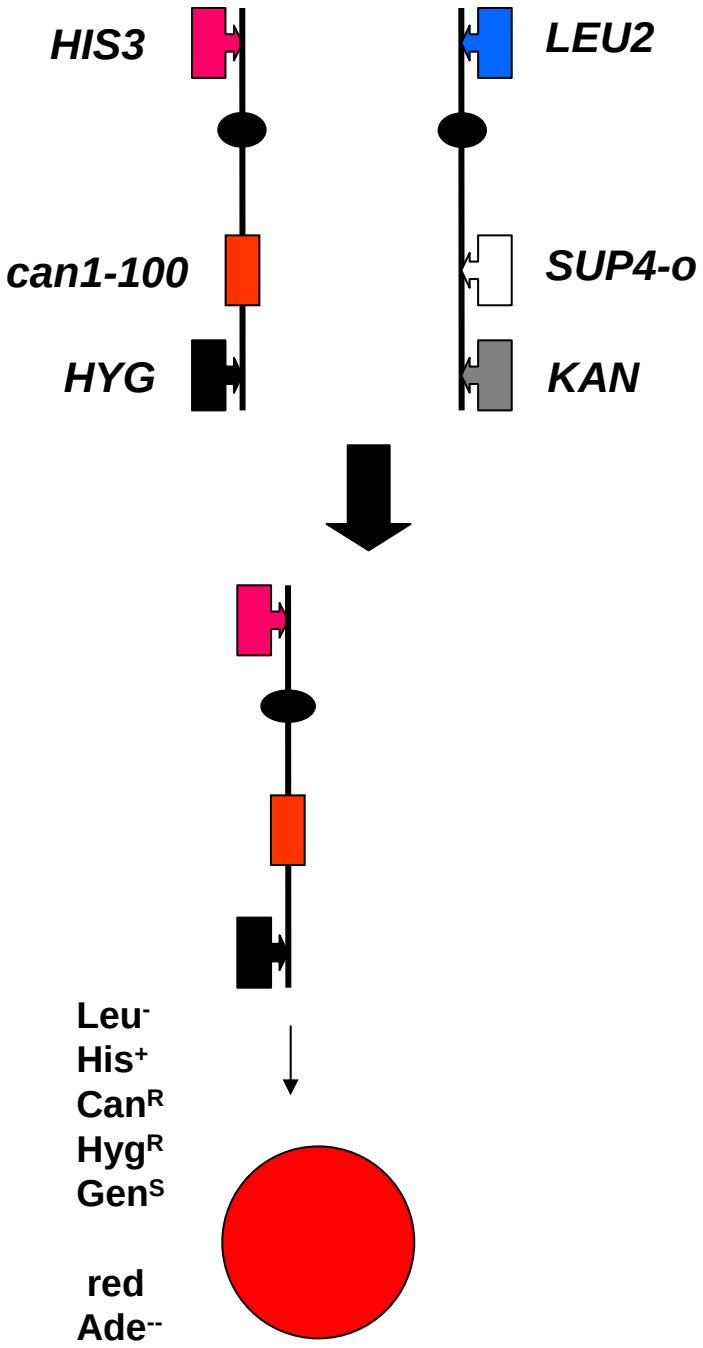
C. Local conversion (Class 3)



D. Local conversion (Class 4)



E. Chromosome loss (Class 5)



F. Chromosome loss (Class 6)

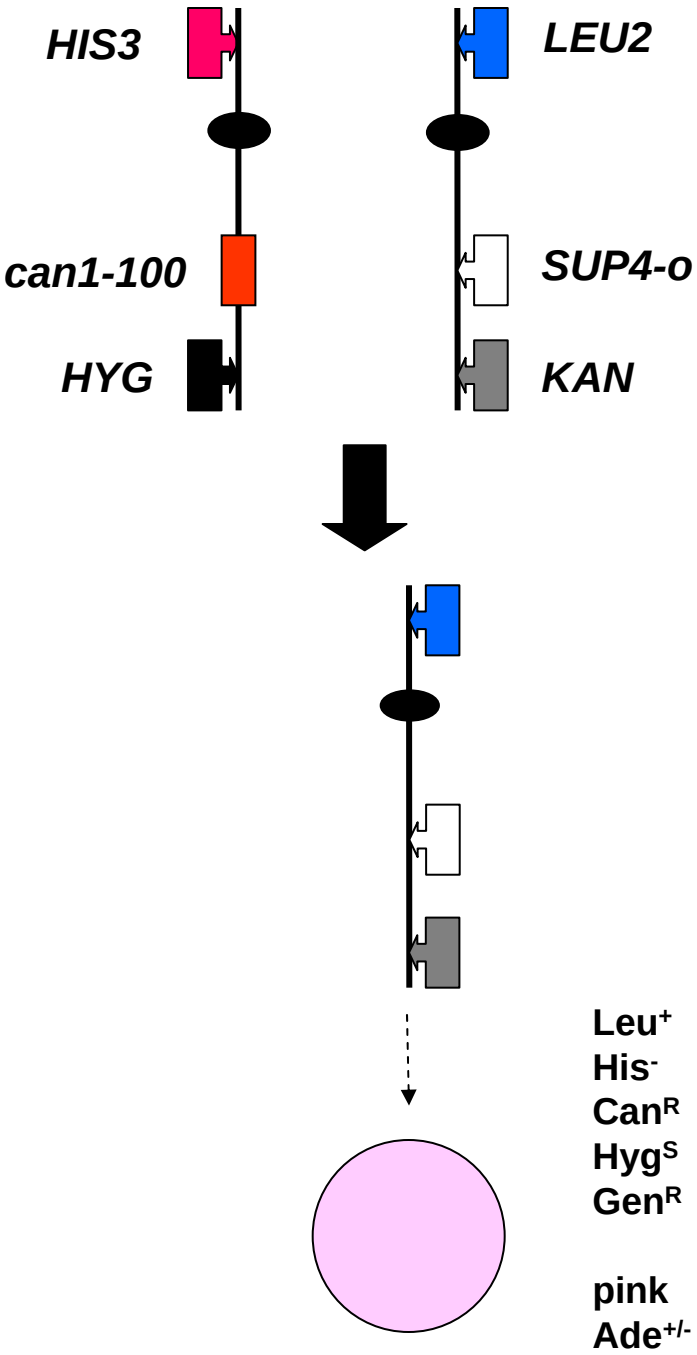
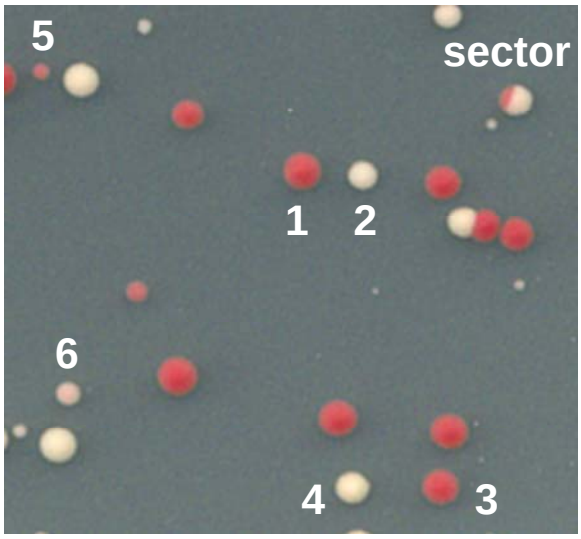
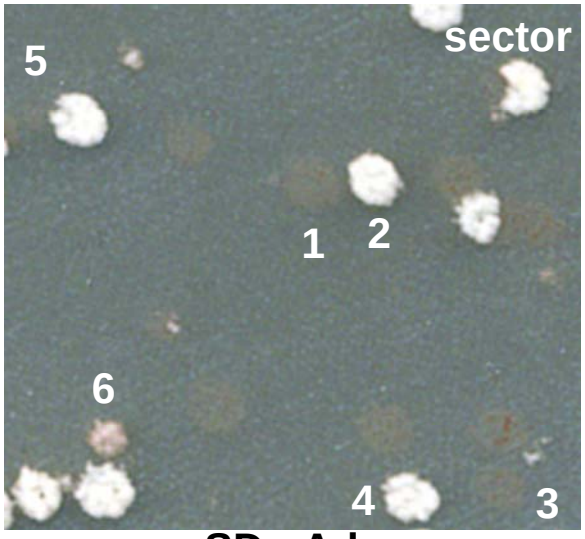


Figure 2.3. Photographs of the different classes of Can^R colonies.

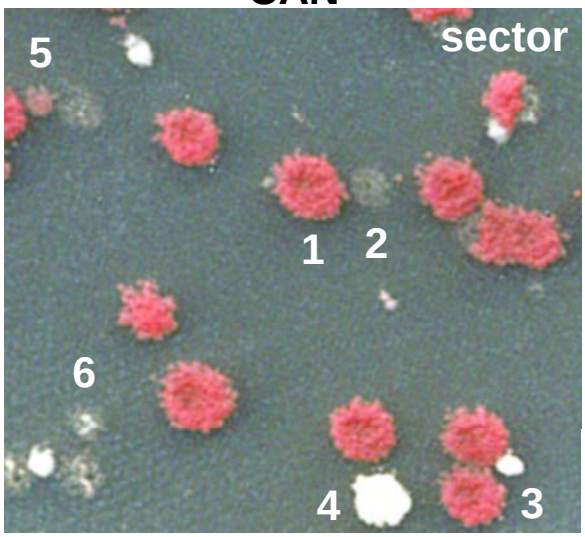
Cells of the MAB6 strain were allowed to form colonies on canavanine-containing medium and were then replica-plated to five different types of diagnostic media; those containing hygromycin or geneticin and those lacking adenine, histidine, or leucine. The colony marked "sector" reflects a reciprocal crossover, and the numbers represent classes 1 – 6.



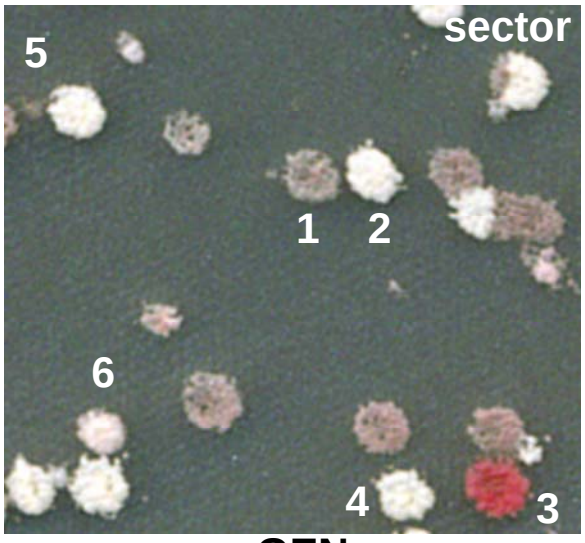
CAN



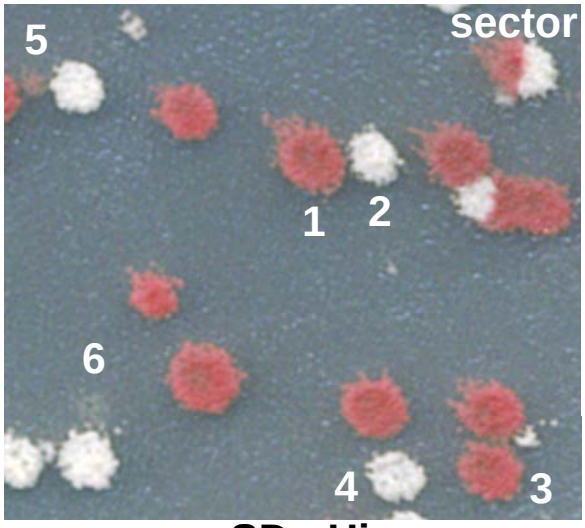
SD - Ade



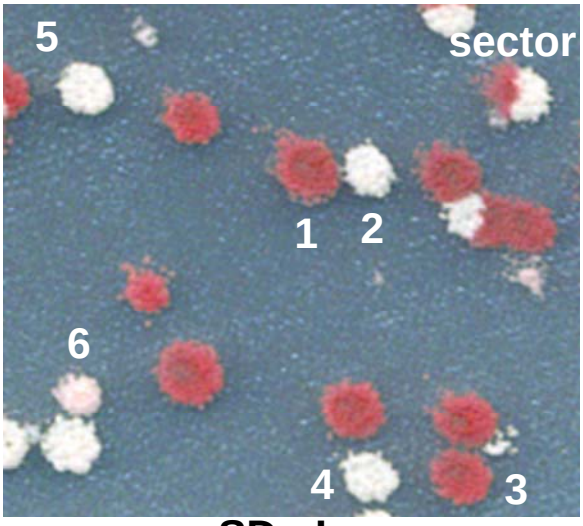
HYG



GEN



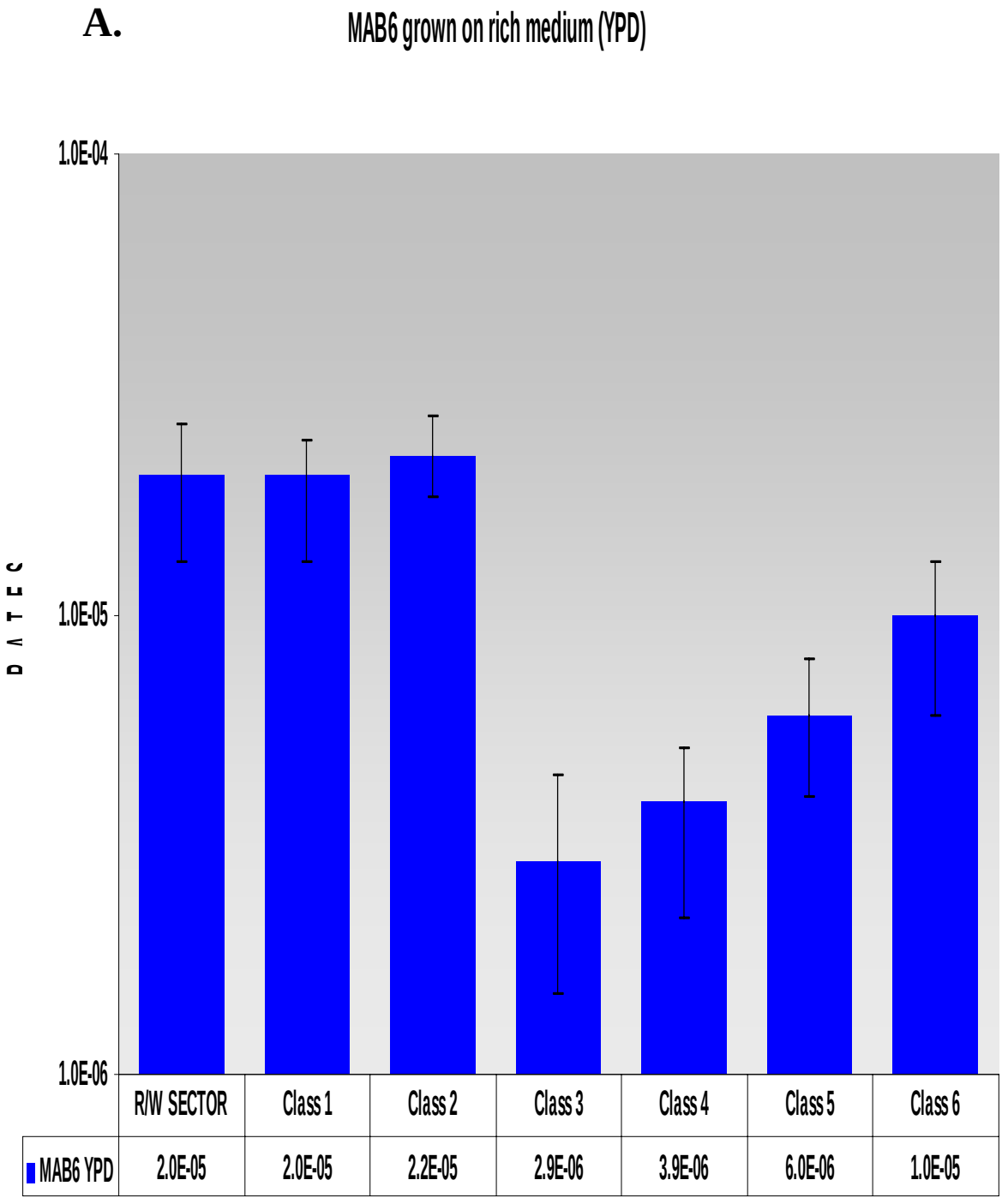
SD - His



SD - Leu

Figure 2.4. Comparison of rates of mitotic recombination and chromosome loss in MAB6. Rates of mitotic recombination and chromosome loss for **A)** MAB6 (*MATa/MATα*) grown on rich medium (YPD) for 2 days, and **B)** MAB6 grown on YPD for 2 days vs 3 days.

A.


MAB6 grown on rich medium (YPD)
1.0E-04
1.0E-05
1.0E-06
R A T E S
R/W SECTOR
Class 1
Class 2
Class 3
Class 4
Class 5
Class 6
MAB6 YPD
2.0E-05
2.0E-05
2.2E-05
2.9E-06
3.9E-06
6.0E-06
1.0E-05

B.

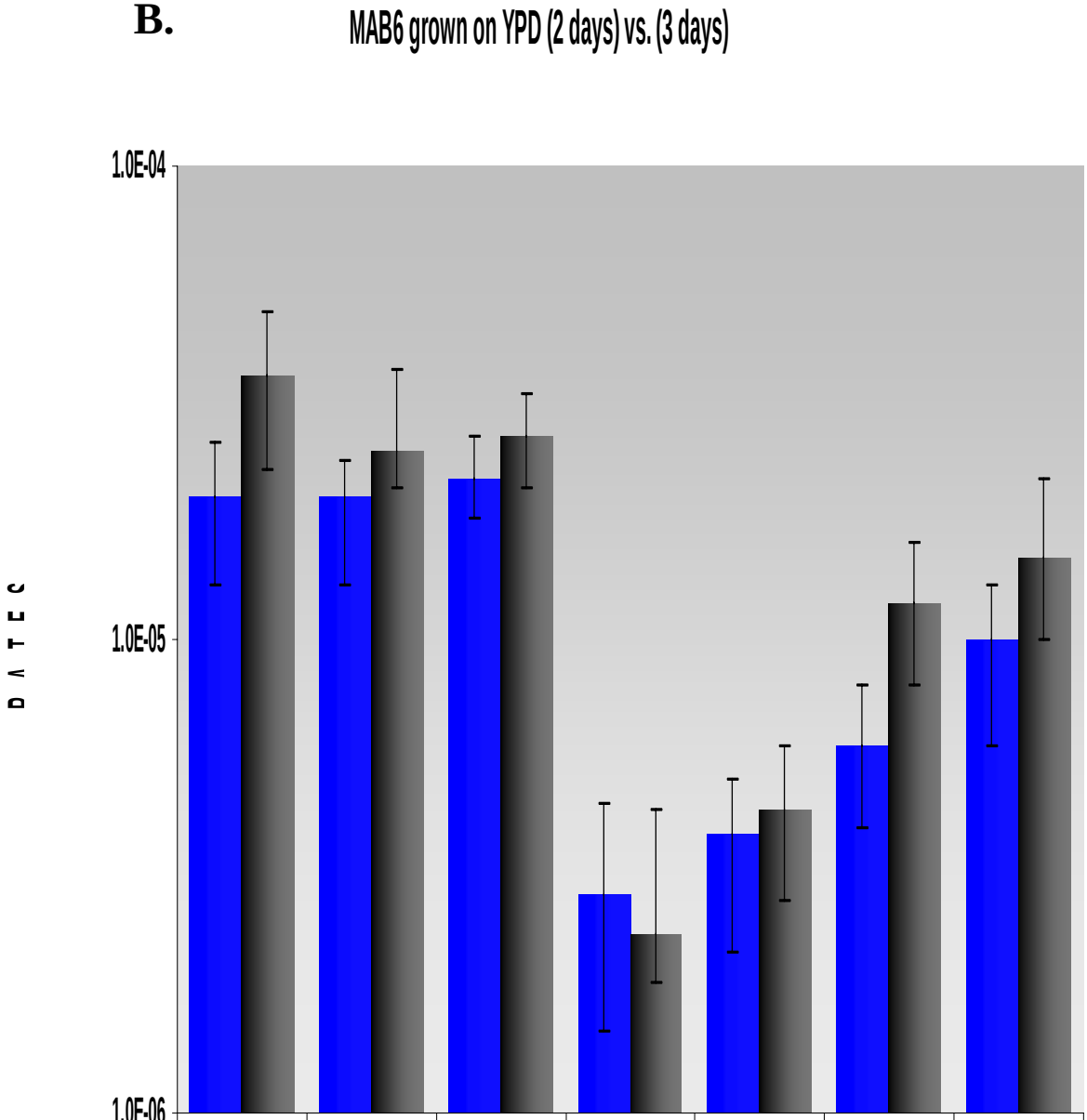

MAB6 grown on YPD (2 days) vs. (3 days)
1.0E-04
1.0E-05
1.0E-06
R/W SECTOR
Class 1
Class 2
Class 3
Class 4
Class 5
Class 6
MAB6 YPD 2days 2.0E-05 2.0E-05 2.2E-05 2.9E-06 3.9E-06 6.0E-06 1.0E-05
MAB6 YPD 3days 3.6E-05 2.5E-05 2.7E-05 2.4E-06 4.4E-06 1.2E-05 1.5E-05

Figure 2.5. Microarray analysis of a class 5 strain. DNA was isolated from a class 5 derivative of MAB6 (MAB6-V2). This DNA was labeled with Cy3-dUTP, and genomic DNA from the progenitor MAB6 strain was labeled with Cy5-dUTP. The two samples were hybridized in competition to DNA microarrays containing all yeast genes. The patterns of hybridization were analyzed using Gene Spring 5.1 software. Individual genes are shown as rectangles. Yellow indicates similar gene dosage in the MAB6-V2 and MAB6, blue indicates a lower gene dosage in MAB6-V2 than in MAB6, and red indicates a higher gene dosage in MAB6-V2 than in MAB6; genes shown in gray (including a region on chromosome XII) had too low a hybridization signal to score or are missing on the microarray. This microarray indicates that chromosome V is monosomic in MAB6-V2.

Chromosome

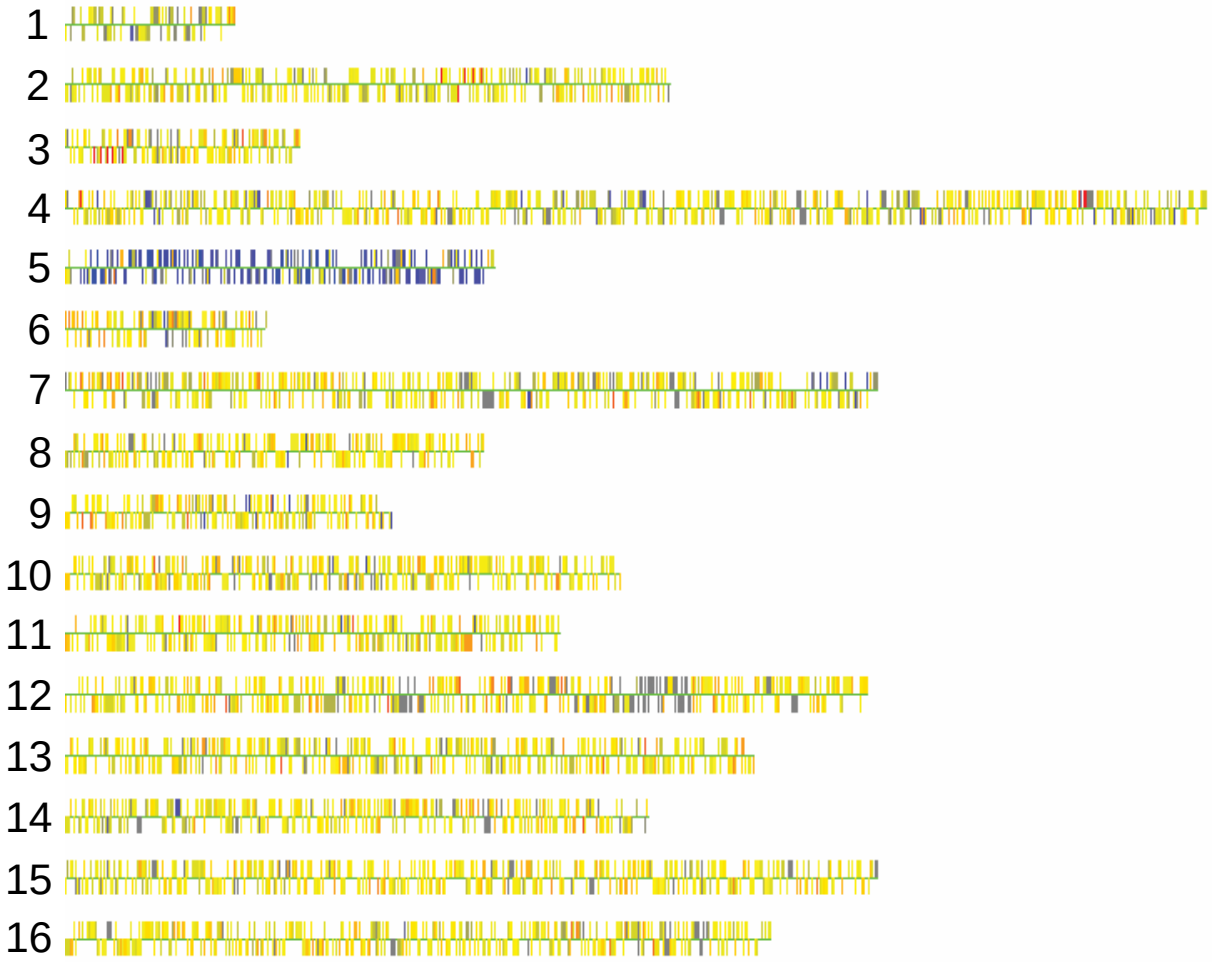
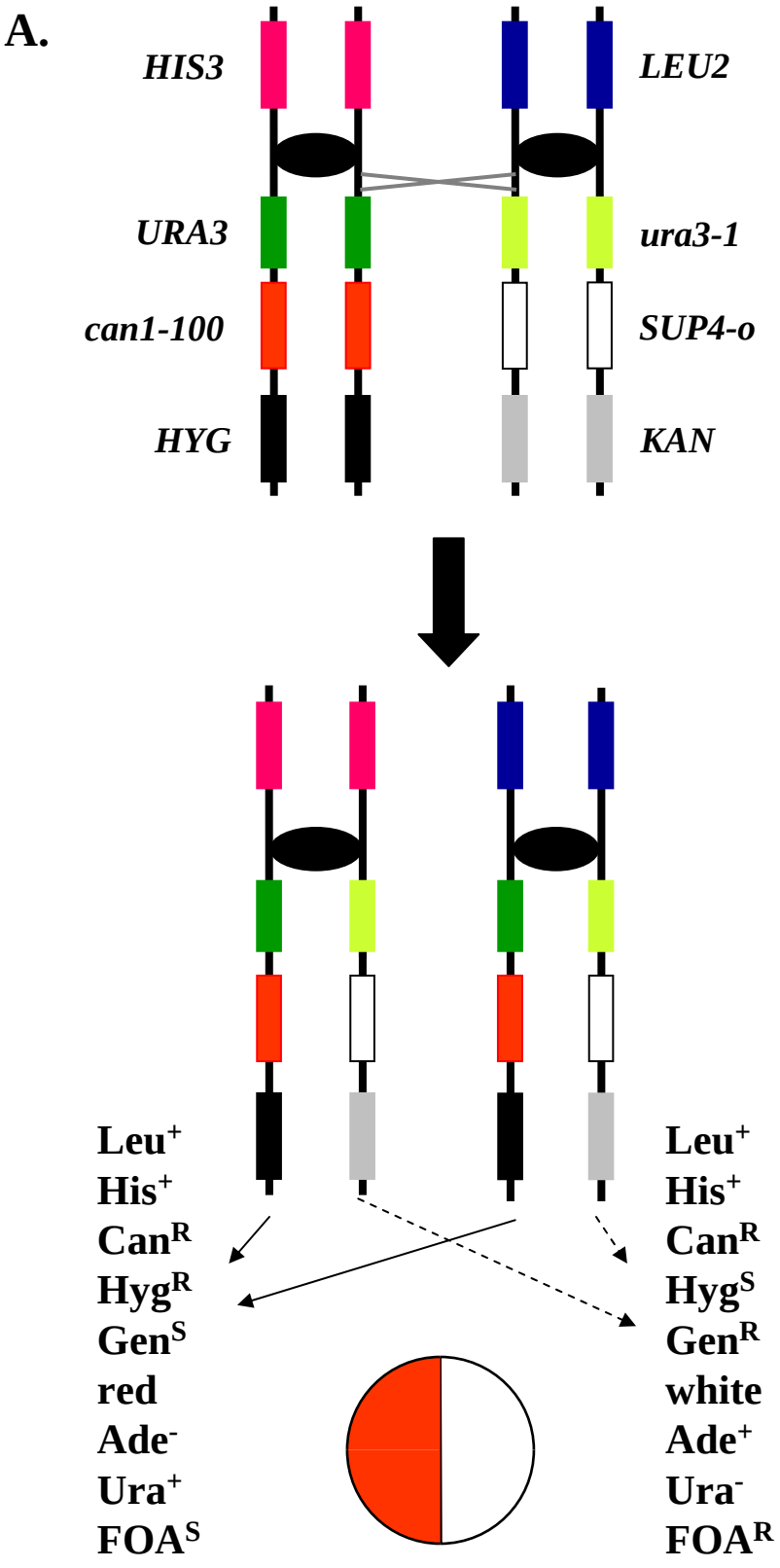


Figure 2.6. Types of sectored colonies resulting from reciprocal crossovers in the *CEN5-URA3* or the *URA3-CAN1* intervals of chromosome V in the diploid MAB13. The starting diploid is phenotypically Can^S Hyg^R Gen^R Ura^+ ($5FOA^S$) His^+ Leu^+ $Ade^{+/-}$ and forms white colonies. The *Saccharomyces* Genome Database coordinates (distance from the left telomere rounded off to the nearest kb) for all markers used in this strain along the 577-kb chromosome V are: *KAN/HYG*^{R} (9 kb), *can1-100/SUP4-o* (32 kb), *URA3* (116 kb), *CEN5* (152 kb), and *HIS3/LEU2* (262 kb). (A) A reciprocal crossover between *CEN5* and *URA3* will result in a sectored colony with sectors of the following phenotypes: Can^R Hyg^R Gen^S Ura^+ ($5FOA^S$) His^+ Leu^+ Ade^- red and Can^R Hyg^S Gen^R Ura^- ($5FOA^R$) His^+ Leu^+ Ade^+ white. (B) A reciprocal crossover between *URA3* and *CAN1* will result in a sectored colony with sectors of the following phenotypes: Can^R Hyg^R Gen^S Ura^+ ($5FOA^S$) His^+ Leu^+ Ade^- red and Can^R Hyg^S Gen^R Ura^+ ($5FOA^S$) His^+ Leu^+ Ade^+ white.


A.
HIS3
LEU2
URA3
ura3-1
can1-100
SUP4-o
HYG
KAN
Leu+
His+
CanR
HygR
GenS
red
Ade-
Ura+
FOAS
Leu+
His+
CanR
HygS
GenR
white
Ade+
Ura-
FOAR

B.

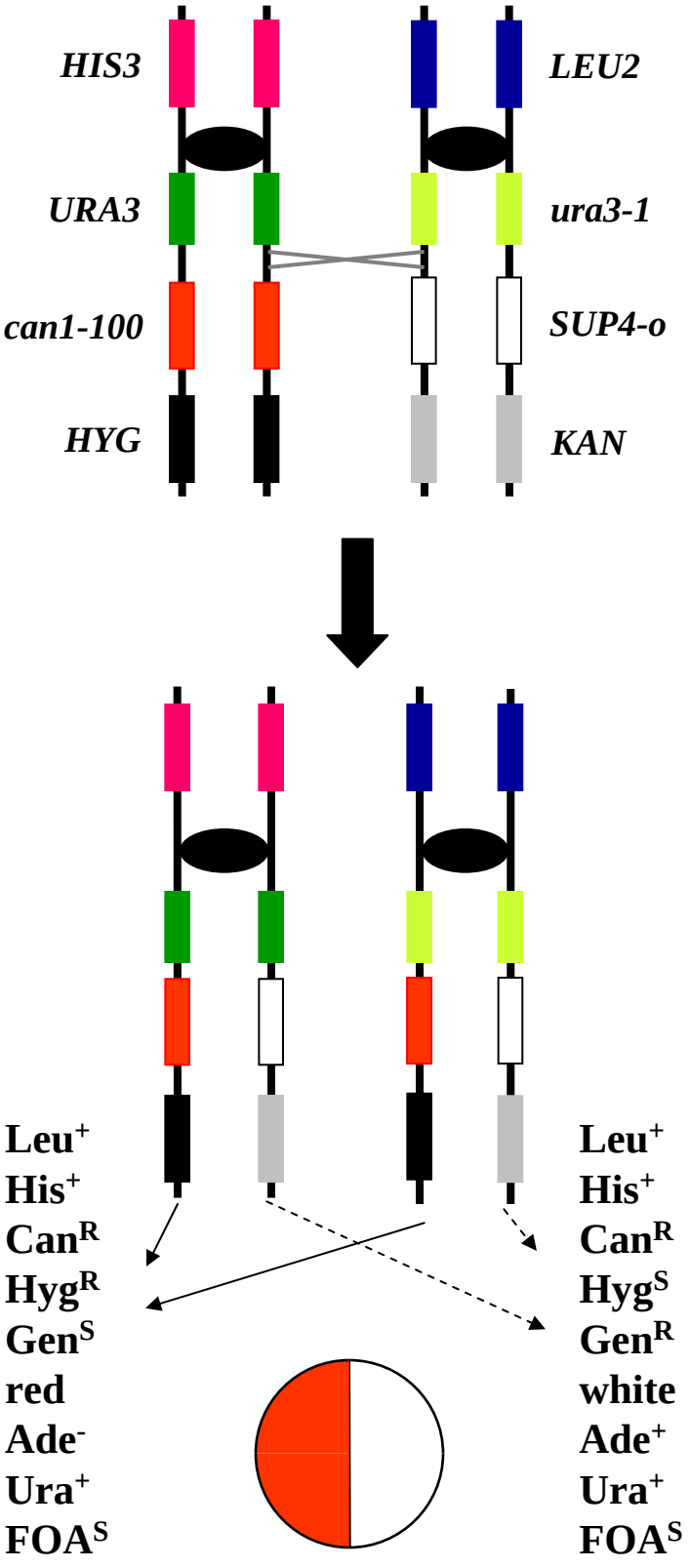

HIS3
LEU2
URA3
ura3-1
can1-100
SUP4-o
HYG
KAN
Leu+
His+
CanR
HygR
GenS
red
Ade-
Ura+
FOAS
Leu+
His+
CanR
HygS
GenR
white
Ade+
Ura+
FOAS

Figure 2.7. Comparison of rates of mitotic and meiotic recombination.

Comparison of the physical and genetic distances for two intervals of chromosome V, *CEN5-URA3* and *URA3-CAN1*. For each interval, the data are shown as a percentage of the distance between *CEN5* and *CAN1*. The physical distances for *CEN5-URA3* and *URA3-CAN1* are 36 and 84 kb, respectively. The meiotic recombination distances (95% confidence limits shown in parentheses) for these same two intervals are 8 cM (7.5-8.4 cM) and 42 cM (41-44 cM) for >1,500 tetrads in *Saccharomyces* Genome Database (SGD), and 7 cM (5-9 cM) and 41 cM (37-45 cM) based on 360 tetrads in our study. The meiotic data shown use the SGD data. The mitotic distances were derived from our analysis of sectors in MAB13 as described in the *Results* section. The 95% confidence limits are indicated for the meiotic and mitotic intervals.

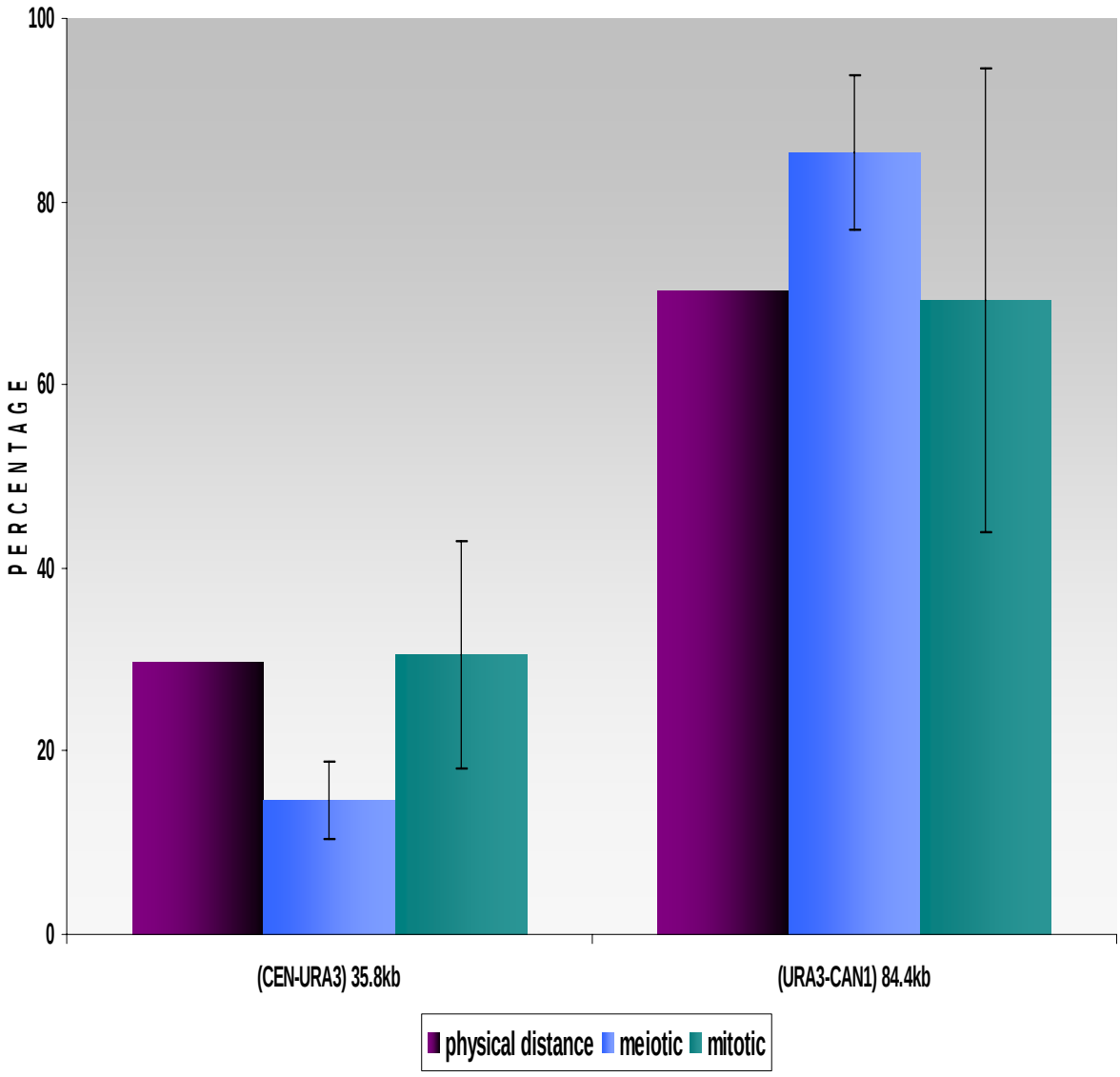
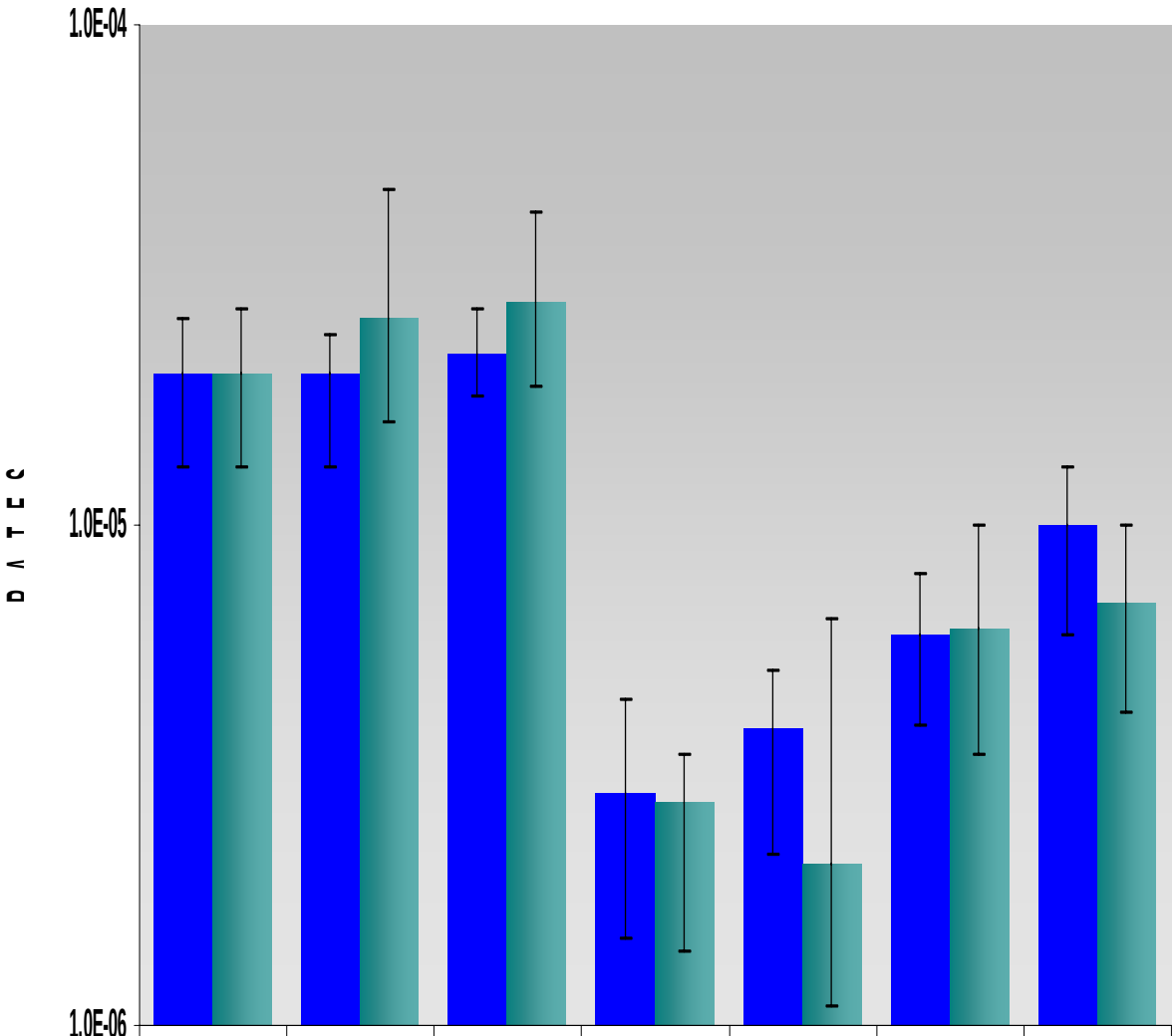

100
80
60
40
20
0
PERCENTAGE
(CEN-URA3) 35.8kb
(URA3-CAN1) 84.4kb
physical distance
meiotic
mitotic

Figure 2.8. Comparison of rates of mitotic recombination and chromosome loss in MAB33. Rates of mitotic recombination and chromosome loss for MAB6 vs MAB33 grown on YPD. The MAB33 strain has a tract of 115 CAG inserted at the *URA3* locus on chromosome V.

MAB6 vs. MAB33 (CAG) grown on YPD

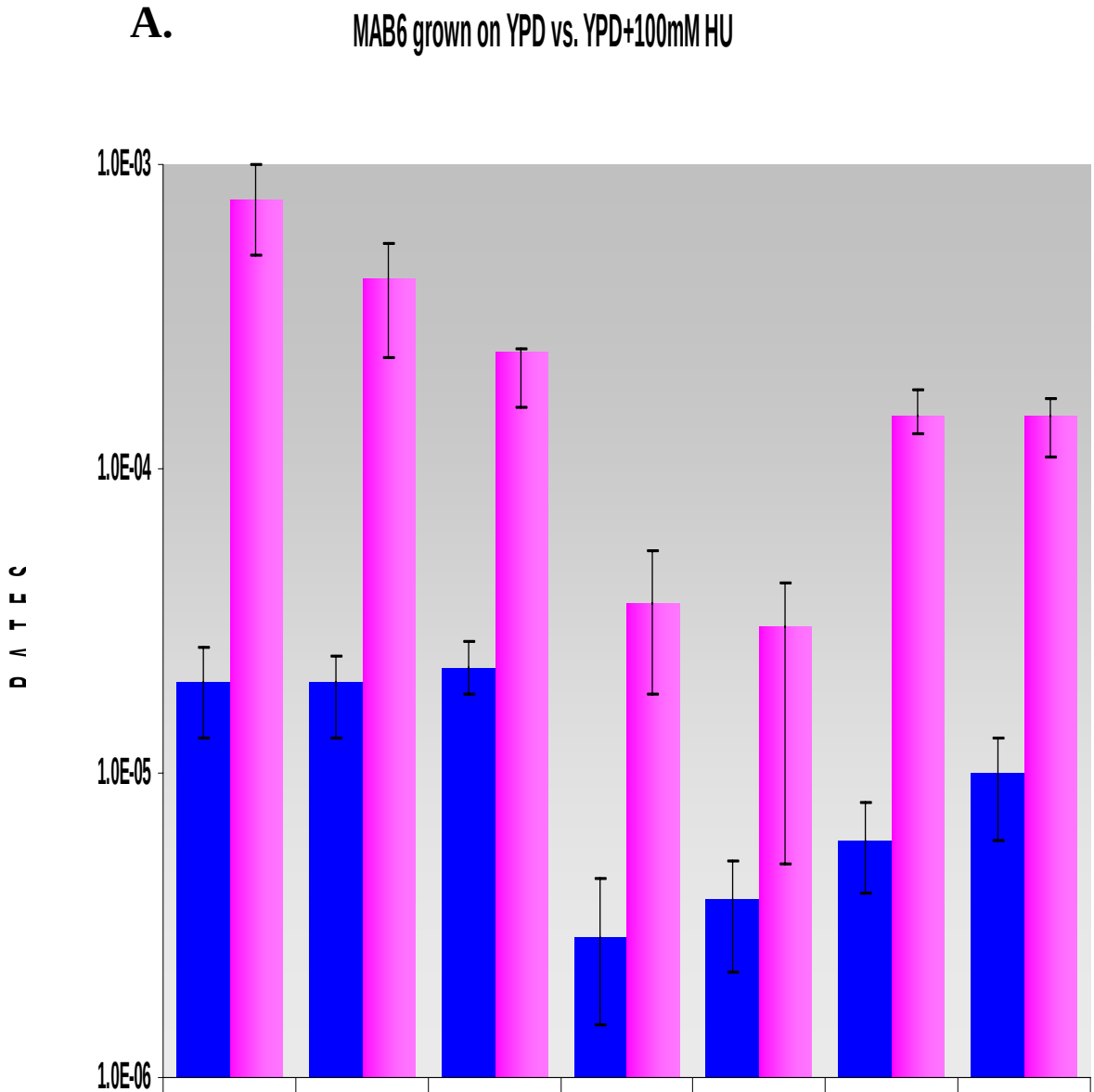


	R/W SECTOR	Class 1	Class 2	Class 3	Class 4	Class 5	Class 6
MAB6 YPD	2.0E-05	2.0E-05	2.2E-05	2.9E-06	3.9E-06	6.0E-06	1.0E-05
MAB33 YPD	2.0E-05	2.6E-05	2.8E-05	2.8E-06	2.1E-06	6.2E-06	7.0E-06

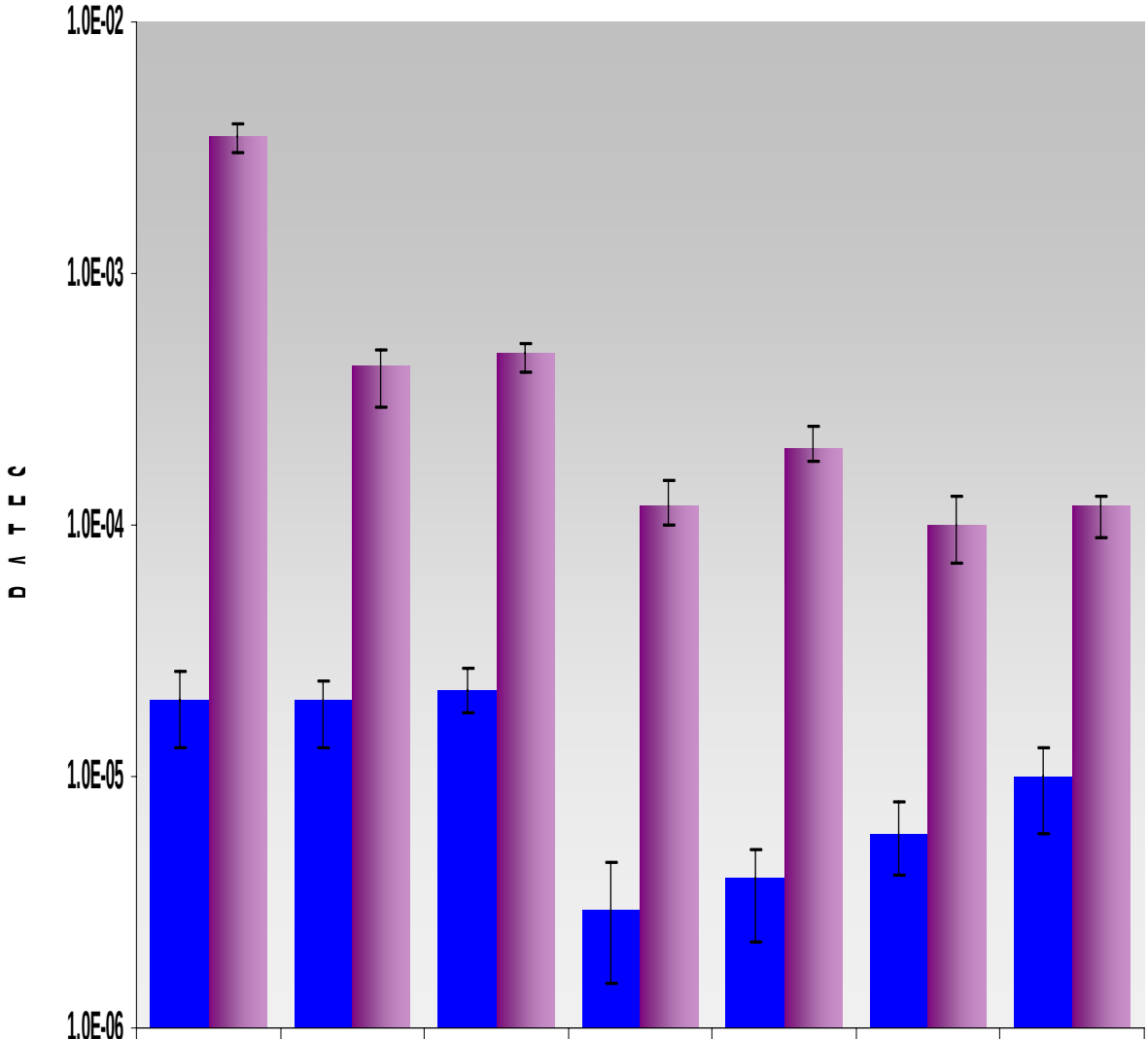
Figure 2.9. Induction of mitotic recombination and chromosome loss by hydroxyurea (HU) and ultraviolet-light (UV) in MAB6.

Rates of mitotic recombination and chromosome loss for **A)** MAB6 grown on rich medium vs MAB6 grown on 100 mM hydroxyurea, and **B)** MAB6 cells unirradiated vs MAB6 cells irradiated with 20 J/m^2 UV.

A.


MAB6 grown on YPD vs. YPD+100mM HU
1.0E-03
1.0E-04
1.0E-05
1.0E-06
R/W SECTOR
Class 1
Class 2
Class 3
Class 4
Class 5
Class 6
MAB6 YPD
2.0E-05
2.0E-05
2.2E-05
2.9E-06
3.9E-06
6.0E-06
1.0E-05
MAB6 YPD+HU
7.6E-04
4.2E-04
2.4E-04
3.6E-05
3.0E-05
1.5E-04
1.5E-04

B. MAB6 grown on YPD (unirradiated) vs. irradiated with 20J/m^2 UV

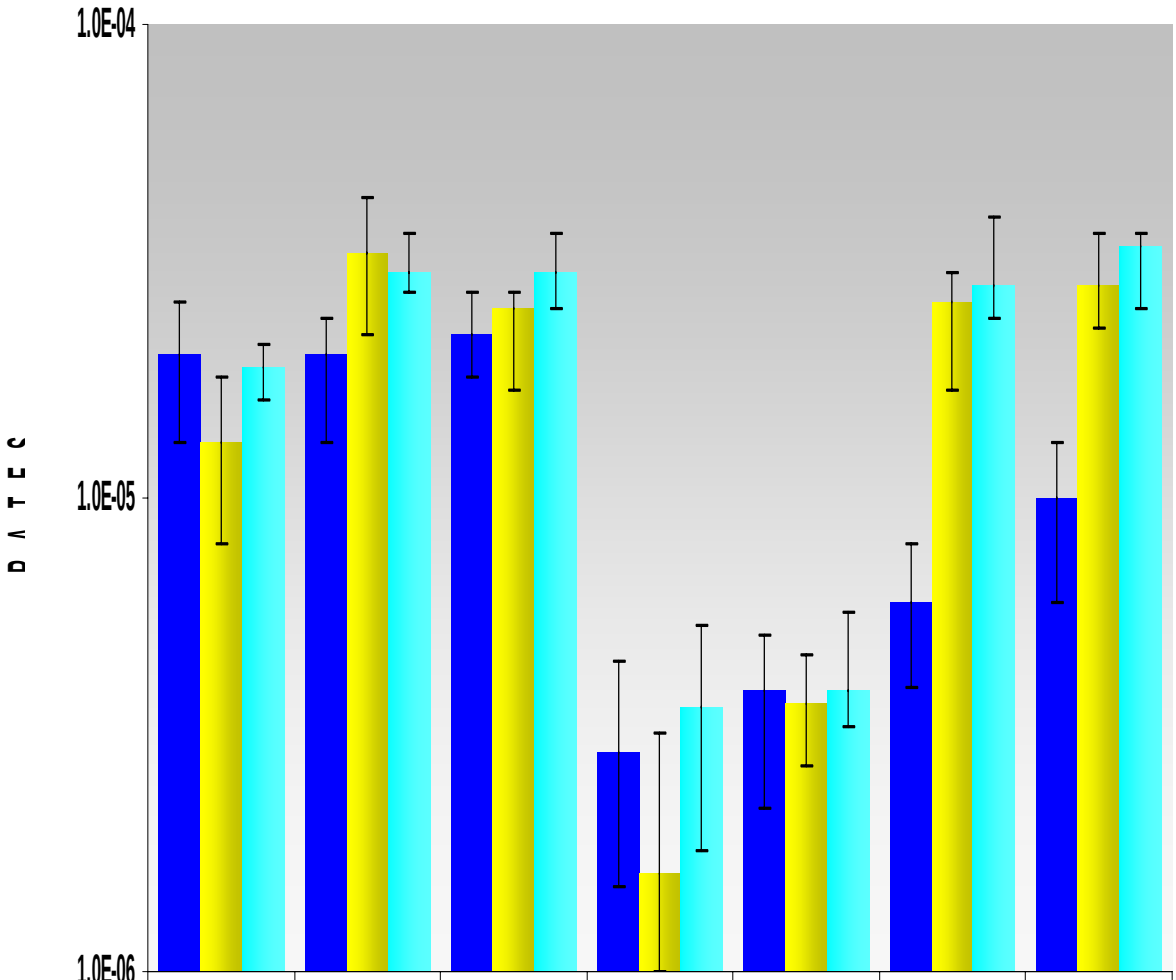


	R/W SECTOR	Class 1	Class 2	Class 3	Class 4	Class 5	Class 6
MAB6 YPD	2.0E-05	2.0E-05	2.2E-05	2.9E-06	3.9E-06	6.0E-06	1.0E-05
MAB6 UV	3.5E-03	4.3E-04	4.8E-04	1.2E-04	2.0E-04	1.0E-04	1.2E-04

Figure 2.10. Effect of mating type heterozygosity on the rates of spontaneous and induced mitotic recombination and chromosome loss.

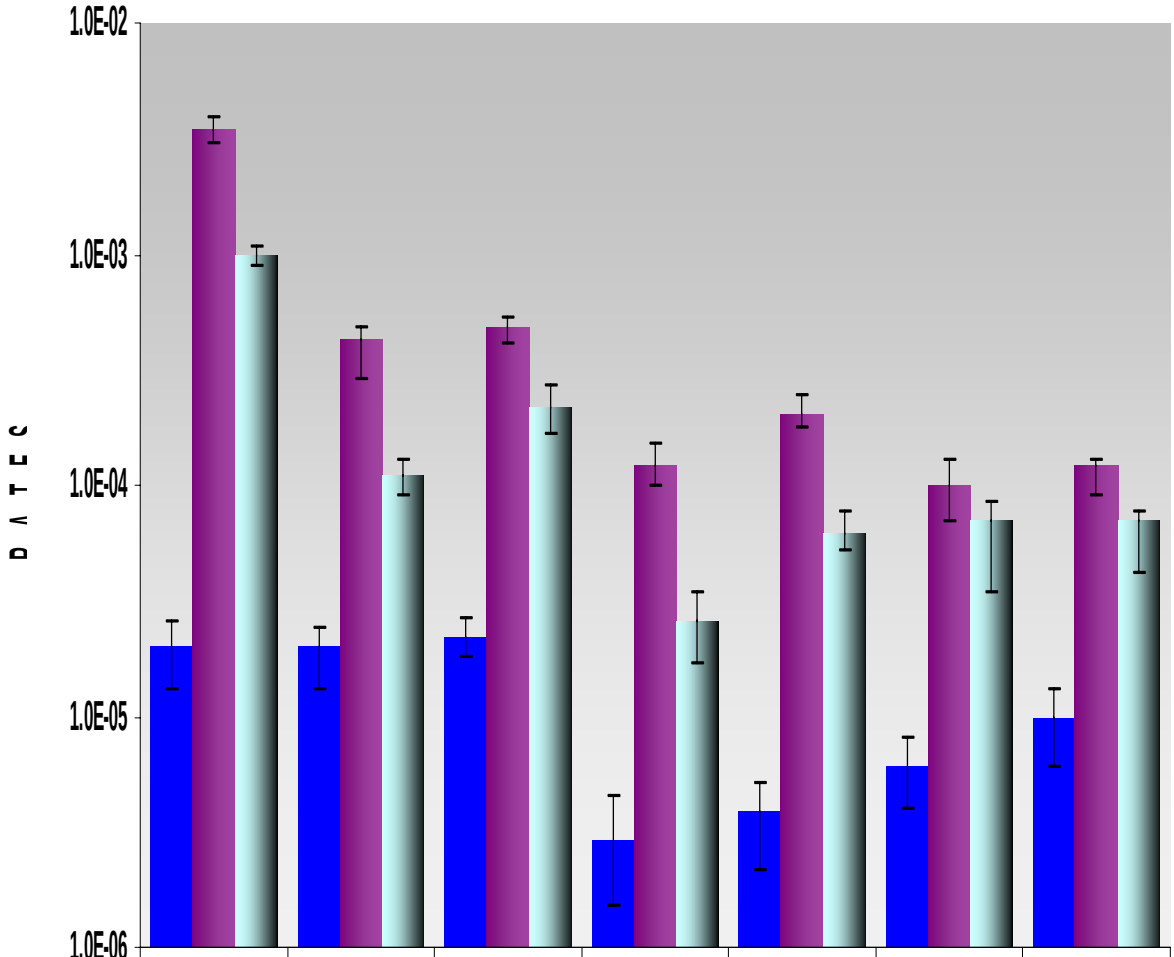
Rates of mitotic recombination and chromosome loss for **A)** MAB6 (*MATa/MATα)* vs MAB35 (*MATa/-*) vs MAB38 (*-/MATα*) grown on YPD, **B)** MAB6 cells unirradiated vs MAB6 irradiated with 20 J/m^2 UV vs MAB35 irradiated with 20 J/m^2 UV, and **C)** MAB6 grown on YPD vs MAB6 grown on 100 mM hydroxyurea vs MAB35 grown on 100mM hydroxyurea.

A. MAB6(a/alpha) vs. MAB35(a/-) vs. MAB38(-/alpha) grown on YPD



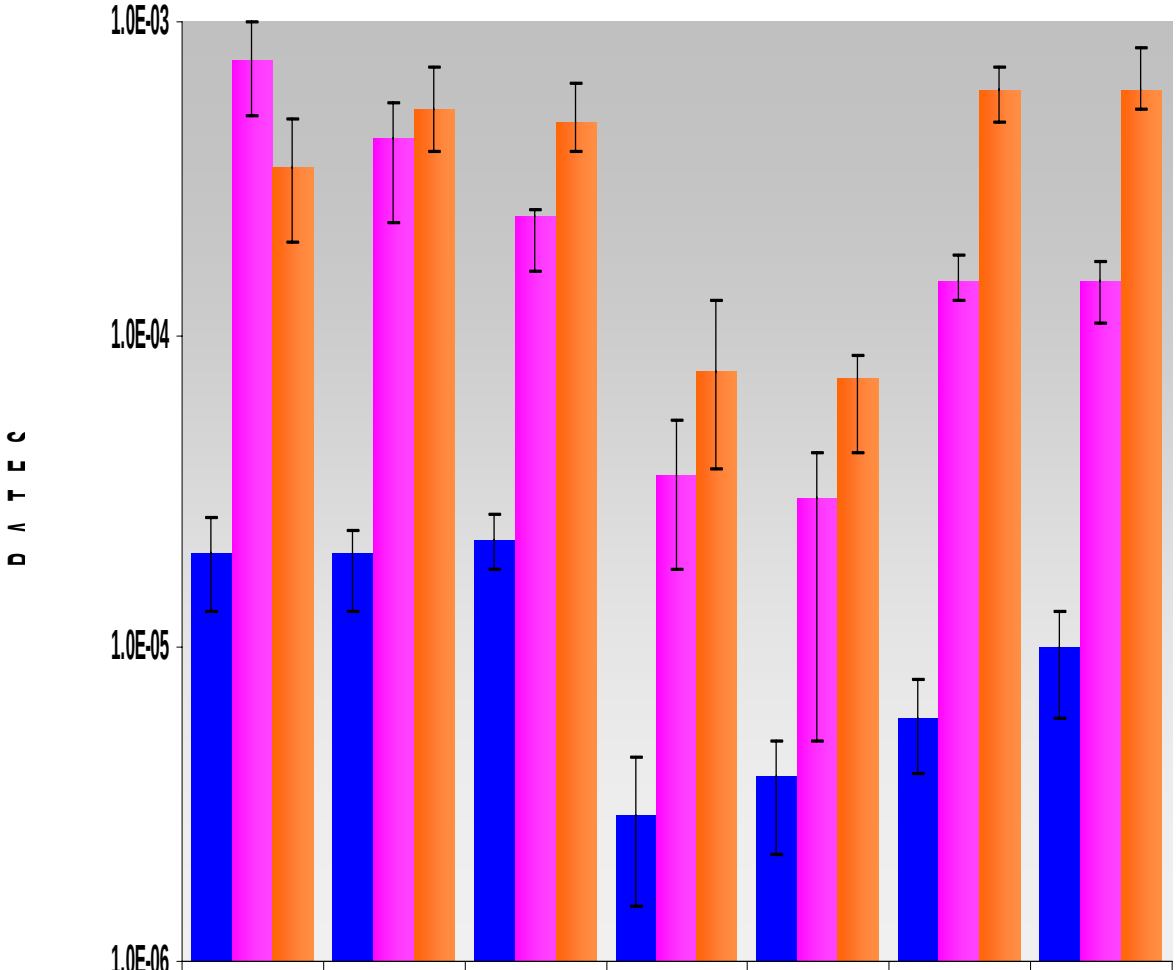
	R/W SECTOR	Class 1	Class 2	Class 3	Class 4	Class 5	Class 6
MAB6 YPD	2.0E-05	2.0E-05	2.2E-05	2.9E-06	3.9E-06	6.0E-06	1.0E-05
MAB35 YPD	1.3E-05	3.3E-05	2.5E-05	1.6E-06	3.7E-06	2.6E-05	2.8E-05
MAB38 YPD	1.9E-05	3.0E-05	3.0E-05	3.6E-06	3.9E-06	2.8E-05	3.4E-05

B. MAB6 unirradiated vs. MAB6(a/alpha) UV 20J/m^2 vs. MAB35(a/-) UV 20J/m^2



	R/W SECTOR	Class 1	Class 2	Class 3	Class 4	Class 5	Class 6
MAB6 YPD	2.0E-05	2.0E-05	2.2E-05	2.9E-06	3.9E-06	6.0E-06	1.0E-05
MAB6 UV	3.5E-03	4.3E-04	4.8E-04	1.2E-04	2.0E-04	1.0E-04	1.2E-04
MAB35 UV	1.0E-03	1.1E-04	2.2E-04	2.6E-05	6.1E-05	7.0E-05	7.0E-05

C. MAB6 grown on YPD vs. MAB6(a/alpha) on 100mM HU vs. MAB35(a/-) on 100mM HU



	R/W SECTOR	Class 1	Class 2	Class 3	Class 4	Class 5	Class 6
MAB6 YPD	2.0E-05	2.0E-05	2.2E-05	2.9E-06	3.9E-06	6.0E-06	1.0E-05
MAB6 YPD+HU	7.6E-04	4.2E-04	2.4E-04	3.6E-05	3.0E-05	1.5E-04	1.5E-04
MAB35 YPD+HU	3.4E-04	5.2E-04	4.8E-04	7.6E-05	7.3E-05	6.0E-04	6.0E-04

Table 2.1. Phenotypes of the different types of genetic events giving rise to canavanine-resistant colonies.

Mitotic event	Phenotype	Class
Reciprocal crossover – R/W SECTOR		
(coincident with plating)----------	His^+ Leu^+ Ade^- Hyg^R Gen^S Can^R Red	sector
	His^+ Leu^+ Ade^+ Hyg^S Gen^R Can^R White	
Reciprocal crossover		
(prior to plating)----------	His^+ Leu^+ Ade^- Hyg^R Gen^S Can^R Red	1
	His^+ Leu^+ Ade^+ Hyg^S Gen^R Can^R White	2
BIR event		
(loss of *SUP4-o* and *KAN*)----------	His^+ Leu^+ Ade^- Hyg^R Gen^S Can^R Red	1
BIR event		
(loss of *can1-100* and *HYG*)----------	His^+ Leu^+ Ade^+ Hyg^S Gen^R Can^R White	2
Local gene conversion		
(*SUP4-o* converted to *can1-100*)---------	His^+ Leu^+ Ade^- Hyg^R Gen^R Can^R Red	3
Local gene conversion		
(*can1-100* converted to *SUP4-o*)----------	His^+ Leu^+ Ade^+ Hyg^R Gen^R Can^R White	4
Loss of *SUP4-o* chromosome----------	His^+ Leu^- Ade^- Hyg^R Gen^S Can^R Red	5
Loss of *can1-100* chromosome----------	His^- Leu^+ Ade^+ Hyg^S Gen^R Can^R White	6

Table 2.2. Rates of the different classes of Can^R colonies in MAB6 (*MATa/MATα*), MAB33 (*MATa/MATα*) $(CTG)_{115}$, MAB35 (*MATa/matαΔ*), and MAB38 (*matαΔ/MATα*) grown in rich medium (YPD).

Rates of each class (95% confidence limits in parentheses); normalized rate in bold-face[1]

Class of colony[2]	**MAB6 (YPD)**	**MAB33 (YPD)**	**MAB35 (YPD)**	**MAB38 (YPD)**
Red/white sectored	$2.0x10^{-5}$ ($1.3\text{-}2.6x10^{-5}$) **1**	$2.0x10^{-5}$ ($1.3\text{-}2.7x10^{-5}$) **1**	$1.3x10^{-5}$ ($0.8\text{-}1.8x10^{-5}$) **0.7**	$1.9x10^{-5}$ ($1.6\text{-}2.1x10^{-5}$) **1**
Class 1	$2.0x10^{-5}$ ($1.3\text{-}2.4x10^{-5}$) **1**	$2.6x10^{-5}$ ($1.6\text{-}4.7x10^{-5}$) **1.3**	$3.3x10^{-5}$ ($2.2\text{-}4.3x10^{-5}$) **1.6**	$3.0x10^{-5}$ ($2.7\text{-}3.6x10^{-5}$) **1.5**
Class 2	$2.2x10^{-5}$ ($1.8\text{-}2.7x10^{-5}$) **1**	$2.8x10^{-5}$ ($1.9\text{-}4.2x10^{-5}$) **1.3**	$2.5x10^{-5}$ ($1.7\text{-}2.7x10^{-5}$) **1.1**	$3.0x10^{-5}$ ($2.5\text{-}3.6x10^{-5}$) **1.3**

Class 3	2.9×10^{-6} ($1.5\text{-}4.5 \times 10^{-6}$) **1**	2.8×10^{-6} ($1.4\text{-}3.5 \times 10^{-6}$) **0.9**	1.6×10^{-6} ($0.8\text{-}3.2 \times 10^{-6}$) **0.6**	3.6×10^{-6} ($1.8\text{-}5.4 \times 10^{-6}$) **1.2**
Class 4	3.9×10^{-6} ($2.2\text{-}5.1 \times 10^{-6}$) **1**	2.1×10^{-6} ($1.1\text{-}6.5 \times 10^{-6}$) **0.5**	3.7×10^{-6} ($2.7\text{-}4.7 \times 10^{-6}$) **0.9**	3.9×10^{-6} ($3.3\text{-}5.7 \times 10^{-6}$) **1**
Class 5	6.0×10^{-6} ($4.0\text{-}8.0 \times 10^{-6}$) **1**	6.2×10^{-6} ($3.5\text{-}10 \times 10^{-6}$) **1**	2.6×10^{-5} ($1.7\text{-}3.0 \times 10^{-5}$) **4.3**	2.8×10^{-5} ($2.4\text{-}3.9 \times 10^{-5}$) **4.7**
Class 6	1.0×10^{-5} ($0.6\text{-}1.3 \times 10^{-5}$) **1**	7.0×10^{-6} ($4.2\text{-}10 \times 10^{-6}$) **0.7**	2.8×10^{-5} ($2.3\text{-}3.6 \times 10^{-5}$) **2.8**	3.4×10^{-5} ($2.5\text{-}3.6 \times 10^{-5}$) **3.4**

[1]The rates of the red/white sectored colonies were calculated directly from the frequencies, and the rates of the other classes were calculated using the method of the median (Lea and Coulson, 1949). The number of independent cultures used for each strain (shown in parentheses) were: MAB6, YPD (40); MAB33, YPD (28); MAB35, YPD (20); MAB38, YPD (20).

95% confidence limits were calculated as described in Materials and Methods, and all rates are normalized to the rates observed in MAB6 grown in YPD.

[2]The phenotypes of the various classes are described in the text and in Table 2-1. The red/white sectored colonies represent reciprocal crossovers (RCO). The genetic events giving rise to the other classes are: Class 1 (BIR and/or RCO event before plating on canavanine-containing medium), Class 2 (BIR + RCO), Class 3 (local gene conversion), Class 4 (local gene conversion), Class 5 (chromosome loss), and Class 6 (chromosome loss).

Table 2.3. Rates of the different classes of Can^R colonies in MAB6 (*MATa/MATα*) and MAB35 (*MATa/matαΔ*) grown in medium with 100 mM hydroxyurea (HU) and irradiated with 20 J/m^2 of UV.

Rates of each class (95% confidence limits in parentheses); normalized rate in bold-face[1]

Class of colony[2]	**MAB6 (YPD + HU)**	**MAB35 (YPD + HU)**	**MAB6 (UV)**	**MAB35 (UV)**
Red/white sectored	7.6×10^{-4} ($5.0\text{-}9.9 \times 10^{-4}$) **38**	3.4×10^{-4} ($2.0\text{-}4.9 \times 10^{-4}$) **17**	3.5×10^{-3} ($3.0\text{-}3.9 \times 10^{-3}$) **175**	1.0×10^{-3} ($0.9\text{-}1.1 \times 10^{-3}$) **50**
Class 1	4.2×10^{-4} ($2.3\text{-}5.5 \times 10^{-4}$) **21**	5.2×10^{-4} ($3.9\text{-}7.2 \times 10^{-4}$) **26**	4.3×10^{-4} ($2.9\text{-}4.9 \times 10^{-4}$) **22**	1.1×10^{-4} ($0.9\text{-}1.3 \times 10^{-4}$) **6**
Class 2	2.4×10^{-4} ($1.6\text{-}2.5 \times 10^{-4}$) **11**	4.8×10^{-4} ($3.9\text{-}6.3 \times 10^{-4}$) **22**	4.8×10^{-4} ($4.1\text{-}5.3 \times 10^{-4}$) **22**	2.2×10^{-4} ($1.7\text{-}2.7 \times 10^{-4}$) **10**

Class 3	3.6×10^{-5} ($1.8\text{-}5.4 \times 10^{-5}$) **11**	7.6×10^{-5} ($3.7\text{-}13 \times 10^{-5}$) **26**	1.2×10^{-4} ($1.0\text{-}1.5 \times 10^{-4}$) **41**	2.6×10^{-5} ($1.7\text{-}3.5 \times 10^{-5}$) **9**
Class 4	3.0×10^{-5} ($0.5\text{-}4.2 \times 10^{-5}$) **7.7**	7.3×10^{-5} ($4.2\text{-}8.6 \times 10^{-5}$) **18**	2.0×10^{-4} ($1.8\text{-}2.5 \times 10^{-4}$) **51**	6.1×10^{-5} ($5.2\text{-}7.7 \times 10^{-5}$) **16**
Class 5	1.5×10^{-4} ($1.3\text{-}1.8 \times 10^{-4}$) **25**	6.0×10^{-4} ($4.8\text{-}7.1 \times 10^{-4}$) **100**	1.0×10^{-4} ($0.7\text{-}1.3 \times 10^{-4}$) **16**	7.0×10^{-5} ($3.4\text{-}8.5 \times 10^{-5}$) **12**
Class 6	1.5×10^{-4} ($1.1\text{-}1.7 \times 10^{-4}$) **15**	6.0×10^{-4} ($5.2\text{-}8.3 \times 10^{-4}$) **60**	1.2×10^{-4} ($0.9\text{-}1.3 \times 10^{-4}$) **12**	7.0×10^{-5} ($4.2\text{-}7.7 \times 10^{-5}$) **7**

[1]The rates of the red/white sectored colonies were calculated directly from the frequencies, and the rates of the other classes were calculated using the method of the median (Lea and Coulson, 1949). The number of independent cultures used for each strain (shown in parentheses) were: MAB6, YPD+HU (40); MAB35, YPD+HU (20); MAB6, UV (29); MAB35, UV (16).

95% confidence limits were calculated as described in Materials and Methods, and all rates are normalized to the rates observed in MAB6 grown in YPD (Table 2.2).

[2]The phenotypes of the various classes are described in the text and Table 2-1. The red/white sectored colonies represent reciprocal crossovers (RCO). The genetic events giving rise to the other classes are: Class 1 (BIR and/or RCO event before plating on canavanine-containing medium), Class 2 (BIR + RCO), Class 3 (local gene conversion), Class 4 (local gene conversion), Class 5 (chromosome loss), and Class 6 (chromosome loss).

Table 2.4. Primer names and sequences used in strain constructions

CHRVF 5’-TCCAGCTGACTCATTTCCTGTATTTCTAAGGGGAAATGCATAGGGCGTAC GCTGCAGGTCGAC
CHRVR 5’-CAGATGTTGATTGCCGTTTCAATCTATCGTCATGAAATTGGTCTTATCGAT GAATTCGAGCTCG
PG/HIS3F 5’-TTCACCTACGATGACTTCAGAAGTGCGCCTATATTTCTTTACTTTGGATCC GCTGCACGGTCCTG
PG/HIS3R 5’-GGCACAAAAATAGCTCTCTCTAAATGGGCGGTGGAAGGGTGTTCAGCCTC GTTCAGAATGACACG
SUP/CANF 5’-ATGACAAATTCAAAAGAAGACGCCGACATAGAGGAGAAGCATATGGGA TCCGGGACCGGATAAT
SUP/CANR 5’-CTATGCTACAACATTCCAAAATTTGTCCCAAAAAGTCTTTGGTTCGGATCC GGAATTCTTGAAAG
MD/LEU2F 5’-TTCACCTACGATGACTTCAGAAGTGCGCCTATATTTCTTTACTTTGCGCTA TCGCACAGAATCAA
MD/LEU2R 5’-GGCACAAAAATAGCTCTCTCTAAATGGGCGGTGGAAGGGTGTTCAAGGA ATCATAGTTTCATGAT

wtURA3F 5’-AAACACCAGAGTCAAACGAC
wtURA3R 5’-ATAGTTCCTTTTTATAAAGG
URA3 upstream HIII F 5’-CAAAGAAGGTTAATGTGGCTGTGGTTTCAGGGTC
URA3 upstream HIII R 5’-CTTATACTGGATCCATTACGACCGAGATTCCCGG
Malpha2/URA3F 5’-GCGAGATAAACTGGTATTCTTCATTAGATTCTCTAGGCCCTTGGAATGT GGCTGTGGTTTCAGG
Malpha1/URA3R 5’-GTTGCGCGAAGTAGTCCCATATTCCGTGCTGCATTTTGTCCGCGTAGATT CCCGGGTAATAACTG
MATA2/URA3F 5’-CGAAACCCAGTTTTTGATTTGAATGCGAGATAAACTGGTATTCTTCGTGG CTGTGGTTTCAGGGTC
MATA1/URA3R 5’-CATACCCAAACTCTTACTTGAAGTGGAGTAATGCCACATTTCTTTGCCAT TACGACCGAGATTCCCGG
TRP1F 5’-ATGTCTGTTATTAATTTCACAGGTAGTTCT
TRP1R 5’-CTATTTCTTAGCATTTTTGACGAAATTTG

VL33517R 5’-CTTCAGACTTCTTAACTCC
VL32915F 5’-GTTCCAGGGCAAAAGTGATTGC
SUP4R 5’-CAACTTGCAAGTCTGGGAAG

Table 2.5. Genotype of haploid strains

Strain name	**Relevant genotype**	**Construction or reference***
W303a	Wild type	Ref. 1
RCY317-9a	*α can1-100 RAD5*	Spore of RCY317†
PG128.1	*α can1-100 V9229::HYG*	Insertion of HYG^R into chromosome V centromere-distal to *can1-100*
MAB1	*α can1-100 V9229::HYG V261553::HIS3*	Insertion of *HIS3* into right arm of chromosome V
MAB10	*α can1-100 V9229::HYG V261553::HIS3 URA3*	*URA3* derivative of MAB1
MAB29	*α can1-100 V9229::HYG V261553::HIS3 ura3-1::pMAB1*	Insertion of plasmid with a $(CAG)_{115}$ tract in the *ura3* locus
MAB50	*α can1-100 V9229::HYG V261553::HIS3 URA3 TRP1*	*TRP1* derivative of MAB10
RCY317-16c	*a can1-100 RAD5*	Spore of RCY317†
MD235	*a can1-100 V9229::KANMX*	Insertion of KAN^R into chromosome V centromere-distal to *can1-100*

MD242-2	*a can1Δ::SUP4-o V9229::KANMX*	Replacement of *can1-100* with *SUP4-o*
MAB4	*a can1Δ::SUP4-o V9229::KANMX V261553::LEU2*	Insertion of *LEU2* into right arm of chromosome V
MAB31	*a can1Δ::SUP4-o V9229::KANMX V261553::LEU2 ura3-1::pMAB1*	Insertion of plasmid with a $(CAG)_{115}$ tract in the *ura3* locus

Only genes that differ from the genotype of W303a (*a leu2-3,112 his3-11,15 ura3-1 ade2-1 trp1-1 can1-100 rad5-535*) are shown.

*Details of the construction are in Materials and Methods.

†The RCY317 diploid is described in ref. 2.

1. Thomas, B. J. & Rothstein, R. (1989) *Genetics* **123,** 725-738.

2. Craven, R. J., Greenwell, P. W., Dominska, M. & Petes, T. D. (2002) *Genetics* **161,** 493-507.

Table 2.6. Genotype of diploid strains

Strain name	**Relevant genotype**	**Construction or reference***
MAB6	*MATa can1Δ::SUP4-o V9229::KANMX V261553::LEU2* *MATα can1-100 V9229::HYG V261553::HIS3*	Cross of MAB1 and MAB4
MAB13	*MATa can1Δ::SUP4-o V9229::KANMX V261553::LEU2* *MATα can1-100 V9229::HYG V261553::HIS3* *ura3-1* *URA3*	Cross of MAB10 and MAB4
MAB54	*MATa can1Δ::SUP4-o V9229::KANMX V261553::LEU2* *MATα can1-100 V9229::HYG V261553::HIS3* *ura3-1 trp1-1* *URA3 TRP1*	Cross of MAB50 and MAB4
MAB33	*MATa can1Δ::SUP4-o V9229::KANMX V261553::LEU2* *MATα can1-100 V9229::HYG V261553::HIS3* *ura3-1::pMAB1* *ura3-1::pMAB1*	Cross of MAB29 and MAB31
MAB35	*MATa can1Δ::SUP4-o V9229::KANMX* *matαΔ::URA3 can1-100 V9229::HYG* *V261553::LEU2* *V261553::HIS3*	Deletion of *MATα* in MAB6
MAB38	*mataΔ::URA3 can1Δ::SUP4-o V9229::KANMX* *MATα can1-100 V9229::HYG* *V261553::LEU2* *V261553::HIS3*	Deletion of *MATa* in MAB6

Only genes that differ from the genotype of W303a (*a leu2-3,112 his3-11,15 ura3-1 ade2-1 trp1-1 can1-100 rad5-535*) are shown. All strains are homozygous for *RAD5*.

*Details of the construction are in Materials and Methods.

CHAPTER III

CONCLUSIONS AND FUTURE DIRECTIONS

The data presented in this dissertation provide several new insights to the field of mitotic recombination. Spontaneous mitotic recombination between homologous chromosomes occurs at rates several orders of magnitude lower than spontaneous meiotic recombination for the same genetic intervals (Fogel and Mortimer, 1971). Because these mitotic events are too infrequent to be analyzed by nonselective techniques, a number of selective methods have been developed for their detection. These selective methods, although useful, have a series of limitations. As part of my project, I developed a novel method in which mitotic events occurring on either of the two homologues can be recovered, eliminating some of the limitations of the previous methods. The distinctive feature of this system is that it is the first system developed that allows the selection of both products result of a spontaneous reciprocal mitotic crossover, providing a fast and accurate assessment of this type of mitotic event. Our system is on chromosome V, but it can be used for any chromosome and any interval by constructing strains in which the *can1-100, SUP4-o*, and the rest of the markers are inserted into the appropriate positions.

We determined the rate of reciprocal exchange between *CEN5* and *CAN1* to be 4.0×10^{-5}. We also calculated the rate for the different classes of unsectored colonies. The rates of class 1 and 2 ($\sim 2.1 \times 10^{-5}$) were about the same as observed for the rate of R/W sectors. The simplest explanation for this result is that probably most of the class 1 and 2 events represent reciprocal crossovers rather than BIR events. McMurray and Gottschling (McMurray and Gottschling, 2003) found that most mitotic recombination events observed in nonselected "young" diploid cells were reciprocal, and our data are consistent with this conclusion. The rates of class 3 and 4 (local gene conversion) were $\sim 3.5 \times 10^{-6}$, 10-fold less frequent than reciprocal crossovers. The rates of chromosome loss (class 5 and 6) were $\sim 0.8 \times 10^{-5}$ per cell division.

Hotspots are genomic regions with unusually high levels of recombination (Lichten and Goldman, 1995; Petes, 2001) and coldspots are regions that display a lower than average frequency of recombination. Global mapping of meiotic recombination hotspots and coldspots in the yeast *Saccharomyces cerevisiae* showed that coldspots were nonrandomly associated with the centromere and telomeres (Gerton *et al.*, 2000). We measured meiotic recombination in two intervals (*CEN5-URA3* and *URA3-CAN1*) in our strain and found substantial suppression of meiotic recombination near *CEN5*. We then investigated whether this suppression near the centromere was also seen in mitotic recombination and found that mitotic crossovers, unlike meiotic crossovers, were not significantly suppressed near *CEN5*. To investigate this issue further and determine whether there are hotspots or coldspots for mitotic reciprocal crossovers, experiments are currently ongoing in the lab to map the position of the mitotic crossovers to an average resolution of 1 kb for the 120 kb interval between *CEN5* and *CAN1*.

Long CTG tracts stimulate spontaneous ectopic mitotic exchange in haploid yeast cells and are associated with DSB formation (Freudenreich *et al.,* 1998). We tested if a $(CTG)_{115}$ tract would also stimulate exchange between the homologous chromosomes in diploid cells, and found that the rates for mitotic recombination between the homologs in the strain carrying the $(CTG)_{115}$ tract (MAB33) were very similar to the rates in the strain without the tract (MAB6). Although a plausible explanation for this result could be the difference in the type of recombination being examined, it would be interesting to see the effect of a tract containing over 200 repeats.

We examined the effect of two DNA-damaging agents (hydroxyurea and ultraviolet light) on mitotic recombination and chromosome loss. Both agents stimulated all the different types of events being examined. Hydroxyurea is a highly specific inhibitor of ribonucleotide reductase, an enzyme that catalyzes the reduction of ribonucleotide to deoxyribonucleotides; this inhibition reduces dNTPs pools affecting DNA synthesis and repair. The metabolism of hydroxyurea acts as a source of reactive oxygen species (ROS). Thus, in addition to the DNA breaks that can occur as a result of stalled replication forks, hydroxyurea can also induce site-specific DNA-damage by forming hydrogen peroxide and nitric oxide. Treatment of cells with 100 mM hydroxyurea stimulated reciprocal mitotic crossovers ~40-fold and classes 1 – 6 ~10- to 20-fold. We also used nonselective methods in hydroxyurea-treated cells to more accurately assay BIR events. In examining 66,464 colonies, we found 67 R/W sectors, 26 with the phenotype expected for a reciprocal crossover and 41 with the phenotype expected for a BIR event. Preliminary data for an experiment where cells were treated with 25 mM hydroxyurea showed that mitotic crossovers were stimulated ~13-fold and classes 1 – 6 ~3- to-6-fold.

The main lesions produced by UV-irradiation are cyclobutane pyrimidine dimers and 6-4 photoproducts that are repaired by the nucleotide excision repair system (Prakash and Prakash, 2000). Surprisingly, we found that a dose of 20 J/m^2 UV-light (90% survival) stimulated reciprocal mitotic crossover 175-fold. The rates for classes 1 and 2 were ~22-fold higher, conversion events (classes 3 and 4) were elevated ~40- to 50-fold, and chromosome loss (classes 5 and 6) were stimulated ~15-fold. As in the experiment with hydroxyurea, we also analyzed these events using nonselective methods. This is an ongoing experiment, but of the 1718 colonies analyzed so far, 11 R/W sectors had the phenotype expected for a reciprocal crossover and 6 had the phenotype expected for a BIR event.

Another extension of these experiments would be to test the effect of other types of DNA-damaging agents on the different types of events. For example, one could introduce a site for a sequence-specific endonuclease (HO or I-*Sce*I) into the *CEN5-CAN1* interval and determine whether a defined DSB increases the rates of all the mitotic events or only specific classes.

We have also studied the genetic regulation of reciprocal mitotic crossovers. We first looked at the role of the mating type alleles on recombination. Several studies have suggested that diploid strains that are heterozygous at the mating-type locus have higher rates of intragenic recombination than strains that are homozygous or hemizygous. We found that heterozygosity at the mating type locus did not affect spontaneous or hydroxyurea-induced mitotic recombination (reciprocal or nonreciprocal). Chromosome loss, however, was elevated several fold in the hemizygous strains. In contrast, in the case of UV, our results show that reciprocal crossovers and classes 1 – 4 were reduced 2- to 5-fold in the

hemizygous strain compared to the heterozygous one, and we did not see any effect on chromosome loss.

An obvious extension of these experiments is to determine the effects of various mutants that affect DNA repair or recombination on mitotic recombination and chromosome loss. The mutants that could be investigated include genes that encode key components of the recombination machinery (such as *rad50, rad51* and *rad52*) as well as mutants that affect the DNA damage checkpoints (such as *mec1*). The exceptional capability of our system to select for mitotic crossovers will be very useful in the examination of these events in mutants in which recombination is expected to be considerably reduced (*rad52* and other recombination mutants).

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