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ANALYSES OF MUTATIONS AFFECTING RECOMBINATION AND MEIOSIS IN SACCHAROMYCES CEREVISIAE

by

Charles W. Edwards, III

Library--Loyola University Medical Center

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Master of Science
April
1986

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VITA

Charles W. Edwards, III was born in Oak Park, Illinois on September 13, 1955. He became a member of the National Honor Society in 1972 while attending Willowbrook Community High School in Villa Park, Illinois. After graduation in 1973, Mr. Edwards enrolled at Northwestern University in Evanston, Illinois and received a Bachelor of Arts Degree from the Department of Biochemistry and Molecular Biology in June 1977. He then attended Michigan State University in East Lansing, Michigan and received a Bachelor of Science Degree from the Department of Microbiology and Public Health in June 1978. Following graduation Mr. Edwards was employed by Difco Laboratories, Detroit, Michigan as a production and quality control technician from July 1978 to July 1981.

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CHAPTER I

INTRODUCTION

Man has long been aware of the existence of genetic heredity in its simplest form, namely like begets like. Darwin altered this dogma with his theory of evolution. He proposed the ability of heredity to gradually change over generations so as to establish new species. Later, Mendel showed that heredity, as defined by physical characteristics is controlled by discrete entities he called genes. The precise and predictable behavior of genes during meiosis led Mendel to conceive the laws of gene segregation and assortment that bear his name. It was not until the 1940's that Avery et al. demonstrated that genes and heredity are encoded within the four nucleotides comprising deoxyribonucleic acid (DNA) (2). The gradual change in heredity required by the theory of evolution can be accounted for by allowing DNA to possess a degree of variability (48).

DNA, or genetic, variability can occur through either mutation or recombination. Mutation is an alteration of the nucleic acid sequences of genes as well as noncoding sequences of DNA. The changes are often deleterious to an organism and often they are neutral, having little or no effect on the organism, but mutations are rarely beneficial. To prevent the harmful effects of mutations organisms have developed repair systems to recognize and correct mutations as they occur. One type of repair system can also be involved in the other source of genetic variability, recombination.

Recombination can be formally divided into two general forms. First, there is intragenic recombination. Using diploid cells with heteroallelic loci, that is different mutations in the same gene one from each parent, Roman demonstrated that nearly all intragenic recombination is the result of a process called gene conversion (110). Secondly, there is intergenic recombination which is referred to as crossing over.

Normally when a cell heterozygous for a given gene (AAaa) undergoes meiosis, each allele will segregate to one of the four meiotic products giving the Mendelian segregation pattern of 2A:2a. Occasionally, there are departures from this segregation pattern. In 1934 Zickler (as described in 134) was working with the fungi Bombardia lunata which produces an eight spored ascus as the result of meiosis and one post-meiotic division. He was using mutations that affected spore color and noticed most asci demonstrated a 4:4 (2:2) segregation pattern for spore color but some asci showed a segregation pattern of 6:2 (3:1). Zickler borrowed the term "conversion" from Winkler (134, 136, 139) to describe his observations. In 1953 Lindgren observed a 3:1 segregation in the yeast Saccharomyces cerevisiae (77, 134).

The second type of recombination that can occur is refered to as crossing over or intergenic recombination between linked genes. Two genes are said to be linked if they usually segregate together during meiosis (134). In 1905 Bateson et al. were studying two linked genes of the sweet pea and noticed that in 12% of the meiotic segregations the linked genes did not segregate together (4, 134). The parental configuration had recombined such that some maternal and paternal information segregated together. Janssens hypothesized that the chiasmata, or nodes, that formed as homologous chromosomes paired in meiosis represented the sites where paternal and maternal chromosomes exchanged material (63, 134). Using the fly, Drosophila melanogaster, Morgan was able to correlate recombination and segregation of linked genes with chromosome segregation and theorized that recombination events involved the actual breakage and rejoining of parts of homologous chromosomes (87, 134). In 1912 Morgan and Cattell coined the term 'crossing over' to identify these exchanges (88, 134). With the additional evidence that linked genes could be arranged in a linear map according to their recombination frequencies (129, 134), the chromosomal theory of inheritance was virtually accepted (134).

Recombination has been studied in prokaryotes, most notably *Escherichia coli* and its bacteriophages, and in eukaryotes including *Drosophila melanogaster*, *Dictyostelium discoideum*, *Lycopersicon esculentum* (tomato), *Zea maize* (corn), and various fungi (for a review see 134). Eukaryotic recombination can occur during either the meiotic or mitotic phases of growth and development. Classically, recombination was first observed as being a part of the meiotic cycle which led to the chromosomal inheritance theory and the discoveries of gene conversion and crossing over (see above and 125, 134). Mitotic recombination has been observed and studied in somatic cells of multicellular organisms such as *D. melanogaster* and *L. esculentum* and during the vegetative life cycles of unicellular organisms such as the fungi *Ascobolus immersus* and *Saccharomyces cerevisiae* (134). Meiotic and mitotic recombination will be discussed separately later in this chapter.

A great deal of recombination research has been done in the fungi because they give the researcher the best of both worlds. Fungi, like bacteria, can be grown to high concentrations easily to allow for detection of rare recombinational events, and like all true eukaryotes, their DNA is bound by histones and distributed among discrete chromosomes that follow Mendel's laws of segregation and assortment (125). They also have well established mitotic and meiotic life cycles and many well characterized mutations for genetic studies (for a review see 125). Information gathered from experiments with *Ascobolus immersus* and *Saccharomyces cerevisiae* will make up the bulk of the following discussion about recombination and possible models of recombination.

MEIOTIC RECOMBINATION

Meiosis and Recombination

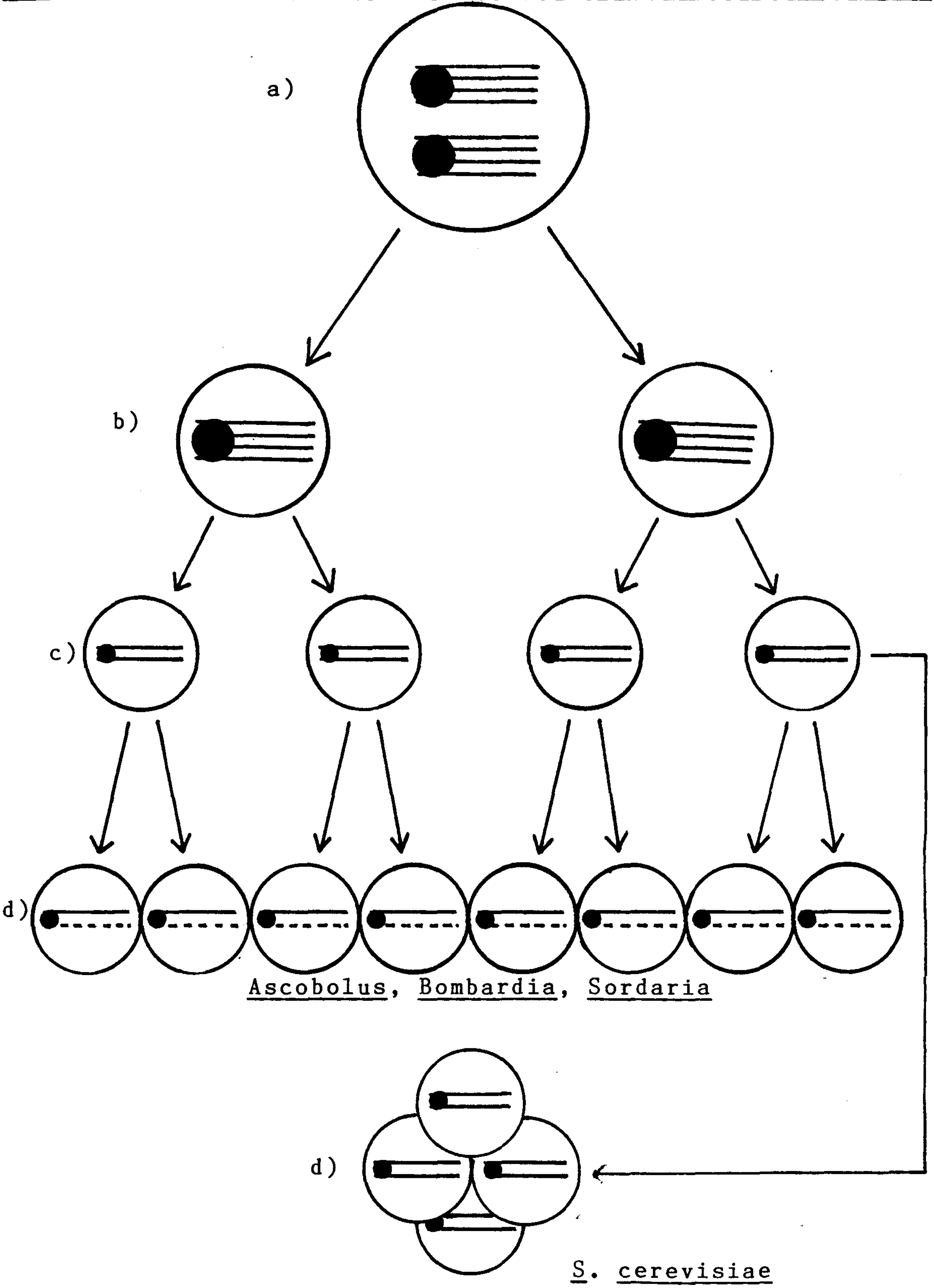
Recombination frequencies in meiosis for known genetic loci are on the order of one thousand fold higher than spontaneous mitotic recombination frequencies for the same loci (95). The fact that many more recombinational events occur in meiosis has led many investigators to use meiotic cells to unravel recombination mechanisms (134). For the purpose of this discussion the basic steps in meiosis and spore formation will be outlined below only briefly to form a scaffold for a more detailed presentation of genetic recombination. A very detailed explanation of meiosis and ascospore development in the yeast _S. cerevisiae_ has been profferred by Esposito and Klapholz (29). The description of recombination that will be used here is essentially the model of Meselson and Radding (86) also known as the Aviemore model (134).

In _Ascobolus_ and _Saccharomyces_ the basic steps of meiosis, at the chromosome level, are essentially the same as shown in Figure 1 (134). First, the cell replicates its DNA thus creating four chromatids for each chromosome with each chromatid containing two strands (one duplex) of DNA.

Figure 1 Meiosis in Fungi.

a) Chromosomes have been duplicated and recombination is complete. Meiosis I reductional division segregates centromeres yielding two nuclear bodies b). Meiosis II equational division segregates chromatids giving four nuclear bodies c). *S. cerevisiae* packages the four haploid nuclear bodies as spores within an ascus d) and *Ascobolus*, *Bombardia*, and *Sordaria* nuclear bodies undergo a postmeiotic mitotic division before packaging eight haploid spores in their asci.

Figure 1 Meiosis in Fungi


a)
b)
c)
d)
Ascobolus, Bombardia, Sordaria
d)
S. cerevisiae

Second, homologous chromosomes pair with each other, synaptonemal complexes form, and recombination ensues. The exact temporal order of these events is not known.

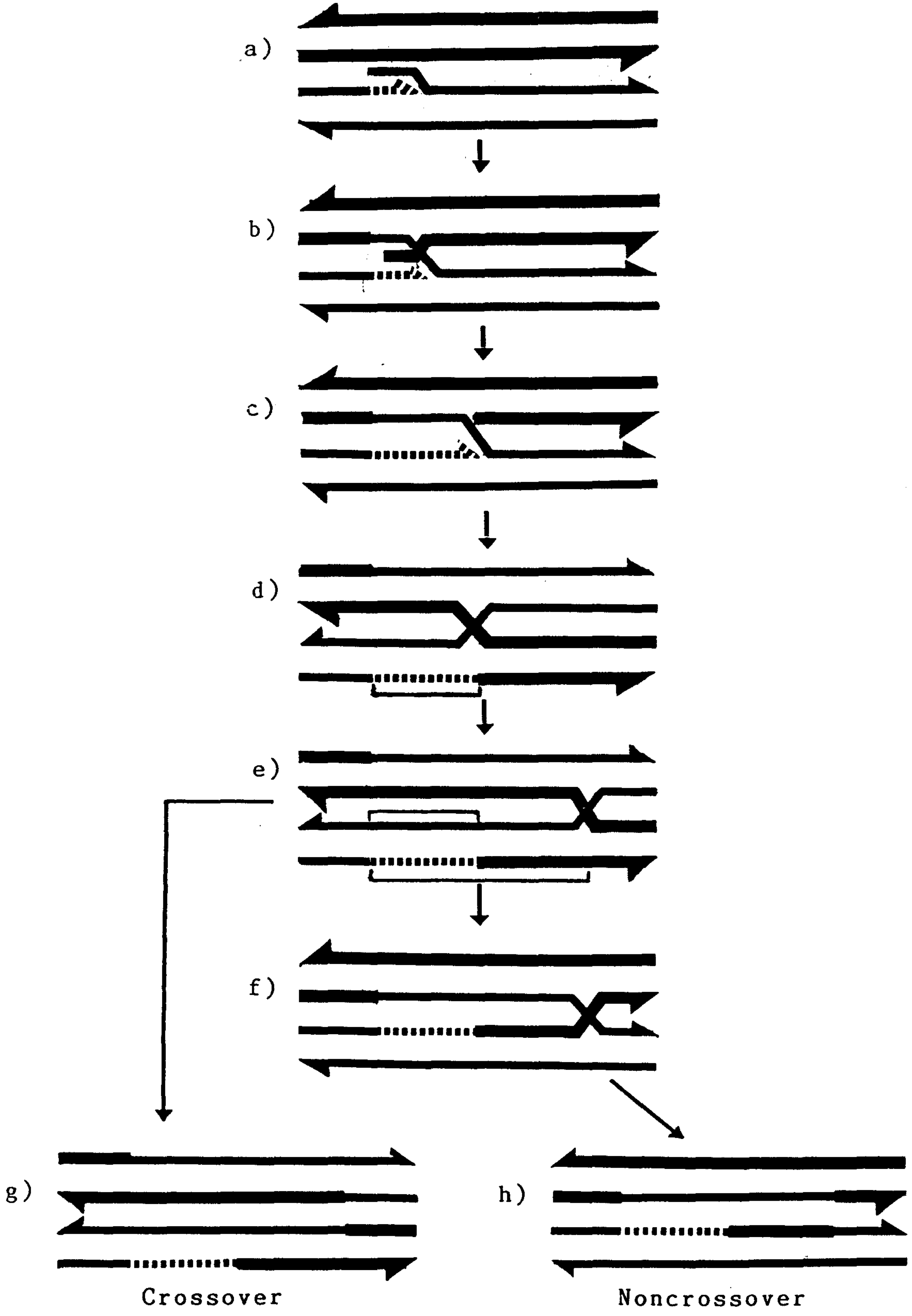
Initiation of recombination in the Meselson-Radding model is the result of a single-strand break in one of the two homologous chromatids. A DNA polymerase can then recognize the single-strand nick and, using the 3' end as a primer replicate the DNA thereby displacing the strand with the 5' end (Figure 2a) (23, 65, 84). The displaced strand pairs with complimentary sequences of the homologous chromatid and induces a single-strand break in the latter (Figure 2b) (86). Strand transfer continues with the concerted action of DNA polymerase-mediated strand displacement and exonucleolytic degradation of the replaced strand (Figure 2c) (17, 18). The DNA duplex formed between the displaced strand and the recipient strand is a hybrid containing one strand from each of the two homologous duplexes. This is heteroduplex DNA. This heteroduplex DNA is formed on only one of the two chromatids and is referred to as asymmetric heteroduplex DNA and is indicated by a bracket in Figure 2c. Heteroduplex formation requires rotation of the chromatids which can be accomplished using energy from the polymerization reaction in strand transfer (86).

Strand transfer ends with the dissociation of the polymerase and the exonuclease. Sigal and Alberts (117) demonstrated that the recombination intermediate can isomerize

Figure 2 The Meselson and Radding Model of Recombination

Initiation is by a single-strand nick. a) DNA polymerase displaces the nicked strand; b) the displaced strand invades a homologous chromatid; c) strand transfer is by DNA polymerase-mediated strand displacement - the displaced strand is degraded by an exonuclease; d) isomerization of the intermediate producing a Holliday structure (see Figure 3); e) branch migration creates symmetric heteroduplex; a second isomerization produces the intermediate in f); endonucleolytic cleavage of f) produces the noncrossover configuration h); cleavage of e) produces the crossover configuration g).

Figure 2 The Meselson and Radding Model for Recombination


a)
b)
c)
d)
e)
f)
g)
h)
Crossover
Noncrossover

producing a second type of intermediate with cross strands (Figure 2d). This structure is known as a Holliday structure after the man who first proposed its existence as part of his model for recombination (58). The Holliday structure is able to migrate in either direction along the axis of the duplexes as the result of rotary diffusion of the two chromatids (14, 66, 76, 85). Because both strands are being transferred through branch migration, heteroduplex DNA can form on both chromatids and this region is referred to as symmetric heteroduplex (86). The symmetric heteroduplex region is where the two brackets overlap in Figure 2e. A unique feature of this structure is that there is a region of asymmetric as well as symmetric heteroduplex. The need for both of these regions will be explained later. This structure is also capable of isomerization producing the structure in Figure 2f (86).

Endonucleolytic cleavage of either the structure in Figures 2e or 2f terminates the exchange event (117). If the structure in 2e is cleaved the result is a reciprocal exchange of the regions flanking the Holliday structure also known as a crossover event (Figure 2g). Cleavage of the structure in 2f produces an exchange of only the inner regions of the duplexes without a crossover (Figure 2h). In this case both the crossover and the noncrossover event lead to some degree of symmetric heteroduplex formation. It should be pointed out that if cleavage occurs before branch migration, as in Figures 2c and 2d, both exchange events would produce only an asymmetric heteroduplex region.

It is not necessary a priori for the DNA strands to be crossed to be cleaved. Holliday's model suggested that the outside strands of the Holliday structure could be cleaved as frequently as the crossed strands (58). Indeed, the isomerization intermediate of Sigal and Alberts provides for an equal opportunity for recognition and cleavage of the two strands as seen in Figure 3 (117). Cleavage can occur in either orientation 1, producing the crossover, or in orientation 2 thereby producing the noncrossover event (Figures 2g and 2h) (86, 117).

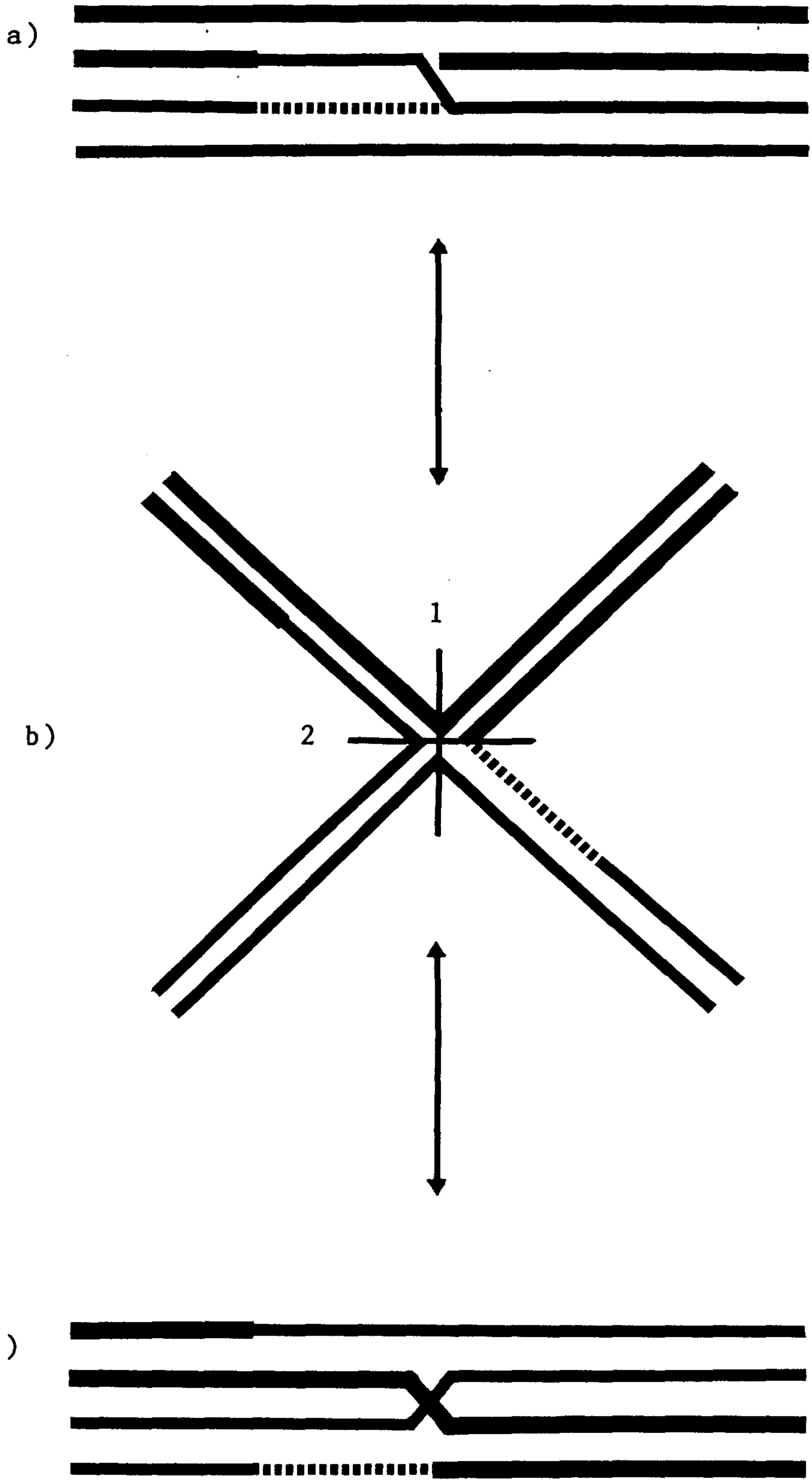
With the termination of the exchange event recombination ends and meiosis continues. The final goal of meiosis is to create haploid gametes or spores from a diploid cell. Prior to meiotic DNA synthesis a diploid cell has two chromatids for each chromosome each with its own centromere. Meiotic DNA synthesis produces a sister chromatid for each existing chromatid and they remain joined at their centromere. There are now four chromatids for every chromosome (Figure 1a) and meiosis must ultimately reduce the number of chromatids such that each haploid product contains only one chromatid for every chromosome. This is accomplished through two separate nuclear divisions.

The first meiotic nuclear division is termed the reductional division since the number of chromatids is reduced

Figure 3 Isomerization of Recombination Intermediates

a) Strand transfer has been completed; isomerization-intermediate chi-structure b); endonucleolytic cleavage at 1 produces the crossover structure of Figure 2g, cleavage at 2 produces the noncrossover structure of Figure 2f, isomerization intermediate can continue through isomerization to produce c); a), b), and c) are all functionally eqivalent.

Figure 3 Isomerization of Recombination Intermediates



by one half in the two resulting nuclear bodies. Homologous chromatids segregate to opposite nuclear bodies thereby assuring each will possess a full chromosome complement (Figure 1b). The second meiotic division is called the equational division because the centromere joining the sister-chromatids is split equally between them as they segregate to opposite nuclear bodies. Again, this division assures each of the four resulting nuclear bodies will have a full complement of chromosomes (Figure 1) (29).

The final stage of fungal meiosis is to package the four nuclear bodies into spores. S. cerevisiae does so immediately and the spores mature in a sac-like structure called an ascus. Ascobolus, Bombardia, and Sordaria, however, allow the four meiotic products to undergo a postmeiotic mitotic division thereby producing eight haploid nuclei which are then assembled into spores within an ascus (Figure 1d). This can facilitate genetic analysis of meiotic recombinational events because events on each DNA strand of a chromatid can be easily detected (see below).

Gene Conversion, Postmeiotic Segregation, and Mismatch Repair

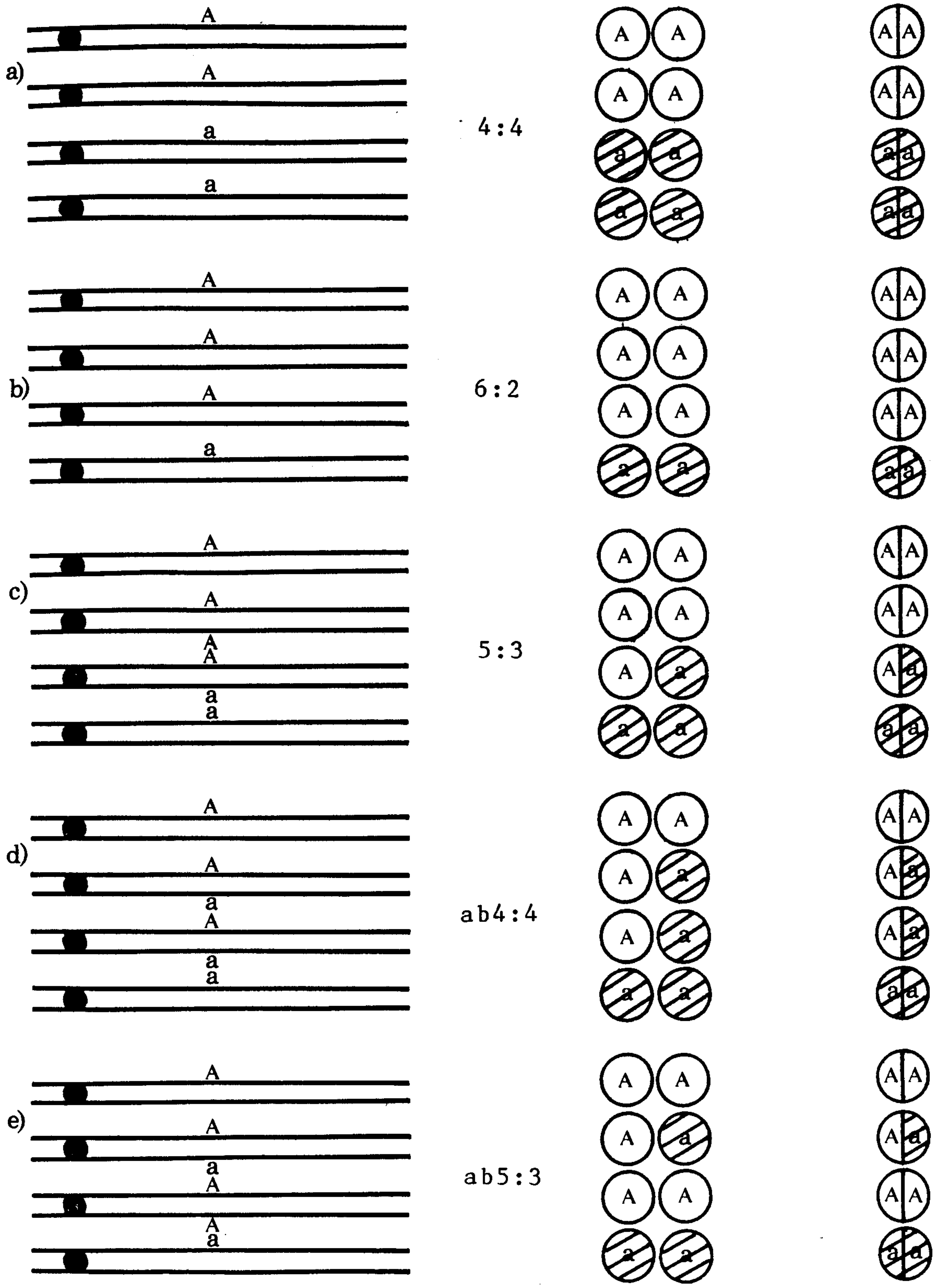
Cells carrying heterozygous auxotrophic mutations, such as LEU1/leu1-12 where leu1-12 lacks the ability to produce leucine, are used to monitor chromosomes and strand segregation through meiosis. Normal segregation in S. cerevisiae is 2:2 and Ascobolus shows a 4:4 segregation pattern due to their one postmeiotic division. Since the two chromatid strands present in S. cerevisiae spores segregate at the first mitotic division, it should be possible to detect 4:4 segregation patterns. For the purpose of this discussion all normal meiotic segregation patterns will be considered to be 4:4.

As stated previously, deviations in the 4:4 segregation pattern occasionally arise (95, 134). These patterns consist of 6:2, 2:6, 5:3, 3:5, aberrant (ab) 5:3, ab 3:5, and ab 4:4 (Figure 4) (95, 134). In the case of the 6:2 and 2:6 patterns it appears the gene on one chromatid has been "converted" to the gene on its homologous chromatid. Roman demonstrated that this example of intragenic recombination was the result of gene conversion (109). He selected eight S. cerevisiae asci showing a 1:3 (2:6) segregation in strains that were homozygous for adenine prototrophy at a specific locus. The spores were separated and the three mutant spores were mated to strains carrying the original adenine mutation. For example, the three mutant spores resulting from a ADE3/ade3-1 diploid were all mated to a strain carrying the ade3-1 allele. If the "third" ade3-? spore had arisen by mutation there was a good chance that it was a different allele from the ade3-1 allele. The ade3-1/ade3-? diploid could then form Ade$^+$ cells through mitotic recombination. If the ade3-? spore was the product of recombination it would be identical to the ade3-1 allele present in

Figure 4 Segregation Patterns for A. immersus and S. cerevisiae

a) A heterozygous marker A/a normally segregates 4:4. b) A gene conversion event results in a 6:2 segregation pattern. c) An unrepaired mismatch on one chromatid gives a 5:3 segregation pattern. d) Unrepaired mismatches on two chromatids gives an ab4:4 segregation pattern. e) Occasionally, one unrepaired mismatch will result in an ab5:3 segregation pattern (see text and Figure 5).

Figure 4 Segregation Patterns for *A. immersus* and *S. cerevisiae*


a)
4:4
b)
6:2
c)
5:3
d)
ab4:4
e)
ab5:3
A
a

the original diploid. Roman found that all of the ade3 mutations were in fact identical to the ade3-1 allele indicating that the 1:3 asci were produced by a recombinational event.

All of the other segregation patterns can only be detected after the chromatid strands segregate at the first postmeiotic mitotic division. These patterns are called postmeiotic segregations. They are easily detected in Ascobolus since its spores contain the segregated strands and they can be genetically tested by separating and testing the spores. In Saccharomyces, the spores must be separated and grown into colonies for genetic testing. Theoretically, the chromatids will segregate at the first division and the resulting mother and daughter cells will each form their own sector of the colony. Postmeiotic segregations are then detected by testing the colonies for the presence of sectors.

Meselson and Radding, as well as Holliday before them, proposed that gene conversion and postmeiotic segregation are the direct result of the transfer of genetic material from one chromatid to its homolog (58, 86, 134). The Meselson and Radding model for recombination has been described and is shown in Figure 2. After the physical exchange of DNA is completed there may be mismatched base pairs in the resultant heteroduplex DNA due to mutations in one or both of the chromatids involved. These mismatches have two possible fates; they can be repaired or they can be left as they are. Repair of the mismatches conceptually requires the removal of the mismatched region on either strand to form a single-strand gap followed by filling in the gap by a DNA polmerase (95, 134).

The Meselson and Radding model for recombination contains two regions of heteroduplex DNA, one symmetric and one asymmetric. These regions are a vital part of the recombinational model because when coupled with the mismatch repair process gene conversion and postmeiotic segregation can be explained.

All of the various mismatch repair possibilities and the predicted segregation ratios for each case are shown in Figure 5 as if exchange had occurred according to the model of Meselson and Radding. The m1 mutation, in Figure 5, lies within the asymmetric heteroduplex region and the m2 mutation lies in the symmetric heteroduplex region. Mismatches in both asymmetric and symmetric heteroduplex regions can be repaired to produce 4:4, 6:2, 2:6, 5:3, and 3:5 segregations but it is important to note that asymmetric and symmetric mismatches have different repair requirements to produce identical segregation ratios. A 6:2 segregation at the asymmetric m1 locus requires repair of the only mismatch from m1/+ to +/+ while a 6:2 segregation at the symmetric m2 locus requires repair of both mismatches present from m2/+ to +/+ (Figures 5b and 5g). It is also important to realize that repair of a mismatch may or may not occur. For exam-

Figure 5 Mismatch Repair and Meiotic Segregation

a) Genetic exchange has occurred as in the Meselson and Radding model. The ml mutation is within the asymmetric heteroduplex region and the m2 mutation is in the symmetric heteroduplex region. Chromatids 1, 2, 7, and 8 are assumed to be present in all other figures and contribute to the segregation patterns.

b) - f) Asymmetric segregation patterns.

b) repair of ml to + on chromatid 3 to give a 6+:2ml segregation pattern.

d) no repair of mismatch giving 5+:3ml segregation.

f) repair of + to ml on chromatid 4 to give the normal 4+:4ml pattern.

c) and e) As if the invading strand in exchange carried the ml mutation instead of the + allele, follow the marker in parentheses.

c) repair of + to ml on chromatid 3 to give the 2+:6ml pattern.

e) no repair of mismatch giving 3+:5ml segregation.

Figure 5 Mismatch Repair and Meiotic Segregation

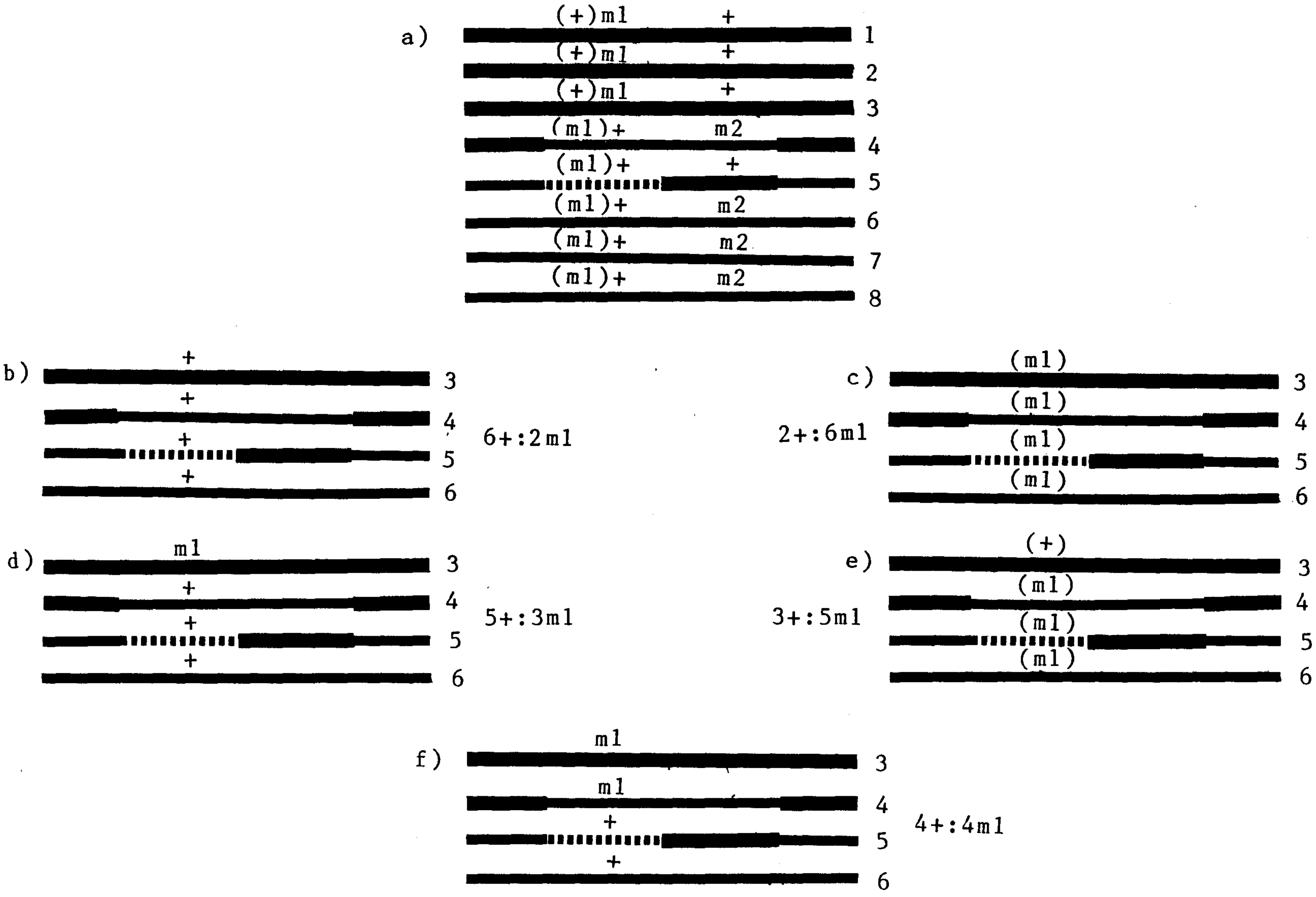

a)
(+)m1 + 1
(+)m1 + 2
(+)m1 + 3
(m1)+ m2 4
(m1)+ + 5
(m1)+ m2 6
(m1)+ m2 7
(m1)+ m2 8
b)
+ 3
+ 4
+ 5
+ 6
6+:2m1
c)
(m1) 3
(m1) 4
(m1) 5
(m1) 6
2+:6m1
d)
m1 3
+ 4
+ 5
+ 6
5+:3m1
e)
(+) 3
(m1) 4
(m1) 5
(m1) 6
3+:5m1
f)
m1 3
m1 4
+ 5
+ 6
4+:4m1

Figure 5 Continued

g) - n) Symmetric segragation patterns.

g) repair of m2 to + on chromatids 4 and 6 giving 6+:2m2 segregation.

h) repair of + to m2 on chromatids 3 and 5 to give 2+:6m2 pattern.

i) no repair on chromatids 5 or 6 coupled with repair of m2 to + on chromatid 4 to give 5+:3m2 segregation.

j) no repair on chromatids 3 or 4 with repair of + to m2 on chromatid 5 to give 3+:5m2 pattern.

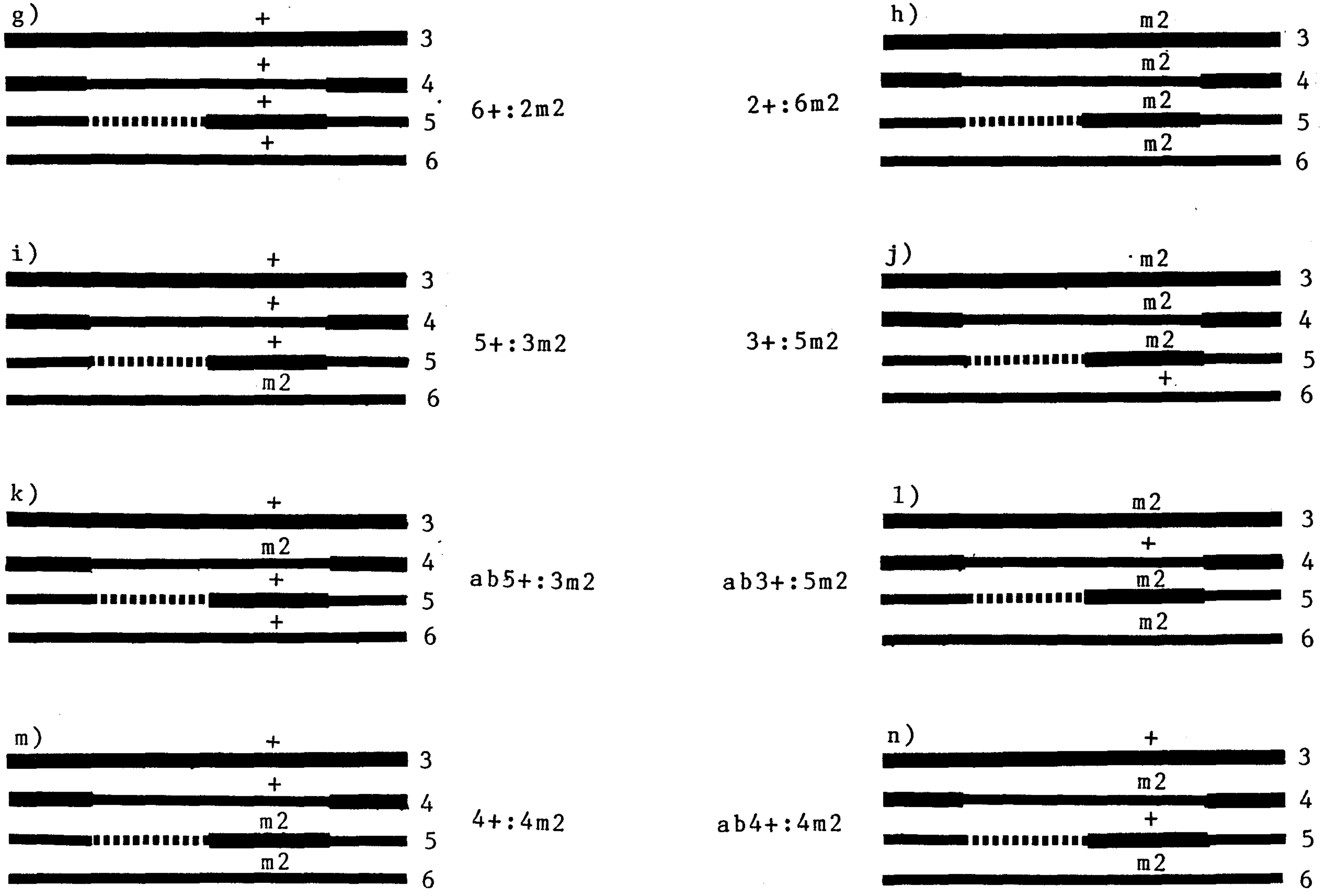
k) no repair on 3 or 4 with repir of m2 to + on 6 to give ab5+:3m2 pattern.

l) no repair on 5 or 6 with repair of + to m2 on 3 to give ab3+:5m2 pattern.

m) repair of m2 to + on 4 with repair of + to m2 on 5 giving normal 4+:4m2 pattern.

n) no repair on 3, 4, 5, or 6 giving ab4+:4m2 segregation

Figure 5 Continued


g)
+
3
+
4
+
5
+
6
6+:2m2
h)
m2
3
m2
4
m2
5
m2
6
2+:6m2
i)
+
3
+
4
+
5
m2
6
5+:3m2
j)
m2
3
m2
4
m2
5
+
6
3+:5m2
k)
+
3
m2
4
+
5
+
6
ab5+:3m2
l)
m2
3
+
4
m2
5
m2
6
ab3+:5m2
m)
+
3
+
4
m2
5
m2
6
4+:4m2
n)
+
3
m2
4
+
5
m2
6
ab4+:4m2

ures 5f and 5m). Even if these conditions are met, 2:6, 3:5, ab5:3, ab3:5, and ab4:4 segregation patterns cannot be derived for the mismatches in the asymmetric heteroduplex region depicted in Figure 5a. The 2:6 and 3:5 segregation patterns can be attained if the invading strand carries the m1 allele instead of the + allele (Figures 5b and 5e). Segregation patterns for symmetric mismatches are unaffected by the chromatid of origin of the invading strand. The ab5:3, ab3:5, and ab4:4 segregation patterns still cannot be derived from asymmetric mismatches and their presence is then indicative of symmetric heteroduplex formation during recombination.

Symmetric Versus Asymmetric Heteroduplex DNA

If the presence of symmetric heteroduplex DNA followed by mismatch repair can account for all of the meiotic gene conversion and postmeiotic segregation events, why does the Meselson and Radding model also incorporate a region of asymmetric heteroduplex DNA? This question is best answered by examining the literature that led up to the Meselson and Radding model.

After Roman demonstrated that gene conversion was a recombination process with high fidelity (109) other investigators also found evidence of 6:2 and 2:6 conversions using other fungi. Case and Giles noticed it at the *pan2* locus in *Neurospora crassa* (16), Strickland found it at the biotin locus in *Aspergillus nidulans* (127), and Olive found it at the *grey* locus in *Sordaria fimicola* (93). *Sordaria* produces an eight-spored ordered ascus meaning the eight spores are in the same relative order as the eight chromatid strands before the first meiotic division (Figure 1). Olive also found evidence for 5:3, 3:5, and ab4:4 segregations at the *grey* locus. A 5:3 or 3:5 segregation was interpreted to mean one chromatid consisted of two strands with different alleles at the same locus and the ab4:4 segregation meant that two chromatids had this type of hybrid configuration. This led Holliday (58) and others (134) to propose that these segregations were due to the presence of hybrid or heteroduplex DNA formed during recombination. Holliday proposed a recombination model to account for the ab4:4 segregation which is similar to the previously discussed Meselson and Radding model except initiation occurs at the same point on both homologous strands which then displace each other and form only symmetric heteroduplex DNA (58).

The Holliday model not only explained the data of Olive (93) and Kitani et al. (68, 69) for *Sordaria* but the data assembled by Leblon and Rossignol (73, 74, 75) with *Ascobolus* also fit the model. Other studies, however, were not as supportive of the idea of symmetric heteroduplex DNA. Fogel and Mortimer used the *ARG4* locus of yeast to examine the frequency of apparent two strand double exchanges, given the genetic configuration *A* *arg4* *B*/*a* *ARG4* *b* where *A* and *B* are linked flanking markers. If symmetric heteroduplex DNA occurs in the ARG4 region and mismatch repair is random for each heteroduplex then in a certain percentage of the cases mismatch correction will be in opposite directions on the two heteroduplexes and in half of these cases the resulting genetic configuration will be an apparent two strand double crossover. The exact theoretical frequency of apparent two strand double exchanges was calculated and applied to the experimental case. They found that the experimental frequency was much less than the theoretical indicating that, at least for the ARG4 locus of yeast, symmetric heteroduplex DNA is not formed and observed gene conversion events are the result of asymmetric heteroduplex DNA (37, 38, 86). DiCaprio and Hastings later confirmed this for the SUP6 region of yeast (22).

The possibility of having asymmetric heteroduplex DNA actually came to light in 1971 when Stadler and Towe demonstrated that for the w17 locus in *Ascobolus* there were few (<2%) ab5:3 or ab3:5 segregations among all 5:3 and 3:5 segregations detected (122). These would be expected to be rare if heteroduplex DNA was predominantly asymmetric (see above). By contrast, the frequency of ab5:3 and ab3:5 segregations was about 1/3 of all 5:3 and 3:5 segregations at the grey locus in *Sordaria fimicola* which does show symmetric heteroduplex DNA by virtue of its ab4:4 segregations (69).

After the Meselson and Radding model was published in 1975 supportive evidence was published. DiCaprio and Hastings published their data showing the lack of symmetric heteroduplex DNA at the SUP6 locus (22). Also, Sang and Whitehouse (115) showed that at the buff locus of Sordaria brevicolis only 6% of the postmeiotic segregations could be attributed to symmetric heteroduplex formation. Additionally, Paquette and Rossignol were able to demonstrate the actual switch from asymmetric to symmetric heteroduplex DNA as predicted by the Meselson and Radding model (see below and 98). Meselson and Radding had realized along with other collegues at the Aviemoe conference in 1974 that in order to account for the segregation data from Sordaria, Ascobolus, and Saccharomyces, a general model for recombination would require the incorporation of both symmetric and asymmetric heteroduplex DNA (98).

Meiotic Gene Conversion and Crossing Over

The Meselson and Radding model allows for gene conversion and crossing over to occur from a common initiation event (86). These crossovers are said to be associated. Kitani et al. in 1962, while working with the grey locus of Sordaria fimicola first uncovered this phenomenon and found that approximately 40% of the gene conversion events and postmeiotic segregations had an associated crossover (68). The frequency of associated crossovers varies from one organism to another and even for different alleles of the same gene (95). The _buff_ locus of _S_. _brevicolis_ shows a low frequency of 20% while the frequency of different _arg4_ alleles in yeast range from 18% to 66% (39, 115). Whereas crossovers associated with homologous chromosome gene conversion can be readily demonstrated, it appears that crossovers are not associated with meiotic intrachromosomal gene conversion (62). Because associated crossovers can be produced through an isomerization process during recombination (Figures 2, 3) and this isomerization is reversible and at equilibrium, many researchers think that ideally associated crossovers should follow a "50% rule". The range of associated crossover frequencies does include 50% but the "50% rule" is not hard and fast and could be ignored.

The Length of Meiotic Heteroduplexes

Studies involving genes with multiple alleles have demonstrated that heteroduplex DNA is a region and not just a point of DNA, and this region can be over one centiMorgan in length (22). These studies have been responsible for identifying the phenomenon known as co-conversion and a related phenomenon called polarity. Polarity has also led to the idea that there may be some specific DNA sequences responsible for the initiation of recombination (95). These three aspects of meiotic recombination all helped in demonstrating the varying lengths of heteroduplex DNA.

Co-conversion was first identified at the _pan2_ locus of *Neurospora crassa* in 1959 by Case and Giles (15). They used a strain carrying two allelic mutations at *pan2* and looked at many unselected asci and found a few cases where both mutations had converted. In 1964 they added a third *pan2* mutation and found cases where all three mutations showed conversion events (16). Two of the adjacent *pan2* alleles also demonstrated coincident postmeiotic segregation patterns indicating that their conversion events were intimately linked (16).

The *ARG4* region of *S. cerevisiae* was examined by Fogel and Mortimer using four different mutant alleles (36). The alleles had been assigned relative positions within the *ARG4* locus using X-ray induced recombination frequencies. Only two of the four alleles were present in any of the strains examined and they were in repulsion (or trans) so coincident convertants could not form prototrophs if a single tract of DNA was responsible for the co-conversion. Two of the *arg4* alleles (*arg4-4* and *arg4-17*) were over 1000 nucleotides apart. Analysis of over 1600 unselected asci revealed instances of co-conversion between each pairwise allelic combination and the degree of co-conversion was inversely related to the distance between the markers (36). This and subsequent studies showed that co-conversion: a) can occur in a region that is over 1000 nucleotides long; b) is the result of direct transfer of a single tract of DNA; and c) frequencies decrease as distance between alleles increase (36, 37, 38). The third case brings us to the property of polarity.

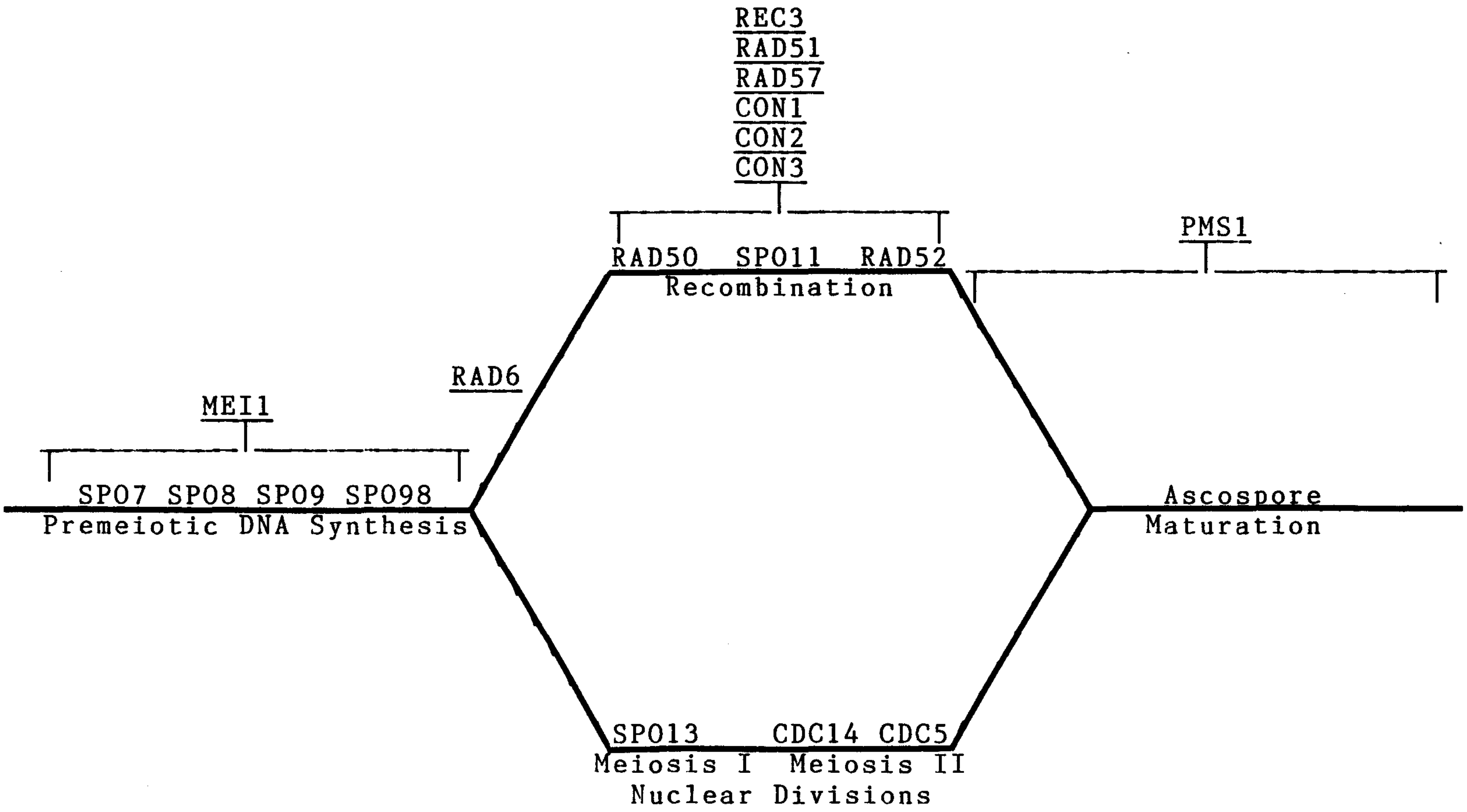
Many genes in many organisms have been studied which have large numbers of mutant alleles. For the most part, gene conversion frequencies are highest for mutant alleles at one end of the gene and the frequencies decrease as the position of the mutation moves toward the other end (for a review see 134). This phenomenon is called polarity. Polarity is thought to depend on the distance between a mutation and a fixed recombination initiation site (95). If there are fixed recombination initiation sites and heteroduplex DNA is then produced and terminated at random by an endonuclease, then the lengths of heteroduplex DNA should form a gradient with shorter lengths predominating. It then follows that a mutation lying relatively close to the initiation site will fall within the heteroduplex DNA region more often than a mutation further away from the initiation site.

Polarity has been extensively studied in Ascobolus immersus by Paquette and Rossignol (98, 111, 112). In one study they used 15 mutations spanning the b2 locus which show mainly 5:3 segregations (98). It was first demonstrated that the mutations showed polarty, and the high and low conversion ends were identified. They then analyzed the various alleles for the frequency of ab4:4 segregations and found that the alleles at the low conversion end had higher frequencies than the high conversion end. This was interpreted to mean that the low conversion end of the gene had a higher frequency of symmetric heteroduplex DNA than the high conversion side of the gene (112). Further experiments with other b2 allelic arrays reinforced their observations and led them to conclude that asymmetric heteroduplex was formed immediately after initiation of recombination and a switch to symmetric heteroduplex DNA was made within the b2 locus (96, 110). This is exactly what the Meselson and Radding model for recombination predicted (85). Co-conversion has also been demonstrated for the b2 locus so the proper temporal order of events is initiation of recombination followed by heteroduplex formation which in turn produces the phenomena of co-conversion and polarity.

Mutations Affecting Meiotic Recombination and Meiosis

In order to understand the molecular aspects of meiosis and recombination, investigators have sought for and utilized mutations that affect the proper functionings of these processes. From a theoretical viewpoint, specific steps within meiosis and recombination can be blocked by mutations in genes that are responsible for proceeding through the steps. Figure 6 is a flow-chart depicting four stages of meiosis which mutations should and have been detected (29) and these steps are premeiotic DNA synthesis, recombination, nuclear divisions, and ascospore development. Premeiotic DNA synthesis is a prerequisite for meiotic re-

Figure 6 Temporal and Spatial Arrangement of Genes Acting in Meiosis


REC3
RAD51
RAD57
CON1
CON2
CON3
RAD50 SPO11 RAD52
Recombination
PMS1
RAD6
MEI1
SPO7 SPO8 SPO9 SPO98
Premeiotic DNA Synthesis
Ascospore
Maturation
SPO13 CDC14 CDC5
Meiosis I Meiosis II
Nuclear Divisions

combination and mutations blocking it will also block recombination and the rest of meiosis (3). Mutations affecting recombination directly should allow premeiotic DNA synthesis but block succeeding steps (29). Mutations in the meiosis I reductional division could bypass the division and produce two diploid spoes as the result of the meiosis II equational division. Similarly, a block in the meiosis II division could also produce two spore asci but these spores would not be true diploids since they would be homozygous for mating type and would be more like haploids following mitotic DNA replication.

A mutation affecting recombination could block one of many points of action. It could block initiation, strand transfer, isomerization, resolution, mismatch repair, or even specifically gene conversion or crossing over (86). Finally, mutations affecting ascospore maturation may affect future germination capabilities, or possibly mismatch repair (29). This section will only be concerned with those mutations appearing in Figure 6 and Table I and how they affect recombination.

In *Saccharomyces cerevisiae* the recessive mutations *spo7*, *spo8*, and *spo9* as well as the dominant mutation *SPO98* all block premeiotic DNA synthesis as well as recombination (29). How and to what extent their respective wild type gene products act in this process is unknown (29). All of the genes depicted in Figure 6 on the recombination fork

Table 1 Recombinational Mutations in S. cerevisiae

Mutation	Meiotic effects	Mitotic Effects
con1	premeiotic DNA synthesis +; no gene conversion; reduced sporulation (40).	None
con2	premeiotic DNA synthesis +; no gene conversion; reduced sporulation; inviable spores (40).	None
con3	premeiotic DNA synthesis +; no gene conversion; reduced sporulation: inviable spores (40).	None
mei1	premeiotic DNA synthesis -; no gene conversion (113)	None
rec1	None	X-ray and UV induced gene conversion absent; X-ray & UV Xing over O.K. (110).
rec3	No sporulation (110).	no induced or spontaneous conversion; induced Xing over normal (110).
rad6	premeiotic DNA synthesis + (44); no recombination (44) no sporulation (21)	elevated Xing over (114); slightly decreased gene conversion (114).
rad50	premeiotic DNA synthesis +; no recombination; reduced sporulation; inviable spores (44)	elevated spontaneous mitotic recombination (44, 80).
rad51	premeiotic DNA synthesis +; reduced sporulation; inviable spores; greatly reduced recombination (89).	decreased spontaneous mitotic recombination (114).
rad52	premeiotic DNA synthesis +; reduced sporulation; inviable spores; greatly reduced gene conversion and Xing over (44, 101)	spontaneous recombination; homothallic interconversion prevented (80).

Table 1 continued

Mutation	Meiotic Effects	Mitotic Effects
rad57	premeiotic DNA synthesis +; reduced sporulation: inviable spores; reduced recombination (44).	reduced spontaneous recombination (114).
spo11	premeiotic DNA synthesis +; reduced sporulation; poor spore viability; no recombination (71).	None
spo13	premeiotic DNA synthesis +; no meiosis I reductional division (70).	None
rem1	None	hypermutable; hyperrecombination (45).
rad3	None	hyperrec. (9).
rad18	None	hyperrec. (9).
cdc9	None	hyperrec. (41).
cdc21	temperature sensitve, at 34 no DNA synthesis or recombination (7)	hyperrec. (41).
pms1	premeiotic DNA synthesis +; poor spore viability; increased gene conversion and post meiotic segregation; normal Xing over (135).	increased spontaneous and mutation (135).

allow premeiotic DNA synthesis but affect recombination to some extent and those appearing on the line have been temporally ordered (79, 81, and Malone personal communication). Those genes appearing above the brackets have not been assigned a relative temporal position in meiosis. Mutations in RAD6 and REC3 prevent sporulation entirely but only rad6 mutations have been shown to allow premeiotic DNA synthesis and prevent recombination (21, 44, 108). CON1, CON2, and CON3 mutants were detected by screening EMS treated cells for a deficiency in meiotic gene conversion. They also have greatly reduced levels of sporulation and the spores produced are inviable (40). This is what would be expected if recombination is necessary for proper chromosome segregation at the meiosis I division (3). Improper chromosome segregation at the meiosis I or meiosis II division is thought to produce aneuploidy in the resulting spores rendering them inviable (3). Mutations in the genes RAD50, RAD51, RAD52, and RAD57 are thought to be necessary for the repair of chromosomal double-strand breaks and they all resemble the CON genes in their meiotic phenotypes (40, 41). The effects of the RAD52 mutations will be discussed in detail in the following chapter and the effects of a RAD50 mutation will be discussed in Chapter IV. A mutation in the MEI1 gene has been shown to be temperature sensitive at 34° C and at this temperature it acts like the CON and RAD50-57 mutations (113). The PMS1 gene probably acts after the recombination mutations since it presumably is involved in mismatch repair after the actual transfer of genetic material (135). Curiously, it also reduces spore viability without reducing recombination unlike previous mutations (135).

The SPO13 gene product is involved in controlling the first meiotic nuclear division because the spo13-1 mutation does not undergo the meiosis I reductional division and proceeds directly to the meiosis II equational division producing two diploid recombinant spores (70). Malone and Esposito and then Malone used the spo13-1 mutation in conjunction with the rad50-1, rad52-1, rad6-1, and spo11-1 mutations hoping to bypass the need for chromosome pairing and recombination (79, 81, and R. Malone personal communication) and then order these mutations temporally relative to each other within the recombination pathway (79, 81). These experiments will be discussed more fully in Chapter II but the results did produce an order of action in recombination which is RAD6, RAD50, SPO11, and RAD52 as depicted in Figure 6 (79, 81). It may be possible to order the other recombination mutations in Figure 6 by using this so-called spo13 bypass system.

MITOTIC RECOMBINATION

Mitotic recombination in the form of reciprocal crossing over was first demonstrated by Stern in 1936 while studying twin spots in *Drosophila melanogaster* (121). Since then mitotic crossing over has been demonstrated in a variety of eukaryotic organisms including fungi, plants, insects, and mammals (for a review see 134).

In 1955 Pritchard constructed a diploid strain of *Aspergillus nidulans* containing two different *adE* mutations in repulsion and two other genes flanking the region (103). He selected for wild type recombinants assuming they would be produced by a reciprocal crossover event which would result in the exchange of the outside markers. In addition to wild type recombinants with exchanged flanking markers he found recombinants with the parental configurations of the flanking markers. Because the production of these configurations would have required a second crossover event he concluded that the crossover between the *adE* alleles had promoted the second crossover and hence negative interference was responsible (103). It was not until Roman performed genetic tests on similarly selected *S. cerevisiae* mitotic recombinants that the recombinants were shown to be the result of a nonreciprocal exchange (109). Roman used a diploid that had the two _ADE5_ mutations _ade5-1_ and _ade5-2_ in repulsion and selected for Ade$^+$ mitotic recombinants. If the Ade$^+$ recombinants were produced by a reciprocal crossover event then a chromatid containing both the _ade5-1_ and _ade5-2_ alleles would be produced . Roman put 88 Ade$^+$ recombinants through meiosis and tested the Ade$^-$ segregants for the presence of the doubly mutant chromosome by mating them with _ade5-1_ and _ade5-2_ parental haploid strains. If the Ade$^-$ segregant was doubly mutant _ade5-1,2_ it would not be able to produce Ade$^+$ recombinants with either parent. None of the 88 Ade$^+$ recombinants tested carried the doubly mutant _ade5-1,2_ allele, thereby demonstrating that mitotic intragenic recombination was the product of a nonreciprocal exchange, and it is now referred to as mitotic gene conversion (109, 134).

The Mitotic Cell Cycle and Recombination

The mitotic cell cycle of _S. cerevisiae_ and other fungi is similar to that of other eukaryotic cells. It is composed of four main phases called G1, S, G2, and M. The G1 phase is metabollically active and the chromosomes are unreplicated i.e. at the two strand state. Chromosomes are replicated in the S or synthesis phase leading to the four strand G2 phase. The M phase is primarily concerned with mitosis and the segregation of chromosomes. Since the research within this thesis deals exclusively with the yeast S. cerevisiae and much is known about mitotic recombination in this organism, the following discussion will only be concerned with S. cerevisiae.

Theoretically, mitotic recombination can occur at the two strand stage (G1) or at the four strand stage (G2). Meiotic recombination occurs at the four strand stage but there have been studies that suggest that the majority of mitotic recombination occurs at the two strand stage. In 1978 Fabre performed an elegant experiment to show that G1 recombination can take place (30). He utilized two allelic temperature sensitve cdc4 mutations that arrest cells in the G1 phase at the restrictive temperature. By holding the cells at the restrictive temperature the cells could only progress out of G1 by relieving the cdc4 block through an intragenic recombination event thereby producing a wild type CDC4 gene. To spur on the recombination event Fabre irradiated the arrested cells with either UV or gamma radiation. He found that cells did progress through the cell cycle and they were CDC4 indicating that recombination had occurred in G1 (30).

Another approach to detect in what stage of mitosis recombination takes place is to take advantage of spontaneously produced sectored colonies. These colonies arise through mitotic recombination and two sectors of a colony will be derived from the recombinant chromatids (95).

Esposito used strains heterozygous for *ADE5* (*ADE5*/*ade5*) and homozygous for the *ADE2* mutation *ade2-1*. This strain is Ade$^-$ and the homozygous *ade2-1* mutation causes the cells to accumulate a red pigment and hence a red colony. If, however, there is a crossover event in the *ADE5* chromosome proimal to the centromere followed by segregation, one sector of the colony will be *ADE5*/*ADE5* *ade2-1*/*ade2-1* and will be red but the other sector will be *ade5*/*ade5* *ade2-1*/*ade2-1*. The homozygous *ade5* mutation blocks adenine biosynthesis before the *ade2-1* mutation, no red pigment is formed, and the second sector is white (24).

Esposito also included heteroallelic mutations at the *LEU1* and *TRP5* loci (*leu1-12*/*leu1-c* *trp5-2*/*trp5-c*) which are centromere proximal to the *ade5* mutation. He then selected for gene conversion at *LEU1* or *TRP5* and an associated crossover would appear as a red-white sectored colony. He then tested the sectors for their genetic configurations at the converted locus. Esposito reasoned that a G2 conversion at *TRP5* can only result in the two sectors having the genotypes (c+/++) and (++/+2). This sectored colony can also arise through a G1 conversion but eight other genotypes can also arise through a G1 event. For example, sectored genotypes (++/c+) and (++/c+) or (++/+d) and (++/+d) are indicative of a G1 event. Analysis of the sectored colonies demonstrated that 70 out of 71 Trp$^+$ convertants and 20 out of 20 Leu$^+$ convertants were produced in G1 (24).

Roman and Fabre also studied prototrophic sectored colonies (110). They exposed cells to X-rays and then arrested some cells in G1 using the temperature sensitive mutation _cly8_, which stops growth at 35° C, and they arrested some cells in G2 using Methylbenzimidol-2-yl Carbomate (MBC). They found that for cells arrested in G1 2 out of 32 prototrophs could have been explained by either G1 or G2 conversion but for G2 arrested cells 12 out of 45 prototrophs were of this class. They concluded that most X-ray induced gene conversion events occur in G1 but some can occur in G2 (110).

Heteroduplex DNA in Mitotic Cells

Like meiosis, there are no mitotic gene conversion resultant genotypes indicative of asymmetric heteroduplex; the two genotypic classes arising through asymmetric heteroduplex DNA can also be explained by a symmetric intermediate (95). There are however, seven genotypic classes that are indicative of symmetric heteroduplex DNA. Using this knowledge, Golin and Esposito were able to calculate minimum percentages of conversion events occurring through symmetric heteroduplex intermediates. They found that at least 27% of _TRP5_ and at least 30% of _LEU1_ mitotic gene convertants resulted from symmetric heteroduplex DNA (45). This is very unlike meiotic heteroduplex DNA in yeast which is almost exclusively asymmetric (95).

Golin and Esposito's study of spontaneous mitotic recombinationn extended into the phenomenon of mitotic co-conversion (46, 47). Further investigation on their part showed that co-conversion at LEU1 and TRP5, which are 18 cM apart, occurs 1200 times more frequently than predicted from their individual frequencies (46, 47). Co-conversion between LEU1 and MET13 (94 cM apart) was enhanced 200 fold indicating that, like meiotic co-conversion, mitotic co-conversion is distance dependent (95). Since the greatest separation of markers shown to co-convert in meiosis is 1.4 cM (22), Golin and Esposito concluded that heteroduplex tracts are much longer in mitosis than in meiosis. The X-ray induced mitotic gene conversion experiments of Roman and Fabre did not reveal any instances of co-conversion between ADE6 and CLY8 which, like LEU1, TRP5, and MET13 are on chromosome VII, are only 20 cM apart (110). This suggests that any heteroduplex formed in response to X-rays is shorter than heteroduplex formed spontaneously.

Another phenomenon related to meiotic heteroduplex DNA is polarity (see above) which is thought to reflect a gene's proximity to a recombination initiation site (95). The HIS1 gene of yeast shows polarity in meiosis but not in mitosis (35, 60) and this is also true for the ILV1 locus (64, 116). The lack of mitotic polarity can be explained by the presence of long tracts of heteroduplex DNA which normally span an entire gene and all of its polar alleles (95).

Another explanation is that meiosis and mitosis may use different initiation sites. This later explanation is supported by evidence that the distribution of recombination events is different in meiosis and mitosis resulting in different map distances (82).

Association of Mitotic Gene Conversion With Crossing Over

The association of crossing over with gene conversion is predicted from the Meselson and Radding model of recombination (86). It was previously mentioned that such an association does exist in meiotic recombination and if recombination in mitosis is mechanistically similar to meiosis there should also be an association in mitosis. Indeed, a number of genes have been investigated in yeast for mitotic recombination and the association of crossing over with gene conversion ranges from 10% to 50% (for a review see 133). That crossing over and gene conversion are associated in mitosis is supported by the fact that both events are stimulated by physical and chemical agents (31, 34).

In the same X-ray induction experiments that Roman and Fabre used to show that gene conversion can occur in the G2 phase of the cell cycle, they showed that 30% of the crossovers associated with a G1 conversion must have occurred in G2 (110). Although these two events may have been independent of one another they still show that gene conversion and crossing over can be physically associated and yet temporally dissociated. Furthermore, some mutations have also been shown to uncouple gene conversion from crossing over. The rad52-1 mutation is required for gene conversion in mitosis but homologous crossing over is reduced to a lesser degree (62, 80). Specific examples of RAD52-independent reciprocal crossing over include reciprocal crossing over between duplications (62), sister chromatid crossing over (102, 138) and under certain conditions circular plasmid integration (see Chapter IV) (96). These observations suggest that some, but certainly not all, reciprocal exchanges are not dependent on gene conversion.

Mitotic Recombination Mutations and Their Effects

Mutations affecting meiotic recombination have been previously described and outlined in Table 1. Some of these mutations are specific for meiotic recombination and some also affect mitotic recombination. In addition, some mutations specifically affect mitotic recombination (Table 1). Both types of mitotic recombination mutations will be discussed here.

The list of mutations affecting both meiotic and mitotic recombination include rec3, rad6, rad50, rad51, rad52, rad57, and pmsl. The rec3, rad51, rad52, and rad57 mutations all reduce spontaneous mitotic recombination (80, 109, 114). As stated earlier, rad52-1 affects mitotic gene conversion to a greater degree than crossing over. Other differences between gene conversion and crossing over are demonstrated by the rec3 mutation which prevents UV or X-ray induced gene conversion but allows induction of crossing over (109). Also, the rad6 mutant, a member of the error-prone repair group, demonstrates elevated levels of spontaneous crossing over but decreased gene conversion (114). The rec1 mutation is similar to rec3 but it does not affect meiotic recombination (81, 108). The other two mutations affecting both meiotic and mitotic recombination both show increased levels of the latter. The rad50-1 mutation abolishes meiotic recombination while increasing mitotic recombination and the pms1 mutation elevates both meiotic and mitotic recombination levels (80, 135).

Mutations specifically affecting mitotic recombination include rec1 (see above), rem1, rad3, rad18, cdc9, and cdc21. All of these mutations produce an increase in mitotic recombination (7, 9, 42, 45, 108). Mutations in rem1 and cdc9 presumably create lesions in DNA requiring the RAD52 recombination repair system since both rem1 rad52 and cdc9 rad52 double mutants are inviable (42, 45). It is also presumed that the hyper-rec phenotype of cdc21 mutations is the result of recombinogenic lesions in DNA (95). RAD3 and RAD18 are members of the excision repair system and the error-prone repair system respectively (9, 41). It is thought that mutations in these genes cause an increase in recombination by eliminating one repair system followed by the chanelling of more DNA lesions into the recombination repair system (41). Table 1 provides more information regarding the effects of mitotic recombination mutations.

Meiotic Versus Mitotic Recombination

It is apparent that there are some very real differences between meiotic and mitotic recombination in yeast. First, spontaneous recombination levels are on the order of 1,000 times higher in meiosis. Second, recombination occurs at the four strand stage in meiosis and at the two strand stage in mitosis. Third, symmetric heteroduplex DNA is quite rare in meiosis but it occurs frequently in mitosis; this may be a reflection of the much longer regions of heteroduplex DNA in mitosis. This may also explain the absence of polarity in mitosis. And finally, gene conversion can be separated from crossing over genetically, by mutations (eg. rad52-1), and temporally, in the cell cycle, in mitosis but not in meiosis.

The differences in recombination between meiosis and mitosis may be due to the priority placed on it by the two growth processes. Recombination is essential to the meiotic process since a successful reductional division requires proper pairing and recombination to prevent the production of aneuploid spores (for a review see 3). Spontaneous mitotic recombination may merely represent the result of DNA repair since many mutations in DNA repair systems abolish or increase mitotic recombination (95). Finally, there may be a RAD52-dependent recombination system for gene conversion and associated crossovers and a RAD52-independent system for other reciprocal crossovers.

AN ALTERNATE RECOMBINATION MECHANISM: THE DOUBLE-STRAND BREAK MODEL

The Meselson and Radding model for recombination was formulated to incorporate observations that could not be easily explained by the previously proposed Holliday model (58, 86). Similarly, Szostak et al. have taken more recent observations concerning recombination and have proposed a new model called the double-strand break model (DSB) (131). In this section observations leading up to the model will be presented followed by the model itself. Finally, a breif discussion will be presented comparing the merits of the two models.

Double-Strand Breaks Are Recombinogenic

Orr-Weaver et al. and Orr-Weaver and Szostak took advantage of the ability of yeast to be transformed with chimeric plasmids containing both *E. coli* and yeast sequences to ask if double-strand breaks stimulate recombination (94, 96, 97). They introduced double-strand breaks and gaps into yeast sequences of circular plasmids and transformed appropriate yeast strains with these linear plasmids. They found that the linear plasmids could a) be repaired to eliminate the break or gap (gene conversion), b) be integrated into the yeast sequence homologous to the plasmid sequence containing the break or gap (crossing over), and with integration the break or gap was repaired correctly (94, 96), and c) gap repair is associated with formation of heteroduplex DNA near the DNA ends of the plasmid. They also found that the transformation frequency, which was dependent on integration in their experimental design, was increased up to 300 fold by the presence of the double-strand break (94). Finally, the repair and integration of a linear plasmid was found to be RAD52-dependent (94). They concluded that a) gene conversion and crossing over could be initiated by a double-strand break, b) the DNA ends produced by the double-strand break interacted directly with homologous sequences, c) double-strand breaks stimulated recombination, and d) although this experimental system used mitotic cells, the requirement for RAD52 suggests that recombination may occur through double-strand breaks in meiotic cells (94, 96, 97). These observations and conclusions led to the formulation of the double-strand break model for recombination.

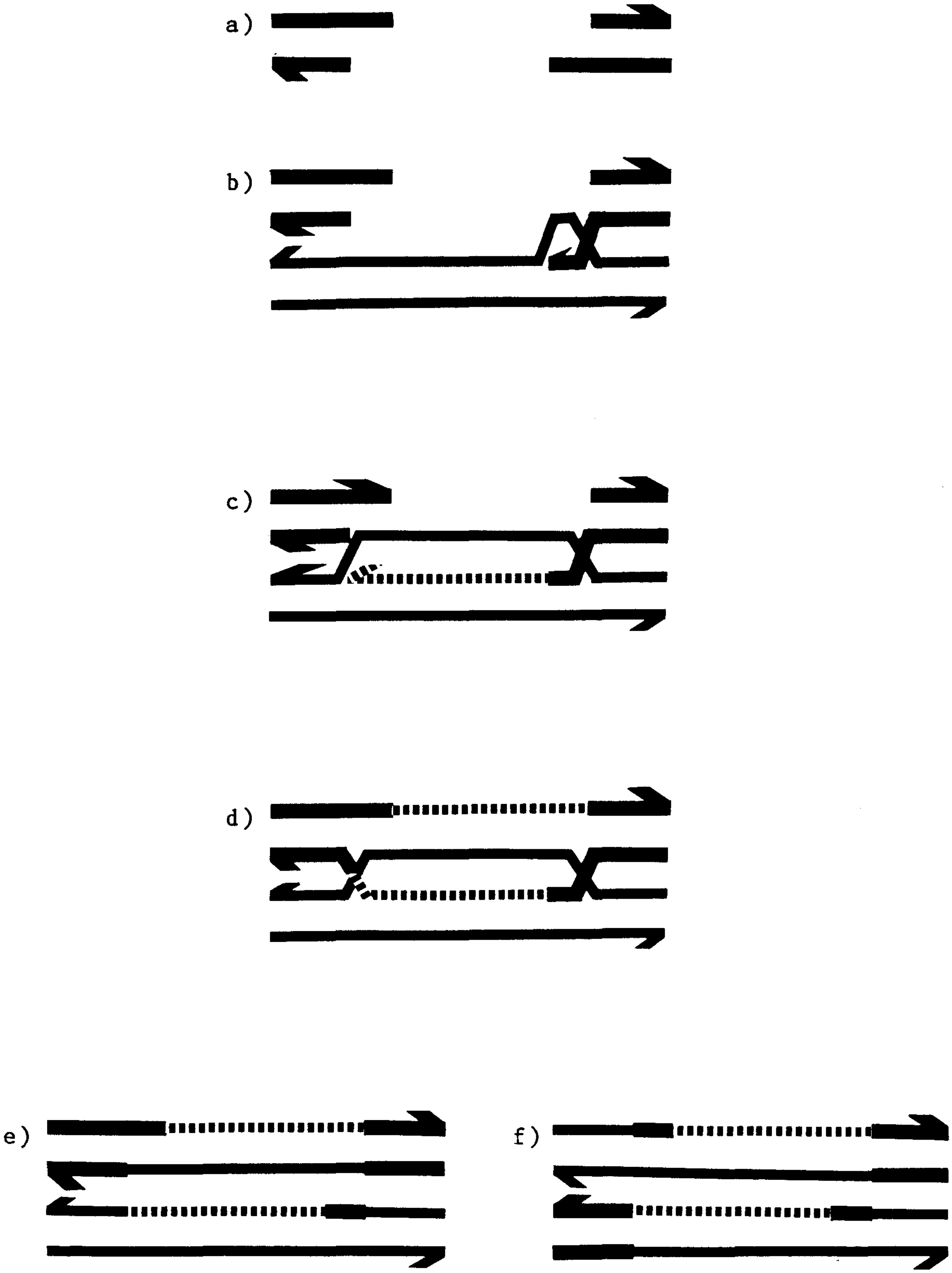
The Double-Strand Break Model

The DSB model for recombination is depicted in Figure 7 and should be referred to during the following description. Initiation of recombination requires the introduction of a double-strand break in the recipient chromatid. The break is then enlarged to a gap and 3' ends are

Figure 7 The Double-Strand Break Model For Recombination

a) A double-strand break is made in one duplex and exonucnucleases enlarge it into a gap. b) One free 3' end invades the homologous duplex displacing a D loop. c) The D loop is enlarged by repair DNA synthesis until the second 3' anneals to it. d) Gap repair is completes with a second round of repair synthesis and branch migration forms two Holliday structures. By cleaving the inner or the outer strands of the Holliday structures either two possible noncrossover e) or two possible crossover f) configurations result (see text).

Figure 7 The Double-Strand Break Model For Recombination



Noncrossover

Crossover

left (Figure 7a). One of the 3' ends invades a homologous region of the donor chromatid thereby displacing a D-loop (Figure 7b). When the enlarging D-loop contains sequences complementary to the second 3' end these two regions will anneal and DNA synthesis can now begin from the 3' end (Figure 7c). The gap is therefore repaired by two sequential DNA synthesis steps and two regions of asymmetric heteroduplex DNA have been formed (Figure 7d). The left hand asymmetric heteroduplex can be extended as in the Meselson and Radding model but the right hand region can only be extended by branch migration if it can occur with two crossed strands present. Branch migration of the left hand region produces a second Holliday structure and branch migration of either Holliday structure will produce symmetric heteroduplex DNA. Here both Holliday structures must be resolved. If they are both resolved in the same sense (i.e. cutting inner or outer strands in both structures) the result is gene conversion without an associated crossover. If the two Holliday structures are resolved in the opposite sense there is gene conversion with an associated crossover. This model allows for associated crossovers to occur on either side of the conversion event unlike the Meselson and Radding model (86, 94).

The DSB model can account for the observed meiotic segregation patterns as follows. If a mutation lies within the region of the gap then the two rounds of DNA replication would produce 6:2 or 2:6 segregation patterns since the ungapped chromatid is the template for both rouncs of synthesis. Postmeiotic segregations would arise if the mutation fell in the regions of heteroduplex DNA without mismatch repair. The appearance of ab5:3 segregation patterns are not easily explained by the DSB model as depicted in Figure 7, but minor modifications can account for them (for detailed explanations see 95, 131). Since yeast has a very large excess of 6:2 over 5:3 segregations it is assumed that the gapped region is large and the heteroduplex regions are small. Ascobolus shows many more postmeiotic segregations so its gapped region is smaller and the heteroduplex regions are larger than those of yeast.

Co-conversion of adjacent loci is easily explained by the DSB model. If the loci both fall into the gapped region they will both be converted. The DSB model also explains polarity since the gapped region can vary in size as can the heteroduplex region of the Meselson and Radding model. This also allows for the existence of specific initiation sites. Therefore, the DSB model can account for the basic requirements of recombination.

Meselson and Radding Versus Double-Strand Break

As described, the DSB model contains some features not found in the Meselson and Radding model. First, the DSB model postulates the existance of two Holliday structures in close proximity in meiosis. Using cells undergoing meiotic recombination, Bell and Byers (6) isolated DNA, cross-linked the strands to prevent branch migration and examined the DNA with an electron microscope. They found that most joined molecules had two or more Holliday structures separated by 100 to 1,000 base pairs. This is the appropriate size for co-conversion events in meiosis (36, 37). Second, the DSB model proposes that associated crossovers may occur on either side of the conversion event. Fogel et al. demonstrated that this was the case for ARG4 convertants in yeast (38). And third, the DSB model postulates that the DNA strand with the initiating event is the recipient of genetic exchange while the Meselson and Radding model postulates that the initiating strand is the donor molecule (86, 131). Evidence in support of the DSB model comes from specific mutations in Schizosaccharomyces pompe and Sordaria brevicolis which exhibit abnormally high levels of gene conversion and a high level of disparity of 6:2 over 2:6 conversions when heterozygous (49, 78, 95). The simplest explanation is that these sites are recognized as initiation sites and then are the recipients of the wild type information from the homologous chromatid (95).

Another observation that lends itself to the DSB model is the parity (equal numbers of 6:2 and 2:6 segregations) of gene conversion events for all classes of mutations in yeast. If the mutation falls in the gapped region then either a 6:2 or 2:6 segregation will result depending on which chromatid is gapped, and both chromatids shpuld be gapped with equal frequency. Also, mismatch repair itself may be a product of the DSB pathway. Hastings has recently proposed that mismatches can be recognized by nucleases that produce a double-strand gap in their place which is then repaired (50). This could help to account for the great predominance of 6:2 over 5:3 segregation patterns in yeast. The potential 5:3 site, which lies in the small heteroduplex region, could then be within a gapped region leading to a 6:2 pattern. In order to extend this idea to *Ascobolus* it must be assumed that the recognition of mismatches in *Ascobolus* is much less efficient than in yeast, and this does seem to be the case (95).

These observations support the DSB model, however, other evidence does not. The DSB model requires double-strand breaks for initiation. Using the double-strand break repair deficient mutation *rad52-1*, Resnick et al. showed that single-strand breaks but not double-strand breaks accumualte in meiosis (107). For the DSB model to accomodate this observation the *RAD52* gene product must somehow be responsible for the production as well as the repair of double-strand breaks (55, 106). Also, evidence presented later in this thesis demonstrates that recombination initiation can occur in strains of yeast homozygous for *rad52* mumutations indicating that double-strand breaks are not involved in this specific instance (see Chapter II). Finally, there is evidence that the DSB model may apply to X-ray induced mitotic recombination and the specific recombination event involved in the interconversion of the mating type in yeast which both presumably have a double-strand break as an intermediate (131). The DSB model for recombination does appear to warrant further detailed investigations.

At this point in time it is impossible to say which current model for recombination is correct or even closer to the actual mechanism. The Meselson and Radding model can account for all of the detailed experimental data generated by Rossignol and his colleagues for the _b2_ locus of _Ascobolus immersus_ (75, 111, 112). Both models can account for the basic requirements of recombination and the DSB model can explain specific instances of recombination where a double-strand break is known to be an intermediate (86, 131). It is also possible that both mechanisms occur in all cells to varying degrees and it is almost certain that the future will bring more modifications and models.

SUMMARY AND CONCLUSIONS

Genetic recombination is a widespread phenomenon occurring in all organisms examined (134). It can occur during both developmental phases of eukaryotes, i.e. mitosis and meiosis. The purposes of recombination are probably three fold: one, to correct mutations as they occur (41); two, to allow for the proper alignment of chromosomes on the meiotic spindle thereby allowing for proper meiotic disjunction and segregation (3); and three, genetic diversity (133). These three purposes can be hard to distinguish and rank in importance because they all may occur by the same mechanism and overlap of purposes undoubtably occurs.

CHAPTER II

MEIOTIC INDUCTION OF RECOMBINATION IN rad52 MUTANTS OF YEAST

INTRODUCTION

The effects of ionizing radiation (X-rays) on the yeast *Saccharomyces cerevisiae* have been extensively studied (41, 43, 51, 106). In addition many mutations causing sensitivity to X-rays have been isolated and characterized by many investigators (17, 89, 104). Richard Snow isolated many X-ray sensitive mutations which remained unpublished until complementation tests were conducted by Game and Mortimer in 1974 (43). They grouped the mutants according to the guidelines adopted at the IV International Yeast Genetics Conference held at Chalk River, Canada in 1970, which states that all loci conferring X-ray sensitivity in the mutant form be denoted by *rad* and those not previously described as UV sensitive be numbered from 50 upward (43). A total of eight complementation groups were found and they were numbered *RAD50* through *RAD57* (43). Of these loci, this chapter will be primarily concerned with the *RAD52* locus.

There are several known mutant alleles of *RAD52*; the ones utilized in these experiments are *rad52-1* and *rad52-2*.

In addition, using recombinant DNA techniques, several gene interruptions have been constructed (eg. rad52::LEU2). The rad52-1 mutation was isolated by Resnick on the basis of its X-ray sensitivity (104) and similarly, Snow isolated the rad52-2 allele (119). The rad52::LEU2 allele was created by Resnick and Nitiss (107). Resnick also showed that a rad52-1 homozygous diploid had decreased levels of sporulation and produced inviable spores when subjected to meiosis inducing conditions (104). The effect of the rad52-1 mutation on meiosis and sporulation was then further examined by Prakash et al. and Game et al. (44, 101). The results of both groups are in agreement and state that in rad52-1 diploids: a) premeiotic DNA synthesis occurs; b) sporulation is decreased; c) inviable spores are produced; and d) intra- and intergenic meiotic recombination is absent. Prakash et al. (101) also demonstrated that spontaneous mitotic recombination is lowered approximately five fold, X-ray and UV induced intragenic mitotic recombination is absent, and spontaneous mitotic reversion rates are elevated 10 to 20 fold over wild type in rad52 diploids.

These observations indicate that RAD52 is required for spontaneous mitotic recombination, meiotic recombination, and the efficient production of viable spores in meiosis. However, the recombination effects were examined at only one or two heteroallelic loci and could be loci specific (80, 101). The effects of the rad52-1 mutation on spontaneous mitotic recombination were extended to several other loci by Malone and Esposito and confirmed the observations of Prakash et al. (80, 101). Extension of the effects of *rad52* mutations on meiotic recombination is the subject of this chapter and will be fully discussed later. The effect of *rad52-1* on spore production is presumably due to faulty recombination which prevents proper chromosome segregation at the meiosis I reductional division (3).

The X-ray sensitive nature of *rad52* mutants suggests that it is required for some type of DNA repair. Double mutant analysis with *rad52-1* and other repair mutations indicated that there are three repair groups that are: a) excision repair, b) error prone repair, and c) recombinational repair (for a review see 41). It was postulated that *RAD52* belongs to the recombinational repair group and this was later confirmed by Prakash et al. (101). Also, Resnick and Martin showed that *rad52* mutants are deficient in DNA double-strand break repair which is probably resposible for the X-ray sensitive phenotype of these mutants (106).

The effects of *rad52* mutations on homologous chromosome meiotic and mitotic recombination led investigators to ask if other types of recombination are also dependent on *RAD52*. Recombination occurring through gene conversion appears to be *RAD52*-dependent (95). These events, other than spontaneous mitotic recombination and meiotic recombination previously mentioned, occur during the mitotic life cycle and include homothallic interconversion of mating type and gene conversion between duplications (62, 80). On the other hand, several types of reciprocal crossing over have been reported to be RAD52-independent (95). Included in this group are crossing over between duplications (62), sister cromatid crossing over (102, 138), and integration of circular integrating plasmids into homologous chromosomal regions (96) (the latter process will be discussed more thoroughly in Chapter IV). Also, although spontaneous mitotic crossing over is greatly reduced by rad52-1, it is less affected by the mutation than mitotic gene conversion (62, 80). Therefore, at least for mitotic recombination, there appears to be a RAD52-dependent recombination process encompassing gene conversion and perhaps associated crossing over, and a RAD52-independent process for some types of reciprocal crossing over (95).

The RAD52 gene certainly plays a pivitol role in mitotic and meiotic recombination in yeast but the function of the gene product and the step(s) at which it acts are still unknown. In order to determine an approximate point of action for RAD52 in meiotic recombination, Malone and Esposito and then Malone used another mutation (rad50-1) deficient in meiotic recombination along with a mutation that altered sporulation, the spo13-1 mutation (79, 81). The spo13-1 mutation eliminates the meiosis I reductional division but other meiotic events including recombination occur normally

(70). Klapholtz showed that recombination is not absolutely required in spo13 mutants, presumably since homologs do not have to pair and segregate away from each other (70). Malone and Esposito found that rad50-1 spo13-1 diploids produced two viable, nonrecombinant, diploid spores but rad52-1 spo13-1 diploids produced two inviable spores (81). Subsequently Malone discovered that the rad50-1 rad52-1 spo13-1 triple mutant produced two viable nonrecombinant, diploid spores similar to the rad50-1 spo13-1 double mutant (79). If RAD50 and RAD52 are acting sequentially along a recombinational pathway then the phenotype of the double mutant rad50-1 rad52-1 should be the same as that of the single mutant which blocks recombination at the earlier step. A mutation that blocks a pathway at an earlier step than another mutation is said to be epistatic to the later mutation. Since the triple mutant rad50-1 rad52-1 spo13-1 has the same phenotype as the rad50-1 spo13-1 double mutant it suggests that RAD50 is epistatic to, or acts before, RAD52 in meiotic recombination.

Resnick et al. showed that rad52 diploids accumulate single-strand breaks in genomic DNA during meiosis (105). The number of breaks corresponds roughly to the number of recombinational events in wild type cells in meiosis (105). No single-strand breaks accumulate in rad50 cells during meiosis. This is in agreement with the hypothesis that rad50 blocks recombination before, and rad52 blocks recombination after, the development of single-strand breaks.

All of the existing data makes it likely that *RAD52* acts at an intermediate step in meiotic recombination (44). It should then be possible to use a procedure like that of Sherman and Roman's to induce meiosis and recombination in *rad52* diploids, return the cells to mitotic conditions before committment to sporulation, and detect recombinants (116). This is essentially the procedure used by Prakash et al. and Game et al. (44, 101). The failure of their studies to detect possible low levels of recombination in meiosis may be attributed to either poor meiotic synchrony, ineffecient meiosis, use of strains heterozygous for other genes affecting meiotic recombination, or examination of cultures which may have had relatively high frequencies of recombination in the starting mitotic population.

This study, therefore, reexamined recombination in various *rad52* mutants using essentially the procedures of Sherman and Roman (116). The strains used generally exhibit more synchronous meiosis than those of previous studies and they have several diagnostic loci to monitor recombination. As previously reported, *rad52-1* and *rad52-2* mutants produce inviable asci and meiotic recombination is greatly reduced. Surprisingly, however, and contrary to previous reports (44, 101), this study demonstrates that *rad52* mutants exhibit a low, but significant, induction of recombination during meiosis. These results indicate that recombination is initiated and recombinants can be detected in rad52 meiotic cells. These results still confirm that the RAD52 gene product is required for the completion of recombination, since the induction of recombination is temporary and recombinants are lost as recombination continues.

MATERIALS AND METHODS

Strains

The *S. cerevisiae* strains used are listed in Table 2. The first four strains are heteroallelic at three diagnostic loci: *URA3*, *TRP5*, and *LEU1*. Strains CE101 and CE102 contain amber and ochre mutations originally present in strains supplied by S. Leibman (University of Illinois, Chicago). Each *rad52* mutation was backcrossed at least three times with good sporulating wild type (RAD52) strains. Diploids were constructed with standard genetic techniques (25, 45) immediately before use since storage of *rad52* diploids can generate a variety of phenotypic suppressors (R. Malone personal communication). The wild type diploid RM104 was obtained from R. Malone. Strains MH32 and MH33 were provided by M. Hoekstra.

Growth and Sporulation

Diploids were streaked out on solid medium containing 3% glycerol, 1% yeast extract (Difco), 2% Bacto peptone (Difco), and 2% Bacto agar (YPG) prior to inoculation into liquid growth medium to enhance growth in medium lacking a fermentable carbon source. The YPG plates were incubated at

Table 2 Genotypes of strains

Strain	Diploid	Relevant Genotype
1	RM104[a]	$\frac{\text{a}}{\alpha}$ $\frac{\text{ura3-1}}{\text{ura3-13}}$ $\frac{\text{CAN1}^{s}}{\text{can1}^{r}}$ $\frac{\text{his7-1}}{\text{his7-2}}$ $\frac{\text{leu1-c}}{\text{leu1-12}}$ $\frac{\text{trp5-c}}{\text{trp5-2}}$
2	RM105	$\frac{\text{a}}{\alpha}$ $\frac{\text{rad52-1}}{\text{rad52-1}}$ $\frac{\text{his1}}{\text{HIS1}}$ $\frac{\text{ura3-13}}{\text{ura3-1}}$ $\frac{\text{can1}^{r}}{\text{CAN1}^{s}}$ $\frac{\text{HIS7}}{\text{his7}}$ $\frac{\text{leu1-12}}{\text{leu1-c}}$ $\frac{\text{trp5-2}}{\text{trp5-c}}$ $\frac{\text{MET13}}{\text{met13-c}}$ $\frac{\text{ade5}}{\text{ADE5}}$
3	RM106	$\frac{\text{a}}{\alpha}$ $\frac{\text{rad52-2}}{\text{rad52-2}}$ $\frac{\text{ura3-1}}{\text{ura3-13}}$ $\frac{\text{CAN1}^{s}}{\text{can1}^{r}}$ $\frac{\text{ade6}}{\text{ADE6}}$ $\frac{\text{leu1-c}}{\text{leu1-12}}$ $\frac{\text{trp5-c}}{\text{trp5-2}}$ $\frac{\text{cyh2}^{r}}{\text{CYH2}^{s}}$ $\frac{\text{met13-c}}{\text{MET13}}$ $\frac{\text{ADE5}}{\text{ade5}}$
4	RM107	$\frac{\text{a}}{\alpha}$ $\frac{\text{rad52-2}}{\text{rad52-1}}$ $\frac{\text{ura3-1}}{\text{ura3-13}}$ $\frac{\text{HIS7}}{\text{his7}}$ $\frac{\text{tyr1-2}}{\text{tyr1-1}}$ $\frac{\text{lys2-2}}{\text{lys2-1}}$ $\frac{\text{ade6}}{\text{ADE6}}$ $\frac{\text{leu1-c}}{\text{leu1-12}}$ $\frac{\text{trp5-c}}{\text{trp5-2}}$ $\frac{\text{cyh2}^{r}}{\text{CYH2}^{s}}$ $\frac{\text{met13-c}}{\text{MET13}}$ $\frac{\text{ADE5}}{\text{ade5}}$
5	MH32[b]	$\frac{\text{a}}{\alpha}$ $\frac{\text{rad52-1}}{\text{rad52-1}}$ $\frac{\text{ura3-13}}{\text{ura3-13}}$ $\frac{\text{leu1-12}}{\text{leu1-12}}$ $\frac{\text{trp5-2}}{\text{trp5-2}}$
6	MH33[b]	$\frac{\text{a}}{\alpha}$ $\frac{\text{rad52-1}}{\text{rad52-1}}$ $\frac{\text{ura3-1}}{\text{ura3-1}}$ $\frac{\text{leu1-c}}{\text{leu1-c}}$ $\frac{\text{trp5-c}}{\text{trp5-c}}$
7	CE101	α rad52-1 his5-c^{oc} leu2-1^{oc} lys2-1^{oc} trp1-1^{am} tyr7-1^{am} ade2-1^{am}
8	CE102	α rad52-2 his5-c^{oc} leu2-1^{oc} lys2-1^{oc} met8-1^{am} trp1-1^{am} tyr7-1^{am} ade2-1^{oc}

[a] Provided by R. Malone; [b] Provided by M. Hoekstra;

^{oc}ochre mutation; ^{am}amber mutation.

30° C for 72 hours. A single colony was picked to 1 ml of sterile 0.2 M dibasic sodium phosphate buffer (Na_2HPO_4), pH 7.5 (PO_4) and the suspension's concentration was determined with a hemeocytometer and a phase contrast microscope. The suspension was used to inoculate 100 mls of liquid medium at a concentration of $1(10)^3$ cells/ml. The medium consisted of 1% potassium acetate, 1% yeast extract (Difco), and 2% Bacto-peptone (YPA). The cultures were incubated with shaking at 30° C.

When the cultures reached a concentration of $2(10)^7$ cells/ml they were harvested by centrifugation and washed twice in sporulation medium which was 2% potassium acetate, pH 7, and supplemented with the necessary amino acids and bases. The cells were resuspended in 50 mls of sporulation medium and incubated at 30° C until sporulation was complete. Sporulation was monitored by removing samples and examining the cells microscopically. Sporulation was complete when the percentage of spores in the culture failed to increase any farther which usually required 48 hours.

Return to Mitotic Growth

The return to mitotic growth procedure is a modification of the one used first by Sherman and Roman (116) to examine the effects of meiosis on intragenic recombination. The sporulating cultures were sampled by removing 2.5 mls approximately every four hours starting at the point of inoculation into sporulation medium. The samples were washed twice with 5 mls of PO_4 buffer and then resuspended in 2.5 mls of PO_4 buffer. From this suspension 0.5 mls was placed in 0.5 mls of 8% formaldehyde to fix a portion of the cells for later enumeration. The rest of the sample was used to inoculate various diagnostic media to follow recombination. These media were solid defined complete media (COM), COM lacking one amino acid or base (drop-out media), media containing either one of the drugs canavinine (U. S. Biochemicals) or cycloheximide (Sigma) (CAN or CYH), YPD, and YPD with 0.1 ul of Methyl methanesulfonate (MMS)/ml (Kodak). MMS mimicks the effects of X-rays and is therefore used to follow rad52 mutations. The drop-out media are identified by the lacking ingredient; LEU drop-out is lacking leucine etc. All of the media has been previously described (45).

The diagnostic media were inoculated with serial dilutions of the cells and the plates were incubated at 30° C until colonies appeared, usually two to three days. The colonies were counted by hand and the concentration of recombinants was calculated and used to determine recombination frequencies which were defined as the recombinant concentration at a given time divided by the total cell concentration at that time as determined from the colonies arising on the YPD or COM plates. Approximately 50 colonies from each diagnostic medium for each sample point for diploid RM107 were picked to YPD master plates for further genetic analysis which were carried out using standard genetic techniques as described by Golin and Esposito (45).

Coreversion Analysis of rad52-1 and rad52-2

Two procedures were used to explore the possibility that either or both rad52-1 or rad52-2 are amber or ochre suppressible mutations.

Procedure A: Strains CE101 and CE102 were streaked out on YPD. Twenty-one colonies of each were picked to master plates with each colony being spread so as to form small square patches. After two days growth the two master plates were replica-plated to the following media: MMS, LEU, TRP, TYR, and HIS. The revertants that grew were then picked to a second master plate which was replica-plated to the same media as before, grown and scored for coreversion.

Procedure B: Master plates containing 21 square patches of either CE101 or CE102 were replica-plated to YPD plates and the latter were irradiated with UV light at a fluence of 20 J/m^2 using 2 General Electric G15T8 Germicidal lamps. The plates were incubated in the dark at 30° C for 48 hours. They were then replica-plated to the following media MMS, ADE, TRP, TYR, HIS, LEU, LYS, and, for strain CE102, to MET. Forty-two MMS revertants and up to 23 revertants from the other media were picked to master plates as square patches and grown. They were then replica-plated to all of the other media, grown, and scored for coreversion.

RESULTS

Sporulation and Viability in *rad52* Mutants After Meiosis

All *rad52* strains tested caused a significant reduction in sporulation abilities as measured by the percentage of cells that formed asci, and all spores produced were inviable (Table 3). This is in agreement with Prakash et al. and Game et al. (44, 101). Analysis of 45 to 48 hour meiotic cultures indicated that up to 50% of the *rad52* cells retained viability. That they were cells and not asci is inferred since spore viability was no more than 1% at 48 hours compared with an overall viability of up to 50%. Standard genetic testing of 48 hour cells indicated that those cells retaining viability and not having a selected recombination event showed no evidence of recombination over mitotic levels. It is assumed that these cells entered meiosis since Resnick, Prakash et al., and Game et al. have previously shown that *rad52-1* diploids exhibit normal levels of premeiotic DNA synthesis (41, 101, 105). It is impossible to say whether or not these cells initiated recombination since recent data from Resnick and Nitiss indicates that recombinants in *rad52* cells are lost when meiotic cells are incubated in rich mitotic medium (e.g. YPD) prior to plating on

Table 3 Sporulation and viability of *rad52* strains after meiosis

Genotype	No. of Cultures	Final Sporulation (ave. %)	Final Survival (ave. %)	Spore Viability	Relative Reduction in Recombination
$\frac{\text{rad52-1}}{\text{rad52-1}}$	4	21 ± 0.5	13 ± 3.5	<0.5[a]	10^{-2}-10^{-3}[b]
$\frac{\text{rad52-2}}{\text{rad52-2}}$	4	25 ± 8.6	50 ± 6.4	0.6	10^{-2}-10^{-3}
$\frac{\text{rad52-1}}{\text{rad52-2}}$	8	10 ± 3.3	48 ± 10.3	1.0	10^{-2}-10^{-3}
$\frac{\text{RAD52}}{\text{RAD52}}$[c]	3	61	96	96	1

All measurements were made after the cells were exposed to liquid sporulation medium for 48 hours with the exception of the *rad52-1*/*rad52-1* cututres. These measurements were made after 45 hours.

[a] Spore viabilities were determined for only one of the cultures of each genotype. Vegetative cell viabilities after exposure to sporulation medium were determined for one culture of *rad52-1*/*rad52-1* and *RAD52*/*RAD52* diploids giving values of 20% and 96% respectively.

[b] Recombination frequencies were compared with those of the *RAD52*/*RAD52* cultures at various diagnostic loci. (See Figures 10-13 and Tables 4-7).

[c] Provided by R. Malone.

selective media. Wild type meiotic cells do not lose recombinants when put through the same regimen (107). Also, vegetative cells remaining in wild type cultures following meiosis show essentially wild type levels of recombination (107). These results are in agreement with previous reports in that a rad52 meiosis is a defective meiosis (44, 101).

Induction of Recombination in rad52 Cells During Meiosis

Exposure to sporulation medium elicits very different responses from rad52 and RAD52 cells (Table 3, Figures 8 and 9). Viability in rad52 cells declines rapidly in sporulation medium whereas in wild type cells it does not (Figure 8), and sporulation is much more efficient in RAD52 cells (Figure 9). Furthermore, meiosis induces a two to three order of magnitude increase in recombination over mitotic levels in wild type cells. In contrast with the earlier reports of Prakash et al. and Game et al. a low but significant increase in recombination over mitotic levels was observed in all rad52 strains tested (44, 101). Tables 4-6 show the actual increases in recombinant cell concentrations through meiosis. The maximum levels of recombination were still on the order of 100 fold less than in RAD52 cells (Table 7). The kinetics of recombination for rad52 strains can be found in Figures 10-13. From these figures it appears that there is a larger induction of recombination in rad52-2/rad52-2 cells. The maximum values are indeed higher

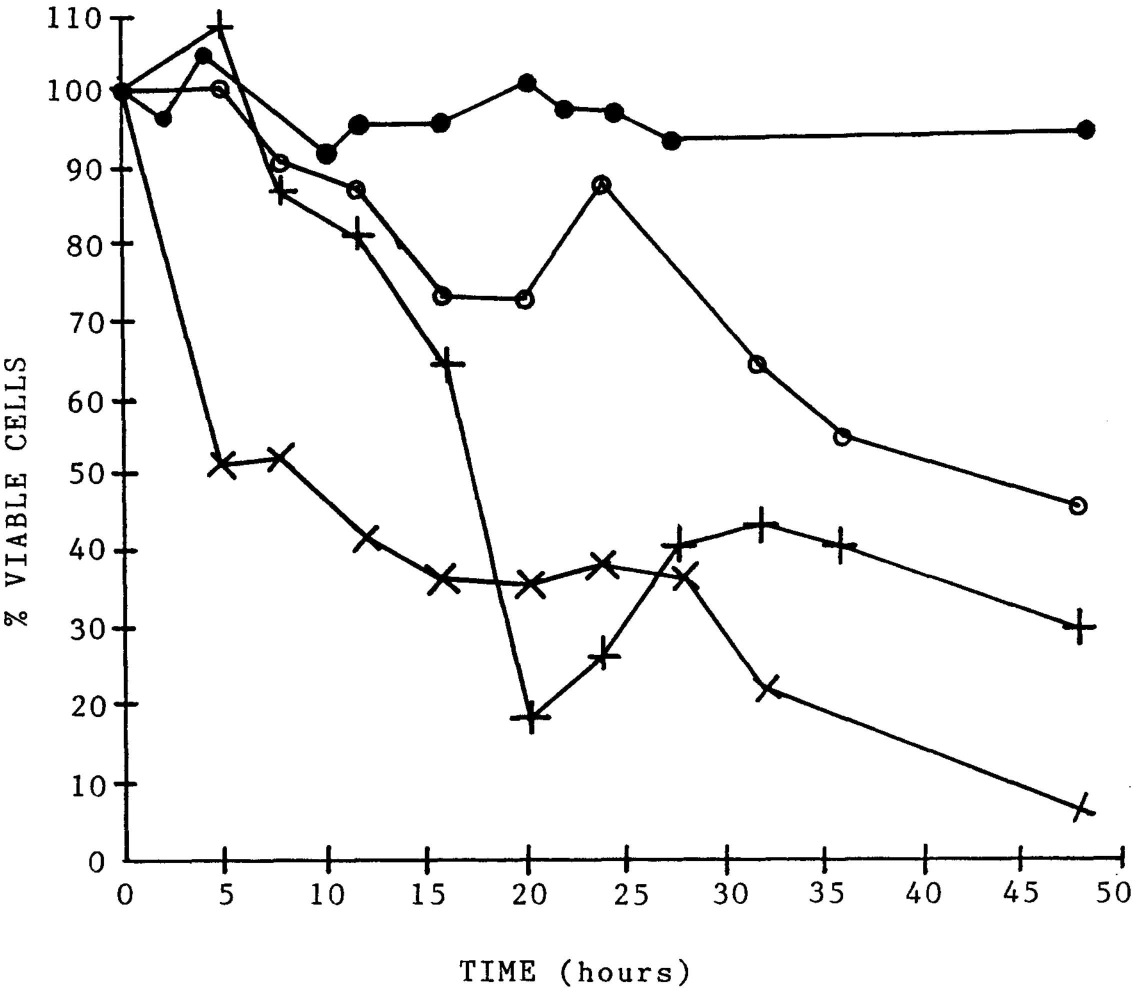


Figure 8 Meiotic viability of RAD52 and rad52 cells. Samples were taken at various times during meiosis and cell viability was determined as described using the following equation:

$$\frac{\text{Cell concentration at the time of sample}}{\text{Cell concentration at 0 hours}} \times 100$$

●, Wild type (RM104); ×, rad52-1/rad52-1 (RM105); +, rad52-1/rad52-2 (RM107); ○, rad52-2/rad52-2 (RM106).

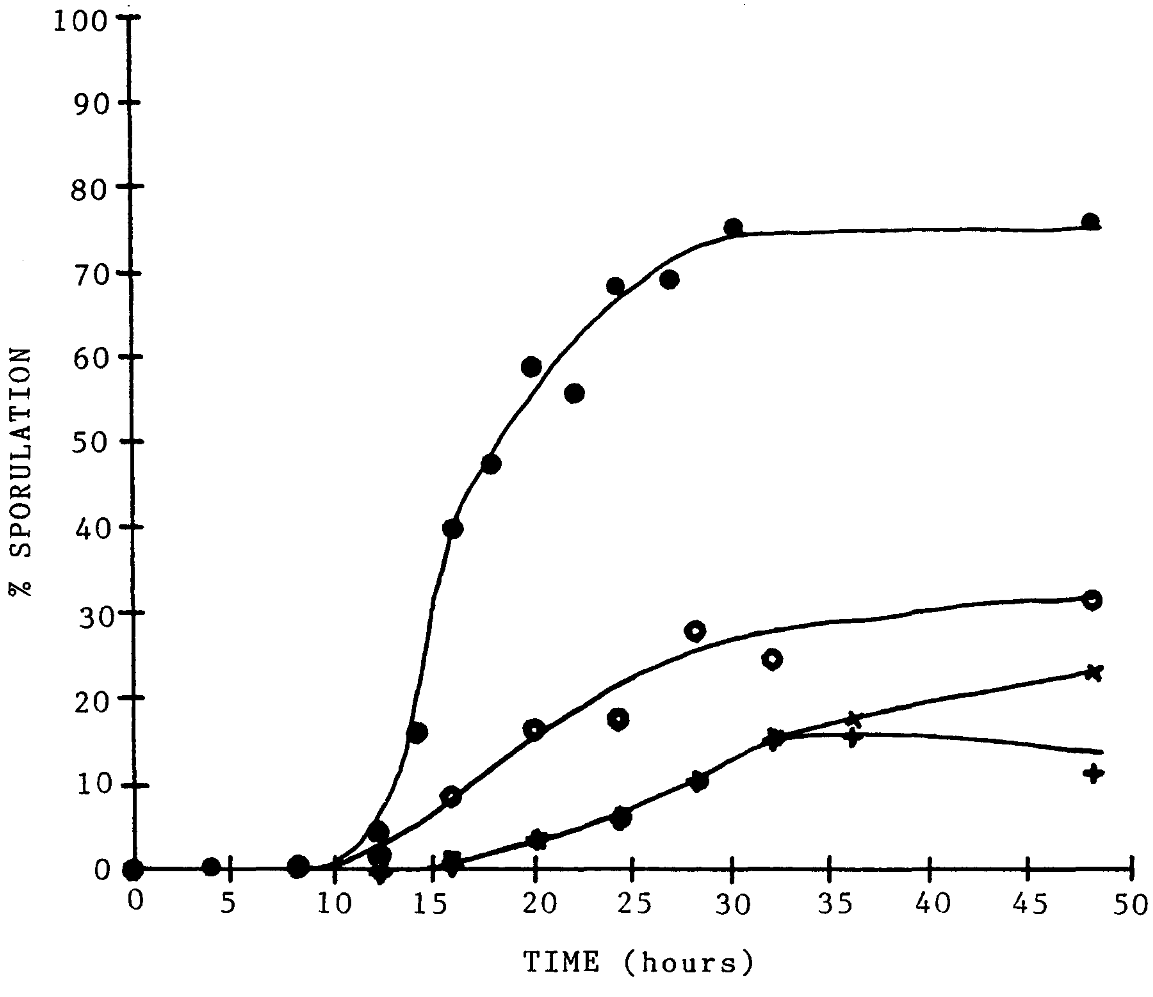


Figure 9 Sporulation of RAD52 and rad52 cells. Samples were taken at various times following inoculation into spor- and sporulation percentages were determined as described. ●, Wild type (RM104); +, rad52-1/rad52-1 (RM105); ×, rad52-1/rad52-2 (RM107); ◎, rad52-2/rad52-2 (RM106).

Table 4 Cell Concentrations During Meiosis

	Total cells/ml X 10^7				Trp$^+$ cells/ml X 10^3			
Time	1	2	3	4	1	2	3	4
0 hours	1.0	2.5	3.0	4.4	0.17	.01	.21	---*
5 hours	1.1	1.3	4.1	4.5	1.9	.07	.42	---
8 hours	2.3	1.3	2.6	4.0	80	.07	.44	---
12 hours	1.2	1.0	2.4	3.8	160	.56	.94	---
16 hours	1.2	0.91	1.95	3.2	200	.49	1.33	---
20 hours	1.3	0.90	.55	3.2	270	.34	1.12	---
24 hours	1.5	1.0	.80	3.9	240	.27	.88	---
28 hours	N.D.	.91	1.23	3.4	N.D.	.27	1.19	---
32 hours	N.D.	.56	1.3	2.8	N.D.	.23	.85	---
36 hours	1.2	N.D.	1.23	2.4	190	N.D.	.76	---
48 hours	N.D.	0.28	.87	2.0	N.D.	.01	.53	---

1 = RAD52/RAD52 (RM104)
2 = rad52-1/rad52-1 (RM105)
3 = rad52-1/rad52-2 (RM107)
4 = rad52-2/rad52-2 (RM106).

*A recombination event at the TRP5 locus occurred early in the mitotic growth phase of diploid RM106 making the calculation of recombinant cell concentration impossible.

N.D. = Not Determined

Table 5 Cell Concentrations During Meiosis

	Leu$^+$ cells/ml X 10^2				Ura$^+$ cells/ml X 10			
Time	1	2	3	4	1	2	3	4
0 hours	2.80	>.01	1.68	8.10	4.20	.5	4.0	7.0
5 hours	15.0	.10	2.78	23.6	12.0	0	6.2	14.5
8 hours	360	.48	2.88	55.8	110	.2	6.3	23.5
12 hours	670	1.88	4.73	81.5	250	.3	8.8	45.5
16 hours	830	1.40	7.48	53.3	200	2.2	9.2	34.8
20 hours	850	1.93	4.40	42.2	310	.8	5.8	23.5
24 hours	1000	.93	4.23	3.11	220	2.8	7.5	22.5
28 hours	N.D.	.60	4.65	25.1	N.D.	1.2	9.8	14.5
32 hours	N.D.	.40	4.20	25.0	N.D.	.3	10	15.5
36 hours	950	N.D.	3.40	30.5	330	N.D.	.8	14.8
48 hours	N.D.	.23	3.25	35.8	N.D.	.2	.23	20.8

1 = RAD52/RAD52 (RM104)
2 = rad52-1/rad52-1 (RM105)
3 = rad52-1/rad52-2 (RM107)
4 = rad52-2/rad52-2 (RM106).

N.D. = Not Determined

Table 6 Cell Concentrations During Meiosis

	Drug resistance X 10^4				
	1	1	2	3	4
Time	CAN	CYH	CAN	CYH	CAN
0 hours	4.9	3.4	6.50	3.20	2.32
5 hours	28.0	20.0	8.50	N.D.	3.20
8 hours	2000	230	9.00	3.32	2.90
12 hours	4800	2300	4.20	3.08	7.70
16 hours	11000	8700	1.80	3.23	7.60
20 hours	12000	9700	2.20	1.53	6.90
24 hours	14000	11000	2.00	1.82	8.50
28 hours	N.D.	N.D.	2.10	2.92	5.40
32 hours	N.D.	N.D.	1.30	1.80	5.50
36 hours	N.D.	N.D.	N.D.	1.73	8.90
48 hours	N.D.	N.D.	0.41	3.15	10.10

1 = RAD52/RAD52 (RM104)
2 = rad52-1/rad52-1 (RM105)
3 = rad52-1/rad52-2 (RM107)
4 = rad52-2/rad52-2 (RM106).

N.D. = Not Determined

Table 7 Meiosis induced recombination in *RAD52* and *rad52* diploids at various loci

	Recombination Frequency											
	ura3 X 10^6			*trp5* X 10^5			*leu1* X 10^5			*rad52* X 10^5		
Genotype	Start	Mid	Final	Start	Mid	Final	Start	Mid	Final	Start	Mid	Final
RAD52 *RAD52*	4.2	210 (12)	280	1.7	1700 (16)	1600	2.8	690 (16)	790	---	---	---
rad52-1 *rad52-1*	0.02	2.5 (16)	0.46	0.04	5.5 (12)	5.8	0.04	1.8 (12)	1.2	---	---	---
rad52-2 *rad52-2*	1.6	12 (12)	10	---	---	---	1.8	21 (12)	18	---	---	---
rad52-1 *rad52-2*	1.3	10.5 (20)	2.6	0.7	20.4 (20)	6.2	0.6	8.0 (20)	3.7	0.5	6.3 (20)	1.0

The diploids were exposed to sporulation medium for various lengths of time and analyzed for the frequency of prototrophs as described in the text. "Start" values refer to the mitotic values at time 0; "final" values are the frequencies at the last time point measured (usually 48 hours); "mid" values refer to the maximum value obtained, or in the case of *RAD52* cells, the value when the final plateau was initially reached (see figures 10-13). The ( ) number below the Mid value indicates the time when the peak or plateau values were reached.

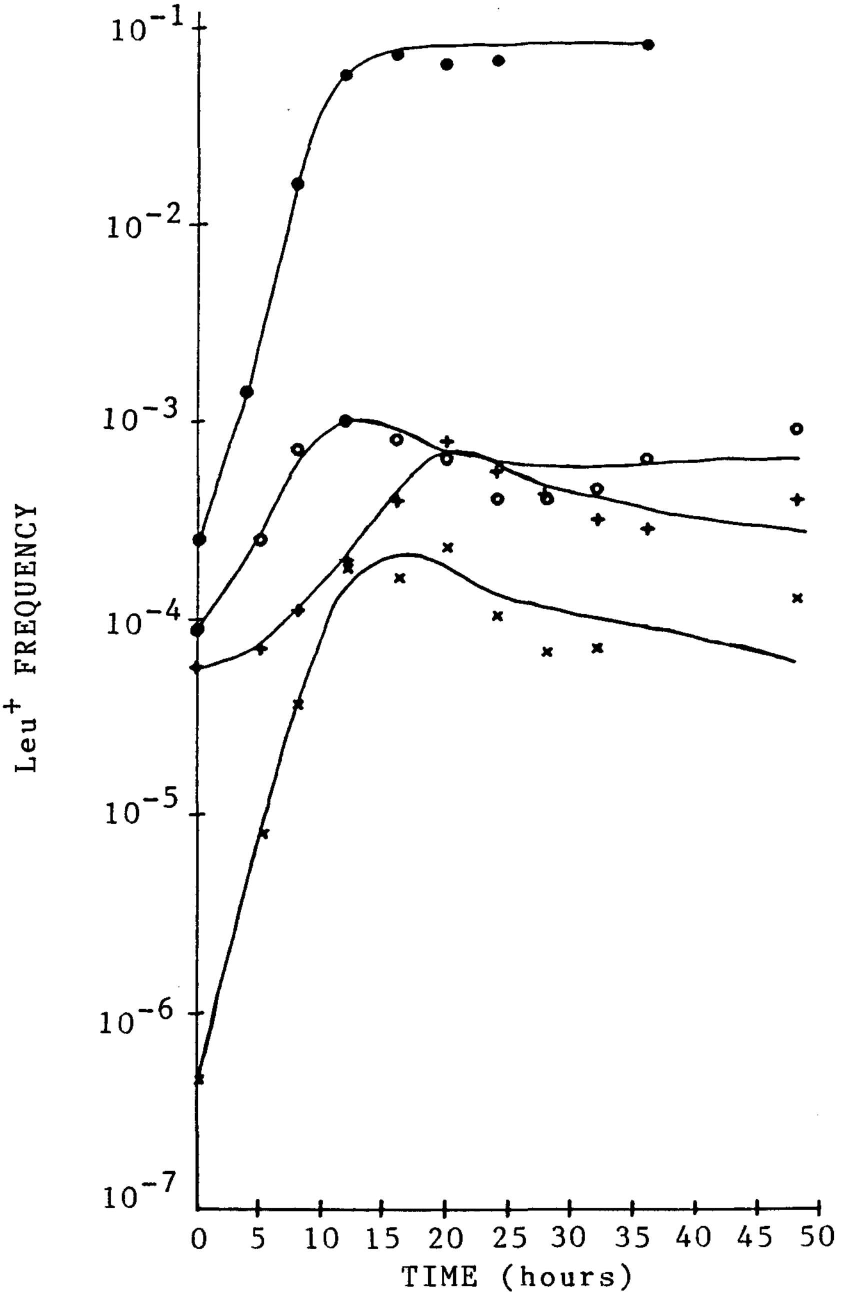


Figure 10 Induction of recombination during meiosis; generation of Leu^+ prototrophs

Samples were taken at various times during meiosis as described and recombination frequencies were determined using the following equation:

$$\frac{\text{Number of } Leu^+ \text{ prototrophs at the time of sample}}{\text{Total number of viable cells at the time of sample}}$$

●, Wild type (RM104); ×, rad52-1/rad52-1 (RM105);
+, rad52-1/rad52-2 (RM107); ○, rad52-2/rad52-2 (RM106).

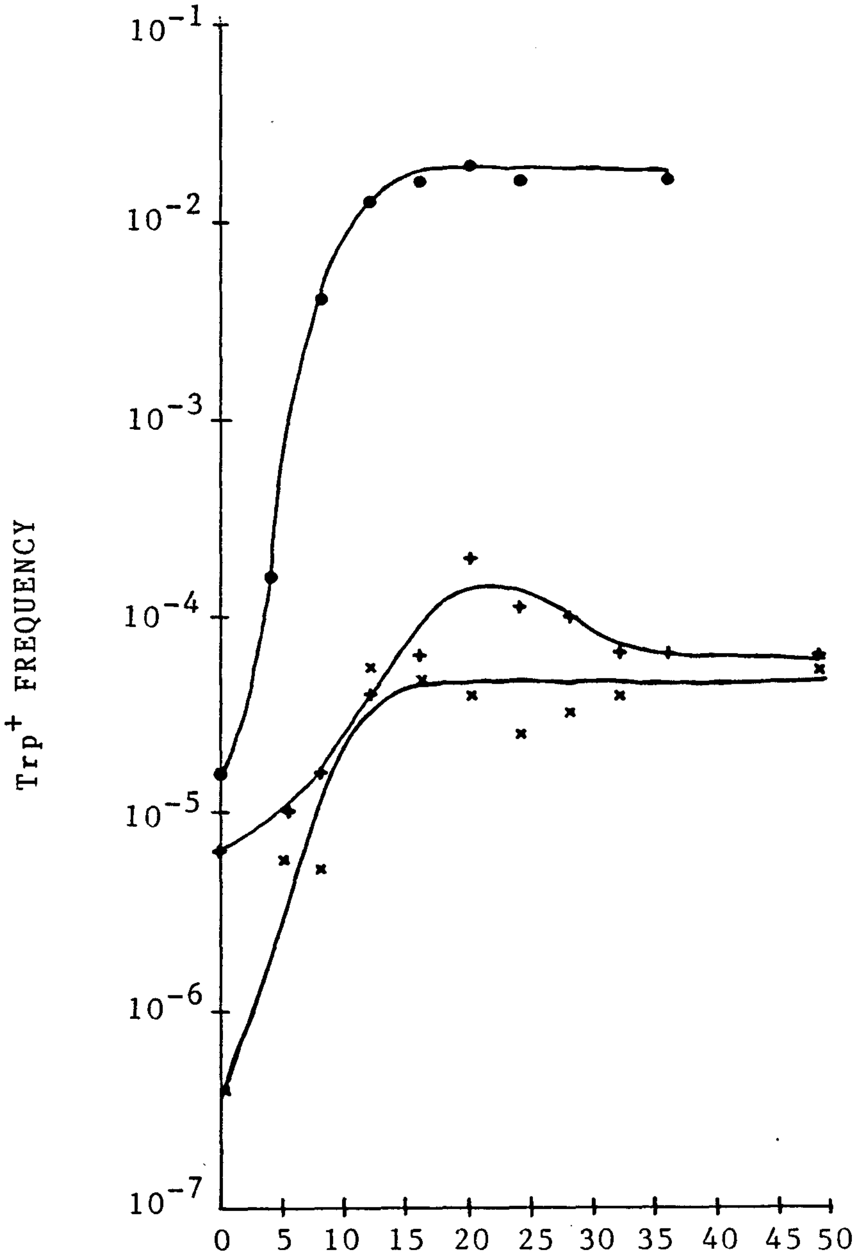


Figure 11 Induction of recombination during meiosis; generation of Trp$^+$ prototrophs

Samples were taken at various times during meiosis as described and recombination frequencies were determined using the following equation:

$$\frac{\text{Number of Trp}^+ \text{ prototrophs at the time of sample}}{\text{Total number of viable cells at the time of sample}}$$

●, Wild type (RM104); +, rad52-1/rad52-1 (RM105);
×, rad52-1/rad52-2 (RM107)

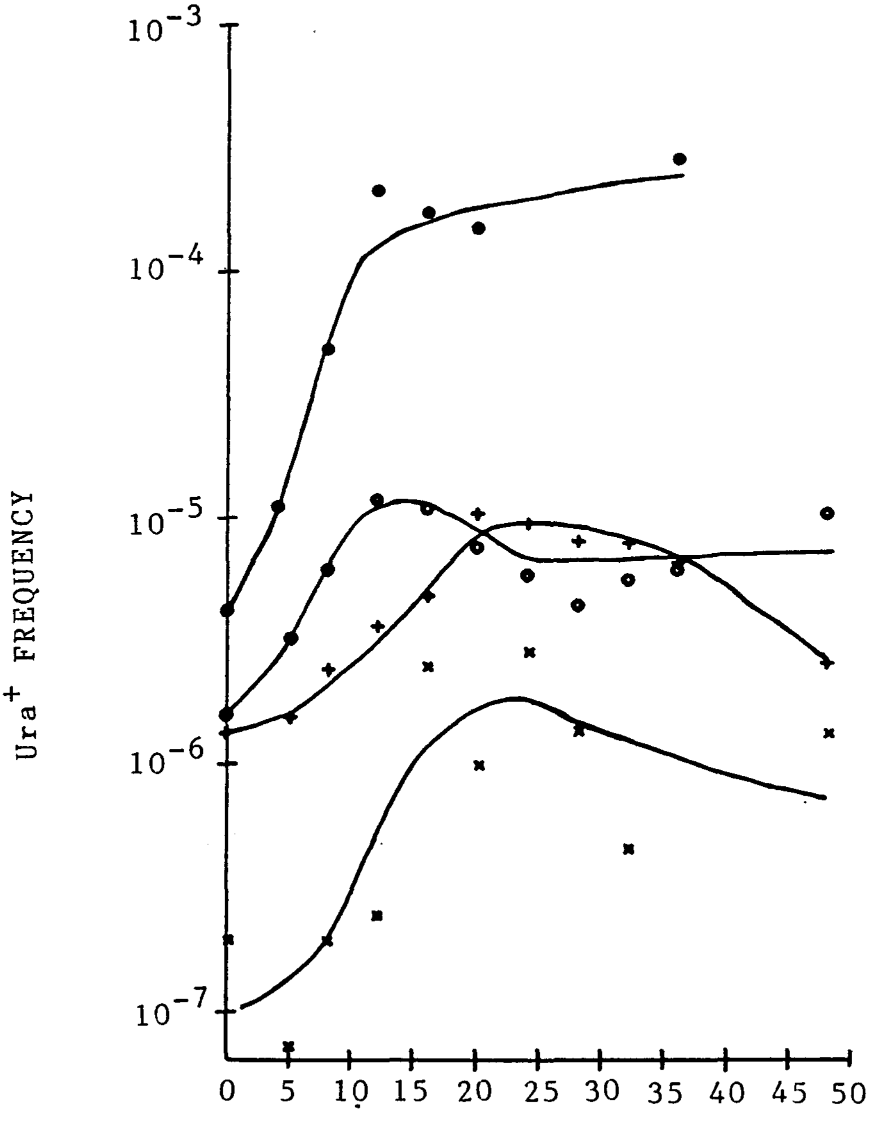


Figure 12 Induction of recombination during meiosis; generation of Ura$^+$ prototrophs

Samples were taken at various times during meiosis as described and recombination frequencies were determined using the following equation:

$$\frac{\text{Number of Ura}^+\text{ prototrophs at the time of sample}}{\text{Total number of viable cells at the time of sample}}$$

●, Wild type (RM104); ×, rad52-1/rad52-1 (RM105);
+, rad52-1/rad52-2 (RM107); ⊙, rad52-2/rad52-2 (RM106).

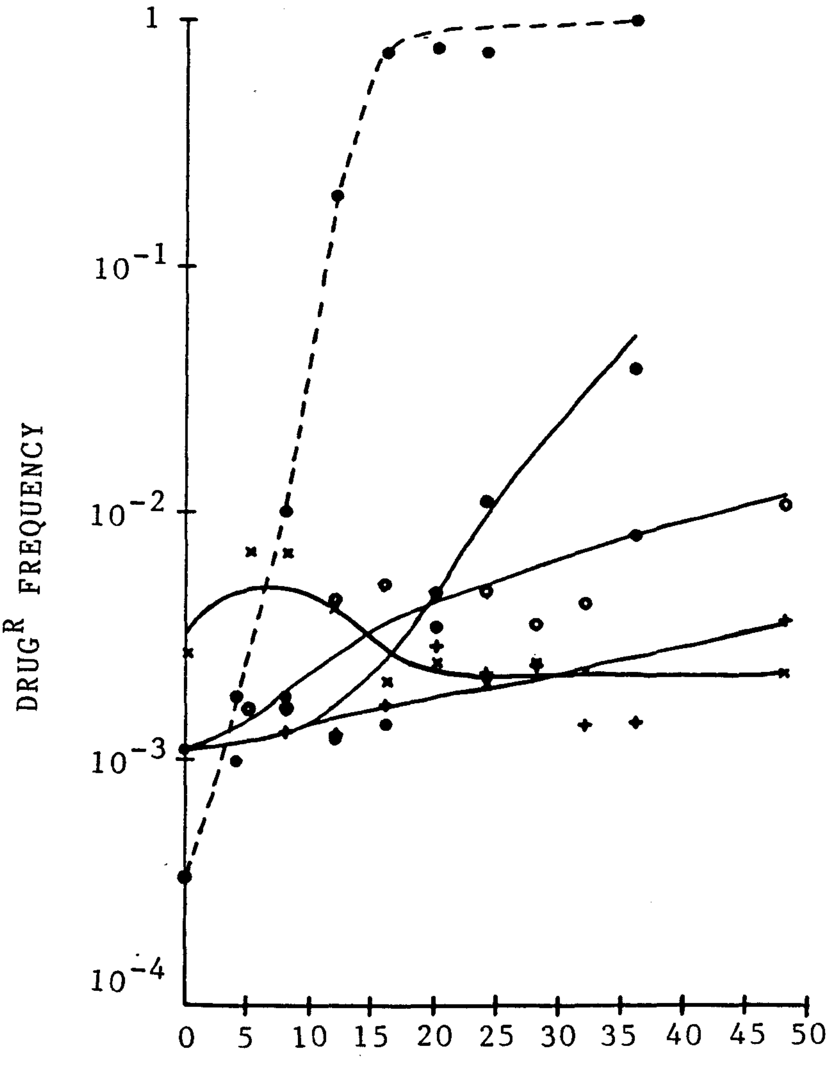


Figure 13 Induction of recombination during meiosis; generation of drug resistant colonies

Samples were taken at various times during meiosis as described and recombination frequencies were determined using the following equation:

$$\frac{\text{Number of drug resistant colonies at time of sample}}{\text{Total number of viable cells at the time of sample}}$$

●——●, Wild type Can^r (RM104); ●- - - - - -●, Wild type Cyh^r (RM104); +——+, rad52-1/rad52-1 Can^r (RM105); ×——×, rad52-1/rad52-2 Cyh^r (RM107); o——o, rad52-2/rad52-2 Can^r (RM106).

in this strain than in the two other mutant strains but there is also a higher mitotic value for rad52-2 cells. Taking the mitotic levels into account, then the amount of induction due to meiosis for the three mutant strains are equivalent.

The scheme for detecting recombinants preferentially selected for particular gene conversion events so the observed induction of recombination could be allele specific and not a genome wide phenomenon. Therefore, prototrophs were picked and tested genetically to determine if there were any other recombinational events that could be detected in the rad52-1/rad52-2 diploid. Since chromosome VII of this diploid was so well marked it was decided to search for possible crossover events occuring within it. Table 8 shows the alignment of the markers on chromosome VII and how putative crossovers were detected. Table 9 displays the actual crossover frequencies discovered as a function of time in meiosis. As can be seen there is a significant amount of crossing over associated with gene conversion as well as nonassociated crossing over throughout meiosis, indicating that the observed induction of recombination was not allele specific nor was it confined to gene conversion.

The Meselson and Radding model for recombination as well as Holliday's model and the double-strand break model predict an approximate 50% association of crossing over with gene conversion, although lately this idea has been criti-

Table 8 Detection of Associated and Nonassociated Crossover Events

Approximately 50 recombinant colonies were picked to YPD master plates from the diagnostic drop-out media for each meiotic time point for diploid RM107. Using standard genetic techniques the genetic configuration of each recombinant was determined. The asterisck (*) occuring by some Trp$^-$ recombinants indicates that the heterozygosity of the TRP5 locus could be determined as follows. The recombinants were mated with tester strains carrying one or the other *trp5* allele. The resulting tetraploids were then sporulated on solid medium for 5 days at 30° C. The sporulation plates were then replicaplated to TRP drop-out media. If the original recombinant was heterozygous for the *trp5* alleles small colonies or papillae would grow out on the TRP drop-out plates from both of the *trp5* tester strains due to rebination. If the original recombinant was homozygous for one of the *trp5* alleles papillae would grow out on the TRP drop-out plate from only the *trp5* tester strain carrying the opposite allele. The position of putative crossover events was determined by comparing the phenotype of the recombinant with the chart in Table 8.

Table 8. Detection of Gene Conversion-Associated and Nonassociated Crossover Events

Starting genetic configuration

ade6 . leu1-c trp5-c $cyh2^r$ met13-c ADE5
ADE6 leu1-12 trp5-2 $CYH2^s$ MET13 ade5

Gene
Conversion

A.
ade6 . leu1-c trp5-c $cyh2^r$ met13-c ADE5
ADE6 LEU1 trp5-2 $CYH2^s$ MET13 ade5
V I II III IV

B.
ade6 . LEU1 trp5-c $cyh2^r$ met13-c ADE5
ADE6 leu1-12 trp5-2 $CYH2^s$ MET13 ade5

Crossover Position	Type	Genotype	Phenotype
I	Associated	ade6 . leu1-c trp5-c $cyh2^r$ met13-c ADE5 ADE6 LEU1 trp5-c $cyh2^r$ met13-c ADE5	Leu^+, Trp^-, Cyh^r, Met^-, Red.*
II	Nonassociated	ade6 . leu1-c trp5-c $cyh2^r$ met13-c ADE5 ADE6 LEU1 trp5-2 $cyh2^r$ met13-c ADE5	Leu^+, Trp^-, Cyh^r, Met^-, Red.*
III	Nonassociated	ade6 . leu1-c trp5-c $cyh2^r$ met13-c ADE5 ADE6 LEU1 trp5-2 $CYH2^s$ met13-c ADE5	Leu^+, Trp^-, Cyh^s, Met^-, Red.*
IV	Nonassociated	ade6 . leu1-c trp5-2 $cyh2^r$ met13-c ADE5 ADE6 LEU1 trp5-c $CYH2^s$ MET13 ADE5	Leu^+, Trp^-, Cyh^s, Met^+, Red.*
V	Nonassociated	ade6 . leu1-c trp5-2 $cyh2^r$ met13-c ADE5 ade6 LEU1 trp5-c $CYH2^r$ MET13 ade5	Leu^+, Trp^-, Cyh^s, Met^+, White.
I & II	Associated & Nonassociated	ade6 . leu1-c trp5-c $cyh2^r$ met13-c ADE5 ADE6 LEU1 trp5-c $CYH2^s$ MET13 ade5	Leu^+, Trp^-, Cyh^s, Met^+, Red.

Table 8. Continued

Crossover Position	Type	Genotype	Phenotype
I & III	Associated & Nonassociated	ade6 . leu1-c trp5-c cyh2^r met13-c ADE5 ADE6 LEU1 trp5-c cyh2^r Met13 ade5	Leu$^+$, Trp$^-$, Cyh^r, Met$^+$, Red.
I & IV	Associated & Nonassociated	ade6 . leu1-c trp5-c cyh2^r met13-c ADE5 ADE6 LEU1 trp5-c cyh2^r met13-c ade5	Leu$^+$, Trp$^-$, Cyh^r, Met$^-$, Red.
I & V	Associated & Nonassociated	ade6 . leu1-c trp5-c cyh2^r met13-c ADE5 ade6 LEU1 trp5-c cyh2^r met13-c ADE5	Leu$^+$, Trp$^-$, Cyh^r, Met$^-$, White.
II & III	Nonassociated	ade6 . leu1-c trp5-c cyh2^r met13-c ADE5 ADE6 LEU1 trp5-2 cyh2^r MET13 ade5	* Leu$^+$, Trp$^-$, Cyh^r, Met$^+$, Red.
II & IV	Nonassociated	ade6 . leu1-c trp5-c cyh2^r met13-c ADE5 ADE6 LEU1 trp5-2 cyh2^r met13-c ade5	* Leu$^+$, Trp$^-$, Cyh^r, Met$^-$, Red.
II & V	Nonassociated	ade6 . leu1-c trp5-c cyh2^r met13-c ADE5 ade6 LEU1 trp5-2 cyh2^r met13-c ADE5	* Leu$^+$, Trp$^-$, Cyh^r, Met$^-$, White.
III & IV	Nonassociated	ade6 . leu1-c trp5-c cyh2^r met13-c ADE5 ADE6 LEU1 trp5-c CYH2^s met13-c ade5	Leu$^+$, Trp$^-$, Cyh^s, Met$^-$, Red.
III & V	Nonassociated	ade6 . leu1-c trp5-c cyh2^r met13-c ADE5 ade6 LEU1 trp5-2 CYH2^s met13-c ADE5	* Leu$^+$, Trp$^-$, Cyh^s, Met$^-$, White.
IV & V	Nonassociated	ade6 . leu1-c trp5-c cyh2^r met13-c ADE5 ade6 LEU1 trp5-2 CYH2^s MET13 ade5	* Leu$^+$, Trp$^-$, Cyh^s, Met$^+$, White.
I, II, & III	Associated & Nonassociated	ade6 . leu1-c trp5-c cyh2^r met13-c ADE5 ADE6 LEU1 trp5-c CYH2^s met13-c ADE5	Leu$^+$, Trp$^-$, Cyh^s, Met$^-$, Red.

Table 9 Evidence for other events occurring in prototrophs arising in rad52 strains during meiosis.

Locus	Chromosome	Type of X-over	Time in meiosis (hours) 0	5	8	12	16	20	24	28	32	36	46	48
LEU1	VII	A	.29	.20	.48			.24	.28	.28	.20			.49
		N	.15	.20	.37			.44	.44	.40	.36			.49
		T	.31	.24	.48			.52	.60	.52	.40			.56
TRP5	VII	A	.30	.40	.40	.24	.40	.36	.32	.36	.44	.40	.48	.44
		N	.11	.28	.16	.28	.20	.40	.40	.36	.40	.44	.52	.54
		T	.32	.40	.40	.40	.52	.60	.56	.56	.56	.56	.60	.61
URA3*	V	T	.31	.00	.00			.24	.28	.60	.48			

A = frequency of associated crossovers

$$= \frac{\text{\# prototrophs with crossover in interval containing converted locus}}{\text{total \# prototrophs}}$$

N = frequency of nonassociated crossovers

$$= \frac{\text{\# prototrophs with crossover in any interval other than in A}}{\text{total \# prototrophs}}$$

T = total frequency of crossovers $= \frac{\text{\# prototrophs with any crossovers}}{\text{total \# prototrophs}}$

A + N does not necessarily equal T since some prototrophs had both associated and nonassociated crossovers.

*There were no markers on chromosome V which allowed a distinction between associated and nonassociated crossovers. "T" refers to total discernable crossovers on all chromosomes.

sized (58, 86, 96). In the rad52 strains used, by the end of meiosis approximately 50% of the gene convertants have an associated crossover suggesting that gene conversion and crossing over are depressed equally by rad52 mutations. The 0 hour values of 30% for associated crossovers neither supports nor refutes the proposition that crossing over is less affected by rad52 mutations in mitosis than gene conversion.

Nonassociated crossovers on the same arm as a selected gene conversion event necessarily require a second recombination event and heteroduplex region according to current models of recombination (58, 86, 131). Since one heteroduplex region in mitosis can extend through an entire arm of a chromosome (46, 47) it is unlikely there would be many nonassociated crossovers given a mitotic gene conversion event. This seems to be the case for the rad52-1/rad52-2 diploid. At the end of mitosis (0 hours), nonassociated crossovers occur 15% of the time at LEU1 and only 11% of the time at TRP5. The LEU1 value should be higher than the TRP5 value since it is more tightly linked to the centromere and hence there is a greater probability that a second initiation event leading to a crossover could occur.

With the beginning of meiotic recombination, and shorter heteroduplex regions, the frequency of nonassociated crossovers should rise. The data in Table 9 is consistant with this idea. In fact, nonassociated crossovers seem to peak at 20 hours the same time gene conversion peaks (Figures 10 and 11). This supports the theory that gene conversion and crossing over are tightly linked in meiosis (58, 86, 131). All of the previous data concerning induction of gene conversion and associated and nonassociated crossovers suggests that although it is affected by rad52 mutations, recombination appears to be occurring normally in meiosis, only at very low levels.

Coreversion Analysis of rad52 Mutations

As mentioned before, rad52 diploids develop phenotypic suppressors during storage. Clearly, one of the most common types of suppressors are suppressor tRNA molecules. If either rad52-1 or rad52-2 were a nonsense mutation, then some of the observed effects could be due to a subpopulation of partially suppressed cells. In order to rule out the possibility that suppressor tRNA molecules were responsible for the levels of meiotic recombination observed, strains were constructed containing either rad52-1 or rad52-2 and several mutations known to be suppressed by altered tRNA molecules. Both amber and ochre nonsense mutations were used and two different approaches were employed. First, spontaneous revertants of the rad52 mutations (Mms^r) and the amber and ochre mutations were selected (Procedure A). These revertants were tested to see if rad52 or the suppressible mutations were coreverted. Tables 10 and 11 show that, with the exception of the ade2-1 mutation, amber

Tables 10 and 11 Coreversion Analysis of rad52-1 and rad52-2

Coreversion analysis was performed as described in text. Of the 42 Mms^r rad52-1 and rad52-2 cells produced by the UV irradiation of Procedure B, only 8/42 of the rad52-1 and 11/42 of the rad52-2 putative MMS revertants were Mms^r upon retesting.

Table 10 Coreversion analysis of rad52-1

Procedure A

Revertant	Possible corevertant loci				
	Mms^{+}	Trp^{+}	Tyr^{+}	His^{+}	Leu^{+}
Mms^{r}	9/ /9	0/ /9	0/ /9	0/ /9	0/ /9
Trp^{r}	0/ /11	11/ /11	11/ /11	0/ /11	0/ /11
Tyr^{r}	0/ /11	11/ /11	11/ /11	0/ /11	0/ /11
His^{r}	0/ /9	0/ /9	0/ /9	9/ /9	9/ /9
Leu^{r}	0/ /11	0/ /11	0/ /11	11/ /11	11/ /11

Procedure B

Revertant	Possible corevertant loci						
	Mms^{+}	Ade^{+}	Trp^{+}	Tyr^{+}	His^{+}	Leu^{+}	Lys^{+}
Mms^{+}	8/ /42	0/ /42	0/ /42	0/ /42	0/ /42	0/ /42	0/ /42
Ade^{+}	0/ /22	22/ /22	0/ /22	0/ /22	0/ /22	0/ /22	0/ /22
Trp^{+}	0/ /18	0/ /18	18/ /18	18/ /18	0/ /18	0/ /18	0/ /18
Tyr^{+}	0/ /23	0/ /23	14/ /23	23/ /23	0/ /23	0/ /23	0/ /23
His^{+}	0/ /20	0/ /20	0/ /20	0/ /20	20/ /20	20/ /20	20/ /20
Leu^{+}	0/ /21	0/ /21	0/ /21	0/ /21	21/ /21	21/ /21	21/ /21
Lys^{+}	0/ /17	0/ /17	0/ /17	0/ /17	17/ /17	17/ /17	17/ /17

The fractions show the number of corevertants (numerator) on a certain media given the number of original revertants (denominator) for the media in the left column.

Table 11 Coreversion analysis of rad52-2

Procedure A

Revertant	Possible corevertant loci				
	Mms$^+$	Trp$^+$	Tyr$^+$	His$^+$	Leu$^+$
Mms$^+$	9/9	0/9	0/9	0/9	0/9
Trp$^+$	0/11	11/11	11/11	0/11	0/11
Tyr$^+$	0/11	11/11	11/11	0/11	0/11
His$^+$	0/9	0/9	0/9	9/9	9/9
Leu$^+$	0/9	0/9	0/9	9/9	9/9

Procedure B

Revertant	Possible corevertant loci							
	Mms$^+$	Ade$^+$	Met$^+$	Trp$^+$	Tyr$^+$	His$^+$	Leu$^+$	Lys$^+$
Mms$^+$	11/42	0/42	0/42	0/42	0/42	0/42	0/42	0/42
Ade$^+$	0/18	18/18	0/18	0/18	0/18	0/18	0/18	0/18
Met$^+$	0/21	0/21	21/21	20/21	20/21	0/21	0/21	0/21
Trp$^+$	0/19	0/19	18/19	19/19	18/19	0/19	0/19	0/19
Tyr$^+$	0/21	0/21	20/21	16/21	21/21	0/21	0/21	0/21
His$^+$	0/19	0/19	0/19	0/19	0/19	19/19	3/19	3/19
Leu$^+$	0/13	0/13	0/13	0/13	0/13	7/13	13/13	7/13
Lys$^+$	0/14	0/14	0/14	0/14	0/14	7/14	7/14	14/14

revertants coreverted other amber mutations and ochre revertants coreverted ochre mutations. None of the amber nor ochre revertants coreverted either rad52 mutation and no rad52 revertant coreverted either amber or ochre mutations, indicating that suppressor tRNA's are almost certainly not responsible for the observed meiotic recombination.

Coreversion procedure B used UV-irradiated cells to induce other species of suppressor tRNA's than those that arose in procedure A. Again, all revertants were tested for their coreversion characteristics and the rad52 revertants did not corevert with nor corevert any other suppressible mutations (Tables 10 and 11). As before, the supposed ochre mutation ade2-1 did not corevert with other ochre mutations and vice versa. It is evident that other suppressor tRNA species were induced since coreversion of amber or ochre mutations were not 100% as in Procedure A. Also, no omnipotent suppressors arose because there were no instances where either amber or ochre coreverted with the other. From the strength of these results it is unlikely that tRNA molecules were responsible for the observed meiotic recombination. In fact Adzuma et al. have recently sequenced rad52-1 and have shown that it is neither an amber nor an ochre nonsense mutation; it is a missense mutation (1).

Induction of Recombination vs. Induction of Reversion

Prakash et al. observed a 10 to 20 fold elevation in the rate of spontaneous mitotic reversion of the trp5-48 allele in rad52-1 diploids (101). Naughton, Hoekstra, and Malone tested other loci and did not find the same reversion levels (personal communication). This uncertainty necessitated the testing of homologous diploids to determine if reversion could explain the observed increases in prototrophs. Strains MH32 and MH33 were kindly provided by M. Hoekstra and contain the same auxotrophic mutations and rad52-1 used previously but in this case the mutations are homozygous. Meiotic reversion of any of the mutations would lead to prototroph formation. Table 12 clearly demonstrates that there was no increase in prototrophic frequency that could not be explained by loss of overall cell viability. Unlike the experiments with the mutations heterozygous, strains with the homozygous mutations did not show an increase in the actual prototroph cell concentration as meiosis progressed. For each of the loci tested, the heterozygous configuration was necessary for the induction of prototroph formation to occur, indicating that recombination and not reversion was responsible.

Table 12 Prototrophic Cell Concentrations During Meiosis in rad52 cells.

Time (Hours)	Total cells/ml $\times 10^7$			Trp$^+$ cells/ml			Leu$^+$ cells/ml			Ura$^+$ cells/ml		
	1	2	3	1	2	3	1	2	3	1	2	3
0	2.5	1.13	2.4	10	1.0	<1	<2	1.0	<1	5.0	10	<1
5	1.3	0.88	0.88	70	10	<1	10	1.0	7.0	<1	5.0	1.0
8	1.3	0.99	0.64	70	3.0	<1	3.0	4.0	1.0	3.0	4.0	1.0
12	1.0	0.64	0.48	560	2.0	<1	3.0	<1	<1	3.0	<1	<1
16	0.91	0.72	0.46	490	1.0	<1	140	<1	1.0	23	1.0	1.0
20	0.90	0.54	0.26	340	1.0	<1	190	1.0	2.0	8.0	<1	<1
24	1.0	0.82	0.24	270	1.0	<1	90	<1	2.0	13	1.0	<1
48	0.28	0.23	0.30	110	<1	1.0	20	<1	1.0	3.0	1.0	<1

1 = *rad52-1*/*rad52-1* *trp5-2*/*trp5-c* *leu1-12*/*leu1-c* *ura3-13*/*ura3-1*

2 = *rad52-1*/*rad52-1* *trp5-2*/*trp5-2* *leu1-12*/*leu1-12* *ura3-13*/*ura3-13*

3 = *rad52-1*/*rad52-1* *trp5-c*/*trp5-c* *leu1-c*/*leu1-c* *ura3-1*/*ura3-1*

DISCUSSION

The results presented in this chapter have reinforced conclusions of previous studies (44, 101) in that the RAD52 gene product is required for normal levels of recombination in meiotic cells. Also, like previous reports, rad52 strains used here produced inviable spores, lower levels of sporulation, and lower levels of recombination for cells at intermediate stages of meiosis than RAD52 strains. However, unlike earlier investigations, these results show that meiosis can induce significant levels of recombination tion at four loci in rad52 strains in terms of frequency and total numbers of recombinants (Tables 4-6).

It was thought possible that the observed prototrophs could have arisen by reversion of one of the heteroalleles. Analysis of homozygous auxotrophic diploids demonstrated that induction of mutation in meiosis was not responsible for the observed prototroph increases. Also, since some phenotypic suppressors of rad52 mutations arise during storage, coreversion analyses were conducted with known suppresible mutations. There was no evidence that amber or ochre specific suppressors were capable of suppressing the Mms^s phenotype of rad52 mutations which correlates with the absence of recombinational repair (12). Therefore, it seems clear that prototrophs were generated by recombination.

The production of prototrophs at heteroallelic loci can occur either by a gene conversion event or by a crossover event between the alleles. It has been shown that gene conversion is the primary mechanism for generating prototrophs in wild type cells in both mitosis and meiosis but there is some evidence that rad52 mutations affect gene conversion more than crossing over (62, 95, 102, 138). Evidence presented here does not directly support nor refute this contention, but whatever mechanism is responsible, genetic recombination is occurring in cells previously described as being devoid of recombination (44, 101).

Since viable recombinants can be recovered from rad52 meiotic cells, I propose that the rad52 meiotic recombination block occurs after initiation of recombination and after heteroduplex formation. Three recent observations support this hypothesis. First, multiple mutant analyses have suggested that rad50 and spo11 are epistatic to rad52 in meiosis (79, 81 and R. Malone personal communication). One interpretation of this observation is that recombination occurs in a stepwise fashion like a biochemical pathway. An early block in the pathway (e.g. rad50 or spo11) would be epistatic to a later block (e.g. rad52). Second, rad52 mutants accumulate single-strand breaks following committment to sporulation and the frequency and time of appearance suggests they might be involved in recombination (105). By contrast, rad50 strains do not exhibit such an accumulation (107). Third, wild type cells exhibit evidence of covalent joining of homologs during meiosis but rad52 cells do not (10). The first two observations are consistent with the hypothesis that rad52 mutations allow initiation and the third observation is consistent with RAD52 acting after heterduplex formation since covalent joining of homologs must occur after strand invasion and heteroduplex formation to form convertants (86, 131).

Other evidence that supports the idea that meiotic recombination is initiated in rad52 cells has been generated by Resnick and Nitiss as part of a collaborative effort done in conjunction with this study (107). When they incubated wild type and rad52 meiotic cells in YPD or synthetic complete medium immediately prior to selective mitotic media wild type cells exhibited induction of recombination but rad52 cells did not. If the cells which had started recombination had completed recombination, it is hard to understand why recombinants would be lost following incubation in rich mitotic medium. If, however, the cells contained recombination intermediates, rapid growth in YPD might preclude their completion. Resnick et al. did find that cells plated on selective mitotic media, without prior incubation in rich medium, required more time for the initial cellular division (if they divided at all) than cells plated on YPD (107). This is a reflection of the fact that the recombination intermediate must be resolved before division, and growth, can occur on selective but not nonselective medium (107). Therefore, intermediates might be resolved in a nonrecombinogenic fashion when DNA replication and/or chromosome segregation proceeds rapidly (107).

An alternative explanation for the observed levels of meiotic recombination is that the *rad52* alleles were leaky, that is they had some residual *RAD52* activity. Two lines of reasoning render this possibility unlikely. First, the *rad52-1*, *rad52-2* and, Resnick and Nitiss' *rad52::LEU2* alleles all behave relatively similarly with respect to spore viability, level of sporulation, radiation sensitivity, and relative meiotic induction of recombination (107). The *rad52::LEU2* allele is made up of the entire *LEU2* gene plus some surrounding DNA inserted at the *Bgl*II site of the *RAD52* gene. The *Bgl*II site is approximately one third of the way in from the 5' end of the proposed *RAD52* coding sequence (1, 107). These mutants are likely to be void of any functional *RAD52* gene product. Second, if recombination was due to leakiness it might be expected that the recombinants would be complete and then would not be lost following incubation in a rich mitotic medium.

DNA strand breaks have been postulated to play a very important role in the initiation of recombination (86, 131). The Meselson and Radding model for recombination proposes that single-strand breaks are necessary for initiation (86). Three experimental observations support this theory. First, Resnick et al. have shown that DNA single-strand breaks accumulate in *rad52-1* cells during meiosis and the number of breaks roughly corresponds with the number of recombination events in wild type cells (107). Second, Hogsett and Oyen have shown that there are no single-strand breaks in the ribosomal RNA genes in meiotic *rad52-1* cells (58). Third, Petes et al. had previously shown that rRNA genes are recombinationally inactive in meiosis in wild type cells (99). Thus, there is a definite correlation between the lack of single-strand breaks in a large segment of meiotic DNA in *rad52-1* cells and the lack of meiotic recombination in this same DNA segment in wild type cells.

The double-strand break model for recombination postulates a double-strand break for initiation of recombination (131). There is evidence that double-strand breaks are not responsible for recombination in *rad52-1* cells. First, Resnick et al. did not detect any double-srand breaks in meiotic *rad52-1* cells (107). Second, unrepaired double-strand breaks would be expected to be as lethal in *rad52* meiotic cells as they are in *rad52* mitotic cells which would preclude double-strand break mediated recombination in these cells (41, 55, 106). Therefore, it appears that single-strand breaks but not double-strand breaks are used for initiation of recombination in rad52 meiotic cells.

It is possible that the lack of double-strand breaks in meiotic rad52 cells is a direct consequence of the mutation. In wild type meiotic cells the RAD52 gene product may act on single-strand breaks to create double-strand breaks. Consistant with this is the fact that Chow and Resnick (20) have shown that rad52-1 cells lack a single-strand endonuclease which could be used to convert single-strand breaks into double-strand breaks, thereby allowing for double-strand break mediated recombination in wild type cells. However, Adzuma et al. have sequenced the RAD52 loci and the presumed open reading frame for the gene product is too small to accomodate the single-strand endonuclease (1, 20).

Whatever role single-strand breaks play in rad52 meiotic cells, the intermediate formed in meiosis that leads to formation of recombinants when removed to mitotic medium must fulfill at least two criteria. First, it must be capable of being resolved mitotically without the RAD52 gene product. Second, it must be reversible by rapid growth in rich medium. The concept of a blocked recombination intermediate is also consistant with the observation that initial events occur in meiosis in rad52 cells.

Using these concepts, the biochemical and genetic results with rad52 cells can be explained in terms of current models of recombination (58, 86, 131). I would like to suggest that meiotic recombination in rad52 cells is initiaed by a Meselson and Radding type of mechanism (86) resulting in heteroduplex DNA and possibly Holliday structure formation. This allows for the formation of recombination intermediates and the occurrance of single-strand breaks, but not double-strand breaks. In the absence of the _RAD52_ gene product, chromosomes undergo initiation but are prevented from completing normal meiotic recombination. Without normal meiotic resolution, the chromosomes may remain intertwined resulting in lethality caused by chromosome breakage at the meiosis I or meiosis II divisions or in the mitotic division following return to rapid growth. This is consistant with the lethality of meiosis in _rad52_ mutants in Spo^+ or in _spo13-1_ strains. Spo^+ cells require completion of recombination before the meiosis I reductional division or else chromosome breakage or absence of desynapsis will create lethal chromosome aneuploidy (3). In _spo13-1_ strains pairing and hence recombination is not required since the meiosis I division is bypassed (70). If recombination has been initiated, however, it must be completed or aneuploidy will result in _spo13-1_ strains from improper segregation at the meiosis II equational division (3). By returning the "blocked" cells to mitotic medium in which they cannot grow, some resolution of recombination intermediates as well as mismatches can occur, thereby allowing recovery of viable recombinants. When cells are returned to rich medium, which allows immediate cell growth there may be insufficient time to resolve the recombination intermediates prior to cell division.

There are two possible ways to explain the long time lags required to observe a recombinant on mitotic selective media after initiation of recombination in meiosis. First, there could be mitotic specific or otherwise alternate enzymes recognizing and processing the intermediate and subsequent mismatches less efficiently than the RAD52 gene product. Second, the RAD52 gene product could be a late control molecule regulating the levels of the enzymes required for normal completion of meiotic recombination. Without the RAD52 control, basal levels of controlled enzymes would necessarily resolve recombination intermediates much more slowly. The latter possibility is intriguing in that it explains how the single-strand endonuclease, described by Chow and Resnick (20) which is absent in rad52 cells could show an increased meiotic activity in RAD52 cells and still be too large to be the actual RAD52 gene product (1). Furthermore, recent investigations by Esposito et al. (27) indicate that the two dimensional protein profile in meiotic rad52 cells has several deviations from wild type cells which may support the idea that the RAD52 gene product is a control molecule that acts to control multiple functions.

The meiotic lethality of rad52 strains can also be discussed in the light of recent studies by Hastings (50). He proposes that mismatch repair proceeds either through an excision and repair mechanism or through the creation of a double-strand gap at the mismatch followed by double-strand break recombinational repair (50, 131). In rad52 cells double-strand break repair is absent and the presence of such breaks should be lethal (55, 105). Again, it should be pointed out that no double-strand breaks have been detected in rad52-1 meiotic cells (105) suggesting that the mismatch correction necessary for the observed meiotic gene conversion events in rad52 cells is not mediated by a double-strand break repair process. This is also further support that the RAD52 gene product is required to create and repair double-strand breaks in meiosis if they occur at all. For these reasons the first explanation for meiotic lethality in rad52 cells is favored.

In order for any mechanism to account for meiosis induced gene conversion, mismatch repair must be able to occur in rad52 cells returned to mitotic medium even though spontaneous mitotic gene conversion is greatly reduced by the rad52-1 mutation. An explanation for this difference is that the rad52-1 mutation may block the initiating events of mitotic gene conversion but allows completion of previously initiated events like those initiated in meiosis and returned to mitotic growth. This implies different initiating events for mitotic and meiotic recombination. Consistent with this is Malone's observation that rad52-1 is epistatic to rad50-1 in mitotic recombination; the opposite of their meiotic phenotypes (79). It is also possible that meiotic cells returned to mitotic growth may have a hybrid recombination system unlike the ones found in either mitotic or meiotic cells. Related to the problem of the role of *RAD52* in spontaneous mitotic recombination is the observation that the 0 hour recombination frequency for the *rad52-2* diploid did not seem depressed as did the other *rad52* diploids. This forms the basis for the experiments and results found in the following chapter.

CHAPTER III

CHARACTERISTICS OF DIPLOID *rad52-2* MITOTIC CELLS

INTRODUCTION

The *rad52-1* mutation of the yeast *Saccharomyces cerevisiae* has been extensively studied and characterized. It is a very pleiotropic mutation (see Table 13) affecting many recombination reactions and is required for the recombinational repair pathway (41). The meiotic phenotypes of the *rad52-1* and *rad52-2* mutations have been discussed thoroughly in Chapter II. This chapter will only be concerned with various mitotic phenotypes of the *rad52-1* and *rad52-2* mutations.

DNA Repair and *rad52* Mutations

The effects of the *rad52-1* mutation on DNA repair are well characterized and will be discussed only breifly. More thorough discussions can be found in reviews by Game and by Haynes and Kunz (41, 51). Resnick not only showed *rad52-1* strains were very sensitive to X-rays but he showed that they were somewhat sensitive to UV irradiation (104). Brendel et al. then correlated X-ray sensitivity to sensi-

Table 13 Phenotypes of rad52-1 Diploids

1. X-ray, gamma-ray, and methyl methanesulfonate sensitive (104, 12).
2. Slightly sensitive to UV irradiation (104).
3. Deficient in DNA double-strand break repair (55, 106).
4. Required for spontaneous mitotic gene conversion (80, 101).
5. Deficient in reciprocal recombination between:
   - a) linear plasmids and homologous chromosomal regions (96, 130),
   - b) circular replicating plasmids and homologous chromosomal regions (Chapter IV).
6. Proficient in reciprocal recombination between:
   - a) duplications (62),
   - b) sister chromatids (102, 138),
   - c) circular integrating plasmids and homologous chromosomal regions (96),
   - d) homologous circular plasmids (130).
7. Required for homothallic interconversion of mating type (80).
8. X-ray- and UV-induced mitotic recombination is absent (101).
9. Meiotic gene conversion and crossing over are greatly reduced but induction of of recombination does occur (44, 101, 107 and Chapter II).
10. Cell viability declines during meiosis (44).
11. Single-strand breaks accumulate during meiosis (106).
12. Sporulation is reduced (44, 101, 104, 107 and Chapter II).
13. Spores are inviable (44, 101, 104, 107 and Chapter II).

tivity to the alkylating agent methyl methanesulfonate (MMS) thereby providing a quick screening procedure for rad52 mutations (12). Using neutral sucrose gradients, Ho and also Resnick and Martin demonstrated that rad52-1 strains were deficient in DNA double-strand break repair since these breaks accumulate after exposure to X-rays (55, 105). Concurrently, multiple mutant analyses using rad52-1 and other DNA repair mutations led to the delineation of three separate repair pathways; excision repair, error-prone repair, and recombinational repair (51, 104). The RAD52 gene was then assigned to the recombinational repair group.

The rad52-2 mutation was first described by Snow as an X-ray sensitive mutation (119). Game and Mortimer showed that Snow's mutation (rs14) was allelic to Resnick's (xs1) and according to the accepted convention they were renamed rad52-2 and rad52-1 respectively (43). While repair phenotypes of the rad52-1 allele were extensively studied, those of the rad52-2 allele were not.

Mitotic Effects of rad52 Mutations

Prakash et al. also examined the mitotic effects of rad52-1 and found: a) UV- and gamma ray-induced mitotic recombination were absent; b) spontaneous reversion rates were elevated 10 to 20 fold in mitosis; and c) spontaneous mitotic recombination was decreased (101). Induced mitotic recombination occurs at higher levels than spontaneous mitotic recombination and induced mitotic heteroduplex DNA regions are shorter, resembling meiotic heteroduplex lengths (95, 110). Also, experiments using heterokaryon diploids (cells with two unfused haploid nuclei) it has been shown that diffusable gene products are actually induced by X-rays and are responsible for the increase in recombination (110). The conclusions of Prakash et al. and also by Game et al. were that the primary defect conferred on cells by the rad52-1 mutation is within the recombinational process (44, 101). Game et al. went further and theorized that the RAD52 gene product was necessary for the successful completion of recombination (44). Consistant with this theory are the results presented in Chapter II and by Resnick et al. (107).

The landmark papers mentioned above laid the groundwork for experiments dealing with other specific types of recombination such as spontaneous mitotic recombination, homothallic interconversion of mating type, recombination between homologous plasmids, and recombination between plasmids and chromosomes. Table 13 lists the established effects rad52-1 has on these processes. This chapter is only interested in the effects of rad52-1 and rad52-2 on spontaneous mitotic recombination and homothallic interconversion of mating type which will be discussed next.

The analysis of spontaneous mitotic recombination of Prakash et al. (101) examined just two loci. Malone and Esposito extended the observation to six other loci and confirmed the findings of Prakash et al. (80). In contrast with the *rad52-1* effects is the observation made in Chapter II that the *rad52-2* mutation may allow significant levels of spontaneous mitotic recombination to occur (107).

Spontaneous mitotic recombination occurs in diploid cells but a specialized form of recombination occurs in haploid mitotic cells carrying the *HO* gene. These cells are capable of interconverting their mating type, i. e. strains having the *a* mating type can interconvert to the *α* mating type and vice versa (52). This switching mechanism allows haploid cells to mate and form normal *a*/*α* diploids and is thought to be the result of a non-reciprocal recombinational event (52). Malone and Esposito introduced this idea by demonstrating that the *RAD52* gene product is required for the interconversion (80). Later, Weiffenbach and Haber showed that lethal chromosome breaks occurred in *rad52-1* cells attempting to interconvert their mating type (133) and these breaks were shown to be double-strand breaks (126). It is now know that the *HO* gene encodes an endonuclease responsible for the double-strand break involved in mating type interconversion and this break is thought to be the initiation event (72, 126).

The effects of *rad52-1* on spontaneous mitotic and meiotic recombination, recombinational repair, and specialized forms of recombination such as mating type interconversion have been well documented. In light of the preliminary findings regarding the effects of rad52-2 on spontaneous mitotic recombination it is prudent to examine this phenomenon more rigorously and at the same time examine the effects of rad52-2 on recombinational repair and mating type interconversion. Results show that rad52-2 strains are indeed capable of wild type levels of spontaneous mitotic recombination unlike rad52-1 cells. In addition, the rad52-2 mutation does not seem to have the same drastic effect on mating type interconversion as the rad52-1 allele and rad52-2 cells are not as sensitive to UV irradiation as rad52-1 cells. However, the response of cells carrying either of the two alleles appear to respond similarly in standard MMS sensitivity assays.

MATERIALS AND METHODS

Strains

The *S. cerevisiae* strains used for these experiments are presented in Table 14. All haploid parental strains carrying either *rad52-1* or *rad52-2* were back-crossed at least three times with *RAD52* strains that demonstrated good growth characteristics. Diploids were constructed using standard techniques immediately prior to use.

Spontaneous Mitotic Recombination Determination

Two procedures were used to assay and determine the spontaneous mitotic recombination characteristics of *rad52* diploids.

Procedure A: Two fresh colonies of diploids 1-4, Table 14, were spread on a YPD plate so there were eight pie wedge shaped patches with like diploids opposite each other. This was allowed to grow at 30° C for two days at which time the plate was replica-plated to LEU, TRP, URA, and MMS media. These plates were incubated as before and after two days any mitotic recombination that may have oc-occurred on the YPD plate would be visible as small colonies called papillae or paps for short. These colonies were

Table 14 Genotypes of Strains Used

Strain	Diploid	Relevant Genotype
1	MH16[a]	a ura3-1 CAN1^{s} / ura3-13 can1^{r} ; his7-1 tyr1-2 lys2-2 / his7-2 tyr1-1 lys2-1 leu1-c trp5-c cyh2^{r} met13-c ADE5 / leu1-12 trp5-2 CYH2^{s} met13-d ade5 ; ade2-1 / ade2-1
2	RM105	a rad52-1 / rad52-1 ; his1 ura3-13 can1^{r} / HIS1 ura3-1 CAN1^{s} ; HIS7 / his7 leu1-12 trp5-2 MET13 ade5 / leu1-c trp5-c met13-c ADE5
3	RM106	a rad52-2 / rad52-2 ; ura3-1 CAN1^{s} / ura3-13 can1^{r} ade6 leu1-c trp5-c cyh2^{r} met13-c ADE5 / ADE6 leu1-12 trp5-2 CYH2^{s} MET13 ade5
4	RM107	a rad52-2 / rad52-1 ; ura3-1 / ura3-13 ; HIS7 tyr1-2 lys2-2 / his7 tyr1-1 lys2-1 ade6 / ADE6 ; leu1-c trp5-c cyh2^{r} met13-c ADE5 / leu1-12 trp5-2 CYH2^{s} MET13 ade5
5	K65[b]	a HO / HO ; RAD52 / RAD52 ; can1^{r} / can1^{r} ; met13-d / met13-d
6	K187[b]	a HO / HO ; RAD52 / RAD52 ; cyh2^{r} met13-c / cyh2^{r} met13-c
7	CE103	α ho rad52-2 can1^{r} cyh2^{r}
8	CE104	α ho rad52-2 can1^{r} cyh2^{r}

---

[a] Provided by M. Hoekstra

[b] Provided by S. Klapholtz; K65 = K65-3D, K187 = K187-24B

counted and the plates were photographed using a Polaroid MP-3 Land camera and Polaroid Type 55 film. The negatives were fixed in 18% sodium sulfite.

Procedure B: Four fresh colonies of diploid RM106 were picked to 1.0 ml PO_4 buffer and enumerated with a hemocytometer. They were used to inoculte 10 mls of YPD liquid medium at a cell concentration of 50 cells/ml. These cultures were incubated with shaking at 30°C untill a cell concentration of approximately $2(10)^7$ cells/ml was attained. After the cutures had reached the proper concentration they were harvested by centrifugation, washed twice with PO_4 buffer, serially diluted, and plated to determine the prototrophic frequency after mitotic growth on YPD, LEU, TRP, URA, and CAN media. These values were compared to wild type values to determine their relative spontaneous mitotic recombination frequencies. It should be noted that this procedure does not differ dramatically from the one described under the Growth and Sporulation and Return to Mitotic Growth headings of the previous chapter. The plating at the point of inoculation into sporulation media corresponds to the final mitotic plating. Prototrophic frequencies obtained using the Return to Mitotic Growth procedure compare favorably to the frequencies obtained with the procedure described here.

Methyl Methanesulfonate Sensitivity Assay

Cells were grown in 20 mls. liquid YPD medium to a concentration of $2(10)^7$ cells/ml. They were then harvested by centrifugation, washed once with sterile distilled water, and resuspended in 5 mls of sterile 0.2 M sodium phosphate buffer at pH 7.5 giving a concentration of $8(10)^7$ cells/ml. The suspension was then placed in a 25°C water bath for 5 minutes. To this cell suspension 50 ul methyl methanesulfonate (MMS) was added in a fume hood. Samples of 100 ul were removed periodically and diluted in 4.9 mls sterile 5% sodium thiosulfate to inactivate the MMS. This suspension was allowed to sit for 3 minutes and was plated on solid YPD medium after serial dilutions were made in 5% sodium thiosulfate. The plates were incubated at 30°C for 48 hours. Immediately prior to the addition of MMS to the cell suspension, a 100 ul sample was removed and treated as all asubsequent samples exposed to MMS. This sample constituted the 0 minute MMS exposure standard. MMS sensitivity was determined using the following equation:

$$\%\ \text{Survivors} = \frac{\text{Cell Concentration at x Minutes}}{\text{Cell Concentration at 0 Minutes}} \times 100$$

where x equals the time of sampling.

UV-Irradiation and Survival

Starting from single colonies, strains MH16, RM105, RM106, and RM107 were grown to a final concentration of

$1(10)^7$ cells/ml. in 10 mls. of YPD medium. The cultures were harvested by centrifugation and washed twice in 0.2 M PO_4 buffer, pH 7.5 and resuspended in 10 mls. of PO_4 buffer. The suspensions were placed in large (150 X 15 mm.) sterile glass petri plates for UV irradiation. The cells were irradiated with a UV dose of 20 $J/M^2/sec$ using two General Electric G15T8 Germicidal lamps. The cells were resuspended by swirling every 10 seconds and 100 ul samples were removed at 20 second intervals and placed in 10 mls of PO_4 buffer. Serial dilutions were plated under red light conditions to prevent photoreactivation and incubated in the dark for 48 hours at 30°C at which time viable cell concentrations were determined. The percentage of cell survivors was calculated using the following equation:

$$\% \text{ Survivors} = \frac{\text{cell concentration at } T = x \text{ sec} \quad X\ 100}{\text{cell concentration at } T = 0 \text{ sec}} .$$

Homothallic Interconversion of Mating Type Analysis

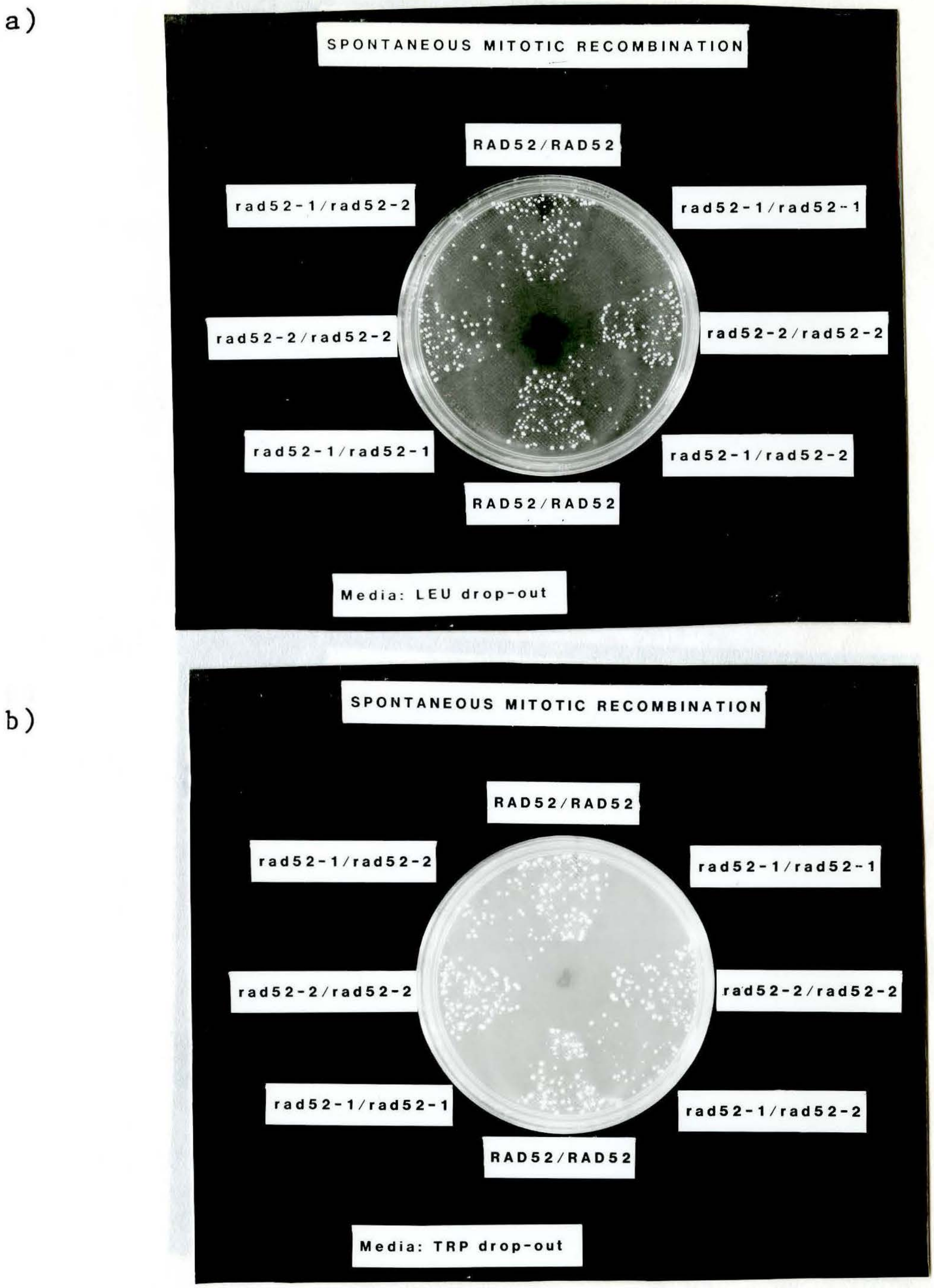
Two separate strains (CE103 and CE104) were mated with spores from the two homothallic strains K65 and K187 by cross replica plating to YPD plates. After incubation at 30°C for 24 hours, diploids were selected for by streaking the mated cells on MET drop-out medium and then screening the Met^+ cells for drug sensitivity. The diploids were sporulated by replica plating a YPD diploid master plate to the sporulation medium SPOIII-22 (2% KAc, .1% glucose, .25% yeast extract, 2% agar, and necessary amino acids and bases) which was incubated at 30° C for five days. At least 10 complete tetrads for each diploid were dissected on YPD plates using a micromanipulator and incubated at 30 $^{\circ}$ C for four days. The resulting colonies were picked to a master plate and incubated as before. The mating type of each segregant was determined by cross-replica plating with *a* and *α* mating type testers, incubated, and then diploids were selected for by utilizing complementing auxotrophic mutations present in the segregants and tester strains. After two days of growth at 30° C the mating type of each segregant was recorded. Segregants were scored for MMS sensitivity, which correlates with the presence of the *rad52-2* mutation, by replica plating the segregants to YPD plates containing 0.01% MMS. These plates wre incubated as before and scored after 24 and 48 hours growth.

RESULTS

Spontaneous Mitotic Recombination in rad52-2 Cells

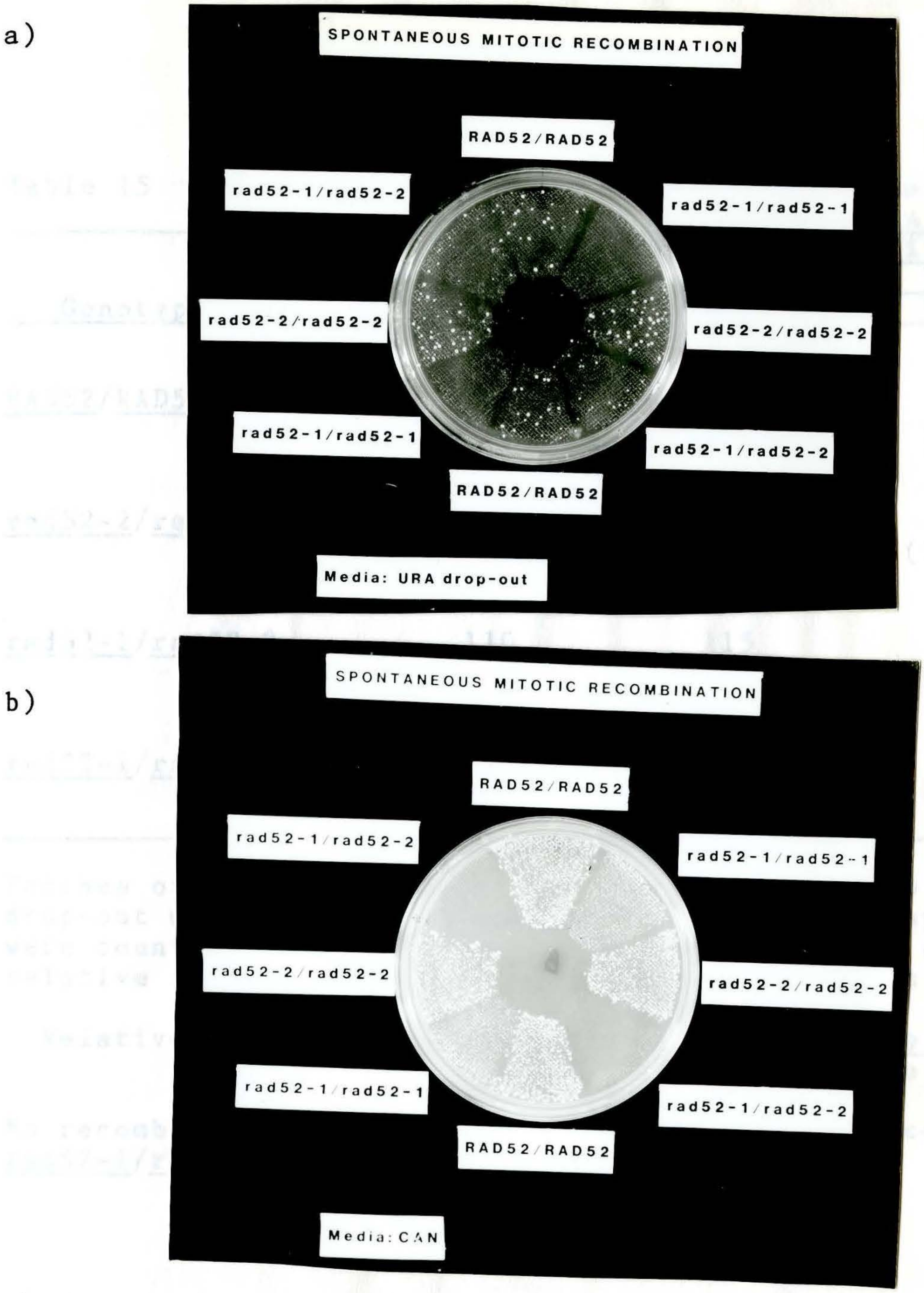
The recombination frequency of rad52-2 diploids measured immediately prior to meiosis, as determined in the previous chapter, indicated that rad52-2 diploids may have appreciable levels of spontaneous mitotic recombination. Therefore, Procedure A, as described above, was performed to determine the feasability of a more rigorous analysis of the effect of rad52-2 on spontaneous mitotic recombination. Figures 14 and 15 show the actual nutrient drop-out and drug plates used to test spontaneous mitotic recombination and Table 15 contains the relative recombination frequencies for each strain. The rad52-2 allele does not appear to greatly inhibit spontaneous mitotic recombination. Although the rad52-2 diploid is resistant to canavanine (and therefore cannot be tested), the rad52-1/rad52-2 diploid displays more Can^r papillae than the rad52-1 diploid. Also, the rad52-2 effect seems to be dependent on gene dosage since the relative recombination frequencies form an order of lowest to highest with rad52-1/rad52-1 < rad52-1/rad52-2 < rad52-2/rad52-2, RAD52/RAD52. From these results it became clear that a more rigorous analysis should be conducted.

Figure 14 Spontaneous Mitotic Recombination in rad52 Cells: Procedure A.


a)
SPONTANEOUS MITOTIC RECOMBINATION
RAD52/RAD52
rad52-1/rad52-2
rad52-1/rad52-1
rad52-2/rad52-2
rad52-2/rad52-2
rad52-1/rad52-1
rad52-1/rad52-2
RAD52/RAD52
Media: LEU drop-out
b)
SPONTANEOUS MITOTIC RECOMBINATION
RAD52/RAD52
rad52-1/rad52-2
rad52-1/rad52-1
rad52-2/rad52-2
rad52-2/rad52-2
rad52-1/rad52-1
rad52-1/rad52-2
RAD52/RAD52
Media: TRP drop-out

a) Leucine drop-out medium. b) Tryptophan drop-out medium. Cells were plated as described. Since the LEU1 and TRP5 loci were heteroallelic for leu1 and trp5 mutations respectively, colonies could only arise through spontaneous mitotic recombination.

Figure 15 Spontaneous Mitotic Recombination in rad52 Cells: Procedure A.


a)
SPONTANEOUS MITOTIC RECOMBINATION
RAD52/RAD52
rad52-1/rad52-2
rad52-1/rad52-1
rad52-2/rad52-2
rad52-2/rad52-2
rad52-1/rad52-1
rad52-1/rad52-2
RAD52/RAD52
Media: URA drop-out
b)
SPONTANEOUS MITOTIC RECOMBINATION
RAD52/RAD52
rad52-1/rad52-2
rad52-1/rad52-1
rad52-2/rad52-2
rad52-2/rad52-2
rad52-1/rad52-1
rad52-1/rad52-2
RAD52/RAD52
Media: CAN

a) Uracil drop-out medium. b) Canavanine drug medium.

Cells were plated as described. Since the URA3 locus was heteroallelic for ura3 mutations, colonies could only arise through spontaneous mitotic recombination. Colonies could only arise on CAN plates following a crossover event (the rad52-2 diploid was resistant to canavanine).

Table 15 Relative Spontaneous Mitotic Recombination Frequencies for rad52 Cells: Procedure A

Genotype	Number of Recombinant Papillae (Estimated Relative Recombination Frequency) LEU1	TRP5	URA3
RAD52/RAD52	598 (1.0)	443 (1.0)	78 (1.0)
rad52-2/rad52-2	452 (0.756)	490 (1.106)	194 (2.487)
rad52-1/rad52-2	110 (0.368)	115 (0.260)	59 (0.756)
rad52-1/rad52-1	1 (0.0017)	1 (0.0023)	0 -----

Patches of the four strains were replica plated to nutrient drop-out media twice as described. Recombinant papillae were counted for each strain at each loci and the estimated relative recombination frequency was determined as follows:

$$\text{Relative Recombination Frequency} = \frac{\#\text{ of papillae rad52}}{\#\text{ of papillae RAD52}}.$$

No recombinant papillae were formed at the ura3 locus in rad52-1/rad52-1 diploids.

Procedure B, as described above, is the accepted method for determining spontaneous mitotic recombination frequencies (80). Tables 16, 17, and 18 demonstrate that the observations made using procedure B are consistent with those made above using procedure A. Table 16 displays the actual number of recombinants for each mitotic culture and Table 17 contains each respective recombination frequency. Table 18 is a summary table with mean spontaneous mitotic recombination frequencies and their standard deviations. Again, it appears that the effect of rad52-2 on spontaneous mitotic recombination is gene dose dependent and the rad52-2 diploid demonstrates essentially wild type levels of mitotic recombination. The lack of spontaneous mitotic recombination in rad52-1 cells has been correlated with high levels of chromosome loss (90). Cyh^r prototrophs can arise through a recombination event or by the loss of the $CYH2^s$ chromosome (90). Chromosome loss occurs very rarely in wild type cells but more frequently in rad52-1 diploids (90). In fact, Mortimer et al. state that the expression of a heterozygous recessive trait in rad52-1 cells is almost entirely due to chromosome loss which may be due to the lack of mitotic recombination (90). If this is the case then the mitotic recombination proficient rad52-2 diploids probably do not experience chromosome loss at the same level as rad52-1 diploids. This is supported by the fact that the rad52-1/rad52-2 diploid has a higher apparent recombination frequen-

Table 16 Mitotic Recombination in rad52 Cells

Genotype	Culture #	Total Cell Conc. X 10^{-7}	LEU1 Recom.	TRP5 Recom.	URA3 Recom.	LYS2 Recom.	CYH2 Recom. X 10^{4}
$\frac{\text{rad52-1}}{\text{rad52-1}}$	1	1.44	168	103	20	---[a]	1.60
	2	1.12	68	85	55	---	1.99
	3	1.28	110	88	40	---	0.74
	4	1.22	83	92	20	---	1.92
	5	1.38	715	120	20	---	0.90
	6	1.46	123	148	42	---	0.75
	7	1.29	85	122	15	---	0.69
	8	1.45	110	110	15	---	0.69
$\frac{\text{rad52-2}}{\text{rad52-2}}$[b]	1	4.42	810	---[c]	700	---	---
	2	2.00	395	488	80	---	---
	3	4.52	435	1622	500	---	---
	4	3.82	388	1055	500	---	---
	5	1.02	392	543	490	149	---
	6	2.06	949	1396	720	355	---
	7	2.14	930	1192	920	351	---
	8	2.27	623	1419	830	223	---

---

[a] --- indicates that these loci were not examined unless otherwise noted.

[b] Cultures 1-4 were the mitotic growth phase of the meiotic cultures examined in Chapter II. Cultures 5-8 were new independent colonies of the same genotype as 1-4.

[c] A recombinational event at this loci early in the growth of the culture made colony enumeration impossible.

Table 17 Mitotic Recombination Frequencies of rad52 Cells

Genotype	Culture #	Total Cell Conc. X 10^{-7}	Recombination Frequencies X 10^5 LEU1	TRP5	URA3	LYS2	CYH2^r
rad52-1 / rad52-2	1	1.44	1.17	0.72	0.14	---[a]	111
	2	1.12	0.61	0.76	0.49	---	178
	3	1.28	0.86	0.69	0.36	---	66.1
	4	1.22	0.68	0.75	0.16	---	157
	5	1.38	5.18	0.86	0.14	---	65.2
	6	1.46	0.84	1.01	0.29	---	51.4
	7	1.29	0.66	0.94	0.12	---	53.5
	8	1.45	0.76	0.76	0.10	---	47.6
rad52-2[b] / rad52-2	1	4.42	1.83	---[c]	1.60	---	---
	2	2.00	1.98	2.44	0.40	---	---
	3	4.52	0.96	3.59	1.10	---	---
	4	3.82	1.02	2.76	1.30	---	---
	5	1.02	3.85	5.33	4.80	1.46	---
	6	2.06	4.61	6.78	3.50	1.72	---
	7	2.14	4.35	5.57	4.30	1.64	---
	8	2.27	2.74	6.25	3.70	0.98	---

---

[a] --- indicates that these loci were not examined unless otherwise noted.

[b] Cultures 1-4 were the mitotic growth phase of the meiotic cultures examined in Chapter II. Cultures 5-8 were new independent colonies of the same genotype as 1-4.

[c] A recombinational event at this loci early in the growth the culture made colony enumeration impossible.

Table 18 Spontaneous mitotic recombination in various rad52 diploids

		Relative Recombination Frequencies (Mean Frequency of Recombinants X 10^5)				
Genotype	No. of cultures	leu1	trp5	ura3	lys2	cyh2
rad52-1[a] rad52-1	6	0.0040 (0.028)	0.014 (0.056)	0.11 (0.069)	0.13 (0.024)	0.078 (7.3)
rad52-2 rad52-2	8	0.305 (2.288)	1.1 (4.370)	3.06 (1.98)	4.05 (1.42)	---
rad52-1 rad52-2	8	0.132 (0.988)	0.206 (0.804)	0.297 (0.193)	---	0.86 (80.4)
RAD52[a] RAD52	2	1.0 (7.5)	1.0 (3.9)	1.0 (0.65)	1.0 (0.35)	1.0 (94)

Diploids were analyzed for spontaneous mitotic recombination as described in the text. Relative recombination frequency refers to the geometric mean of all cultures of the *rad52* strain divided by the geometric mean for all cultures of the wild type for each locus.

[a] Data taken from Malone and Esposito (80).

cy at the CYH2 locus than the rad52-1 diploid and the rad52-2 diploid would be expected to be even higher.

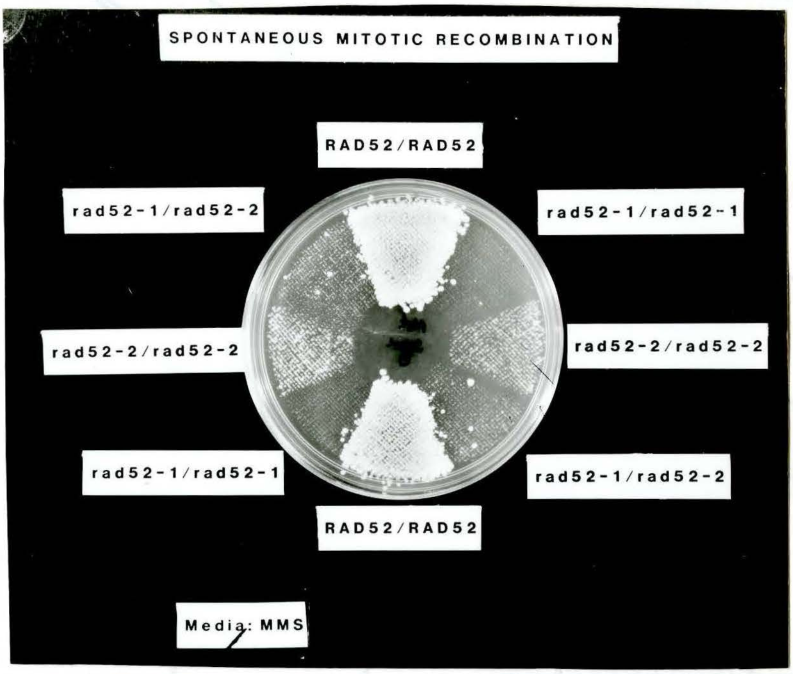
The dose dependent effect of rad52-2 suggests that there is some mitotic recombinational activity associated with the rad52-2 gene product. This activity may be as high as that of the wild type RAD52 gene product for mitotic recombination since the RAD52/rad52-1 heterozygote also shows an intermediate level of spontaneous mitotic recombination like the rad52-1/rad52-2 heteozygote (101). However, this data was obtained by examining only one locus and further experiments should be performed for verification. The rad52-2 mutation, unlike the wild type gene, is defective in meiotic recombination, thereby indicating that the rad52-2 gene product has only partial activity. The question then becomes: does the rad52-2 gene product have sufficient activity to prevent other cellular defects attributed to the rad52-1 mutation?

MMS Sensitivity of rad52 Strains

It has been postulated that the repair of DNA damage induced by MMS is accomplished by the recombinational repair pathway (12). Therefore, if there is some mitotic recombinational activity associated with rad52-2, then the response of rad52-2 diploids to MMS may be different than that of wild type or rad52-1 diploids. Some support for this hypothesis was demonstrated when strains MH16, RM105, RM106, and RM107 were replica plated to a YPD plate containing 0.01% MMS as part of procedure A of the spontaneous mitotic recombination analysis. Figure 16 shows the actual plate and reveals an apparent gene dosage relationship between rad52-2 and MMS sensitivity. The large colonies appearing in the patches of the rad52-1/rad52-2 diploid may have arisen as the result of mitotic recombination, but the faint growth of the entire patch, (which was even more pronounced for the rad52-2 diploid) may reflect a low level resistance to MMS. Because of this a more thorough MMS sensitivity assay was conducted.

MMS sensitivity was determined for strains K65, RM105, RM106, and RM107 as described and Figure 17 depicts the results. As can be seen, there is no evidence for partial resistance, or a gene dosage relationship, of the rad52-2 allele. All three of the rad52 strains responded similarly to MMS exposure. The apparent gene dosage relationship observed before could be due to the procedure employed. The cells replica plated to the YPD-MMS plate grew in the presence of MMS, a mutagen, and further selected for more MMS resistant cells. In the MMS exposure experiment, on the other hand, the MMS was neutralized with sodium thiosulfate before growth, so the MMS did not exert any selective pressure on the cells during growth. Another problem with the YPD-MMS plate observation is that MMS breaks down and can also evaporate from the plate thereby

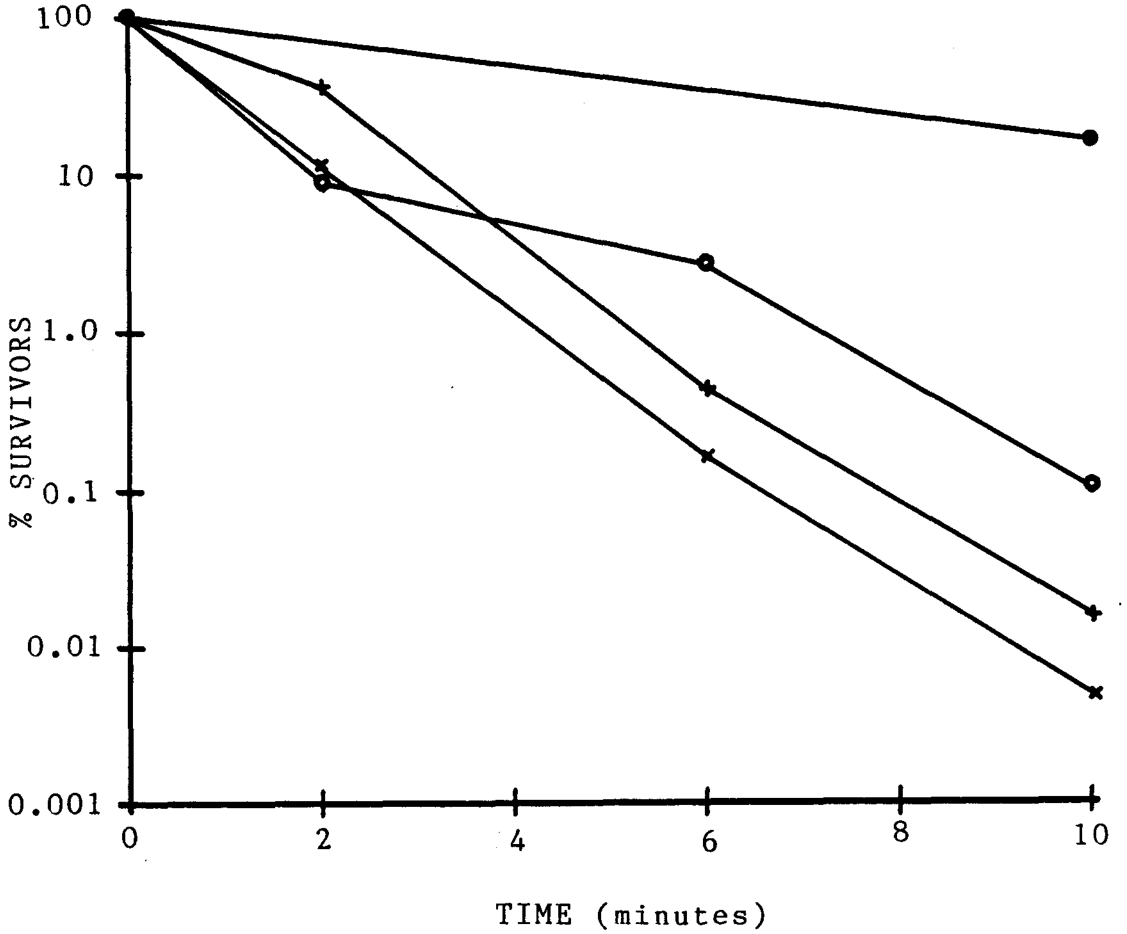
Figure 16 MMS Sensitivity of rad52 Strains


SPONTANEOUS MITOTIC RECOMBINATION
RAD52/RAD52
rad52-1/rad52-2
rad52-1/rad52-1
rad52-2/rad52-2
rad52-2/rad52-2
rad52-1/rad52-1
rad52-1/rad52-2
RAD52/RAD52
Media: MMS

Cells were plated on YPD plates containing 0.01% MMS as described for the analysis of spontaneous mitotic recombination, procedure A. Large colonies appearing in the region of the rad52-1/rad52-2 diploid patch probably arose as the result of spontaneous mitotic recombination.

RAD52/RAD52 = MH16; rad52-1/rad52-1 = RM105;
rad52-1/rad52-2 = RM107; rad52-2/rad52-2 = RM106.

Figure 17 MMS Sensitivity of rad52 Strains



Cells were treated as described. ●, RAD52/RAD52 (K65); ×, rad52-1/rad52-1 (RM105); +, rad52-1/rad52-2 (RM107); o, rad52-2/rad52-2 (RM106).

allowing for those cells not killed by the MMS to grow when the MMS reaches a low enough concentration to permit growth. But all of the rad52 strains should have experienced the same MMS concentration at all times following replica plating meaning that the rad52-2 cells may be only marginally more resistant to MMS than rad52-1 cells. Therefore, it can be concluded that the overall response to MMS is similar for all three rad52 strains tested, however, rad52-2 may be marginally more resistant to low concentrations of MMS than rad52-1. This can be tested by using different and lower concentrations of MMS in the MMS exposure assay.

UV Sensitivity of rad52 Strains

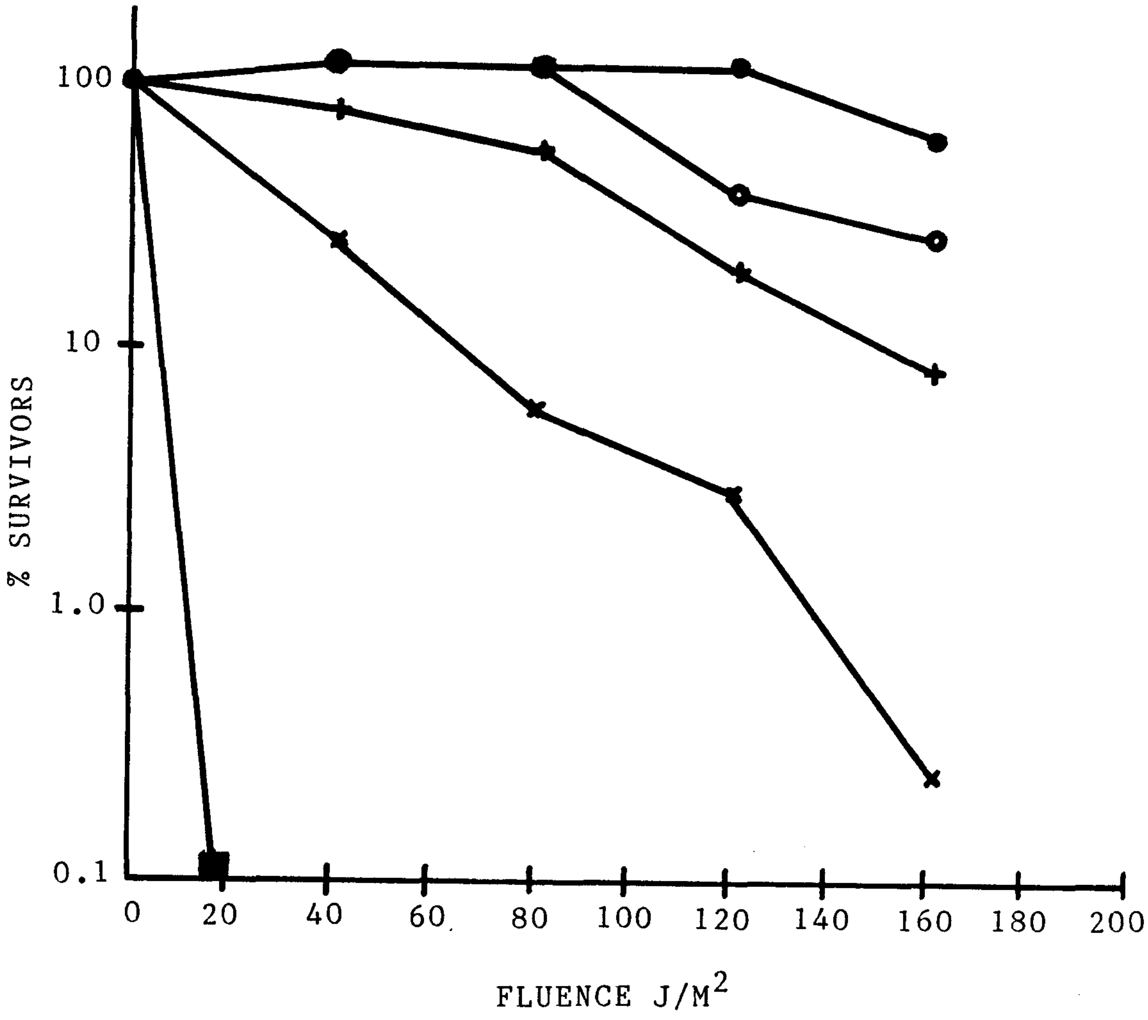
Compared to other UV sensitive mutations (e.g. rad3-2) the rad52-1 mutation confers a slight sensitivity to UV irradiation (41, 51). Resnick originally demonstrated that 10-20% of a rad52-1 haploid cell population survived a UV dose of 1800 ergs/mm^2 (=180 J/M^2) (104). Later, Prakash et al. tested a rad52-1 diploid strain and found that approximately 20% of the population suvived a UV dose of 40 J/M^2 (101). When compared to a 0.1% survival for rad3-2 diploids at a dose of less than 20 J/M^2, rad52-1 diploids are much more resistant to UV irradiation (M. Hoekstra personal communication).

The fact that rad52-1 diploids are somewhat sensitive to UV irradiation suggests that the recombinational repair pathway does play a role in the repair of UV-induced DNA damage. In light of the mitotic recombination activity observed for the *rad52-2* allele it was thought that this allele may also affect UV sensitivity differently than the *rad52-1* mutation. Therefore, all of the strains used above were tested for their sensitivity to UV radiation.

Strains MH16, RM105, RM106, and RM107 were irradiated with UV as described above and Figure 18 plots the log % survivors against UV fluence in J/M^2. The graph demonstrates that there is a difference between *rad52-1* and *rad52-2* cells in their response to UV-induced DNA damage and the UV response observed for the *rad52-1* diploid is in good agreement with the observations of Prakash et al. (101). The difference also appears to be gene dose related and UV resistance, again can be ranked from lowest to highest with *rad52-1*/*rad52-1* < *rad52-1*/*rad52-2* < *rad52-2*/*rad52-2* for UV doses up to 160 J/M^2. In addition, *rad52-2*/*rad52-2* cells appear to respond as wild type cells up to a UV dose of 80 J/M^2, but above this dose *rad52-2*/*rad52-2* cells can no longer repair UV damage as efficiently as wild type cells. Since this sensitivity assay was only conducted once it should be repeated to strengthen the observations.

Compared to a highly UV sensitive strain (*rad3*/*rad3*) all *rad52* strains repair UV damage much more efficiently. The actual differences between the three *rad52* strains is less than their differences with *rad3-2*. If the differences

Figure 18 UV Sensitivity of rad52 Strains



Cells were treated as described. ● , RAD52/RAD52 (K65); ✕ , rad52-1/rad52-1 (RM105); + , rad52-1/rad52-2 (RM107); ○ , rad52-2/rad52-2 (RM106); ■ , rad3-2/rad3-2 (MH16). The rad3-2/rad3-2 data was kindly provided by M. Hoekstra.

between the *rad52* strains are real it lends credence to the previous observations that *rad52-2* cells may be slightly more resistant to MMS than *rad52-1* cells; there may be some residual activity in mitotic cells.

Homothallic Interconversion of Mating Type in rad52-2 Cells

Homothallic strains of yeast carry the *HO* gene and are capable of interconverting their mating type from *a* to *α* or from *α* to *a* allowing strains literally to mate with themselves. As stated previously, haploid strains carrying both the *HO* and *rad52-1* genes are prevented from interconverting and, with some of alleles of the *MAT* locus, those that attempt it produce a lethal double-strand break at the *MAT* locus (80, 133). When a diploid strain heterozygous for *HO* (*HO*/*ho*) and *rad52-1* (*rad52-1*/*RAD52*) is sporulated, four equally probable genetypes will be produced: they are *HO RAD52*, *ho RAD52*, *HO rad52-1*, and *ho rad52-1*. Two of these genotypes cannot interconvert their mating type because they carry the *ho* allele, and each should be produced with equal numbers of *a* and *α* mating types capable of mating (maters). One genotype (*HO RAD52*) should always interconvert during growth and the progeny then mate forming diploids incapable of mating (nonmaters or N). The last genotype (*HO rad52-1*) was shown to prevent interconversion but there was an excess of spores with the *a* mating type over the *α* mating type (80). This was taken to indicate that some *MATα HO rad52-1* cells tried to interconvert and subsequently died due to a doubl-strand break (80, 133). Malone and Esposito did not find a single case where a *HO* *rad52-1* cell successfully interconverted its mating type (80). Since *rad52-2* strains have been shown to allow spontaneous mitotic recombination and may have slightly more recombinational repair activity than *rad52-1* strains, it was decided to investigate the effect of *rad52-2* on mating type interconversion.

The procedures described in Materials and Methods were followed and the results are in Table 19. Unlike the *rad52-1* results of Malone and Esposito, there were six spores that contained the *rad52-2* gene, as evidenced by their sensitivity to MMS, and were nonmaters indicating that they had interconverted their mating type and were therefore *HO* *rad52-2* spores originally. However, like Malone and Esposito, there was an excess of spores of the *MATa* *rad52-2* genotype compared to the *MATα* *rad52-2* genotype. Malone and Esposito attributed the excess to *MATα* cells attempting to interconvert and producing a lethal double-strand break in the process. Here, it appears that some of the *MATα* *rad52-2* cells attempted interconversion and were successful. It therefore appears that 1) the *rad52-2* allele prevents most interconversion of *HO* cells; 2) the interconversion which does occur seems likely to have been done in *MATα* cells given the moderate excess of *MATa* over *MATα* cells; and 3) *MATa* *HO* *rad52-2* cells may be stable and nonconverting

Table 19 Homothallic Interconversion in rad52-2 Strains

Diploid	# of Spores	RAD52			rad52-2		
		N	a	α	N	a	α
CE105[a]	38	15	5	1	3	6	8
CE106[b]	35	10	3	4	1	11	6
CE107[c]	28	9	2	5	1	7	4
CE108[d]	31	7	4	6	1	8	5
Total	132	41	14	16	6	32	23
	%	31.1	10.6	12.1	4.5	24.2	17.4
	Expected %*	25	12.5	12.5	0	25	25
	Expected #	33	16.5	16.5	0	33	33

*Expected % assuming that there is no interconversion =
% RAD52 N = % RAD52 HO = .50 X .50 = .25 = 25%
% RAD52 a ho = % RAD52 α ho = .50 X .50 X .50 = .125 = 12.5%
% rad52-2 N = % rad52-2 HO = 0 (no interconversion)
% rad52-2 a = % rad52-2 α = .50 X .50 = .25 = 25%

[a] CE105 = CE103 X K65; [b] CE106 = CE103 X K187;

[c] CE107 = CE104 X K65; [d] CE108 = CE104 X K187.

since the observed number, 33, is exactly what is expected, 32, if both MATa HO rad52-2 and MATa ho rad52-2 cells are MATa maters. The small amount of interconversion in rad52-2 cells again suggests a residual activity of the rad52-2 gene product, and is consistant with the observations on MMS sensitivity, UV sensitivity, and spontaneous mitotic recombination.

DISCUSSION

The experiments within this chapter stemmed out of an observation made in Chapter II. The meiotic 0 hour time point data suggested that rad52-2 strains allowed spontaneous mitotic recombination, unlike the rad52-1 allele. Two separate procedures were used to extend this observation and they demonstrated that rad52-2 cells do allow levels of spontaneous mitotic recombination similar to that of wild type cells. In addition, rad52-1/rad52-2 cells allow spontaneous mitotic recombination at a level intermediate between wild type and rad52-1 levels. This is also observed with a RAD52/rad52-1 heterozygote and suggests that the recombinational activity in rad52-2 cells is due to its gene product. Whereas rad52-2 cells have enough recombinational activity to perform spontaneous mitotic recombination, there is not enough activity to allow normal meiotic recombination. This is probably because there are many more recombination events in meiosis than in mitosis and the level of activity supplied by the rad52-2 gene product is inadequate.

Related to the differences between mitotic and meiotic recombination are the differences between the repair of UV- and MMS-induced DNA damage. Some UV-induced DNA damage is repaired by recombination which is manifested as a slight sensitivity to UV irradiation for rad52-1 cells. It was thought that the mitotic recombination activity found in rad52-2 cells might affect UV sensitivity. This was found to be the case for low levels of UV irradiation. And as before, the rad52-2 effect was dose dependent since rad52-1/rad52-2 cells had a UV response intermediate to those of rad52-1 and rad52-2 diploids. Because rad52-2 cells could not repair DNA lesions produced at higher UV doses as efficiently as wild type cells it appears that there are more lesions at these higher doses than there are normal spontaneous mitotic recombination initiation sites.

The repair of DNA lesions caused by the alkylating agent methyl methanesulfonate (MMS) is reportedly the same as for X-ray-induced damage which requires the recombinational repair pathway exclusively. This is why rad52 cells are very sensitive to both of these agents (12, 41). The slight recombinational repair activity of rad52-2 may not be detectable since just one unrepaired DNA lesion could be lethal (41). In fact, the standard MMS sensitivity assay performed in this chapter revealed that rad52-1 and rad52-2 cells behaved similarly. However, in the less stringent replica plating test rad52-2 cells appeared to be somewhat more resistant than rad52-1 cells. It may be that rad52-2 cells are more resistant to MMS in low concentrations since the MMS concentration is 100 fold higher in the MMS exposure procedure than in the YPD-MMS replica plate procedure.

The final *rad52-1* phenotype tested was the ability or inability of *rad52-2* cells to interconvert their mating type in the presence of the *HO* gene. Mating type interconversion is prevented in *rad52-1* cells and lethality can result in *MATα* *HO* *rad52-1* cells that attempt to interconvert because of the development of a double-strand break at the *MAT* locus (80, 133). By contrast, *rad52-2* do inhibit, but apparantly does not absolutely prevent, the interconversion and it may be that those cells that successfully completed the interconversion were originally of the *α* mating type. This is consistant with the other phenotypes of *rad52-2* in that there is a higher recombinational activity associated with the *rad52-2* gene product than there is for the *rad52-1* gene product.

This chapter and the preceeding one have compared the phenotypes of the alleles *rad52-1* and *rad52-2*. One conclusion that can be made is that the *rad52-2* allele is not as deleterious to the cell as the *rad52-1* allele. Both of these alleles appear to have a similar response to MMS- and X-ray-induced damage (J. Game personal communication) and their reduction of meiotic recombination levels is also similar. However, the alleles differ in other phenotypic ways. These phenotypes were all previously attributed to *rad52-1* and two were considered absolutely dependent on the *RAD52* gene product. Now, all of the phenotypes tested for both alleles can be arranged in an order that reflects the activity of the *rad52-2* gene product which also reflects the absolute dependency of the various cellular processes on the *RAD52* gene product. For example, spontaneous mitotic recombination occurs at essentially wild type levels in *rad52-2* cells indicating that this process is the least dependent on the *RAD52* gene product. The phenotypic order from least dependent to the most dependent is: spontaneous mitotic recombination < UV resistance < interconversion of mating type < MMS resistance and meiotic recombination. The phenotypic order also reflects the number of DNA lesions that must be repaired recombinationally. A "lesion" can be either a DNA break or gap or be the result of initiation of recombination. Theoretically, there should be only one DNA double-strand break associated with mating type interconversion in each G1 phase of the mitotic cell cycle (72). But this occurs in a haploid cell and there is evidence that *RAD52* activity is partly dependent on heterozygosity at the mating type locus (38, 41). The *rad52-2* activity would then be even lower in a haploid cell than it would be in a diploid cell and this might help account for the observed inefficiency of mating type interconversion.

The reason behind the observed *rad52-2* gene product activity is most easily explained by assuming that the allele is leaky. A leaky allele is one that has some level of residual activity of its gene product and the affected cellular processes occur normally mechanistically but at a lower rate. This concept is very amenable with the idea of different *RAD52* activity requirements for the processes discussed above. The fewer recombinational lesions associated with spontaneous mitotic recombination and UV irradiation would be repairable with a low gene product activity whereas the more numerous lesions found from MMS exposure and from meiotic recombination initiation would not be repairable. A leaky allele could also function as a control molecule since if its control activity is altered then any controlled gene products may also show an altered activity. There is evidence that the *RAD52* gene product is a control molecule. Chow and Resnick have described a nuclease that appears to be under the control of *RAD52* (20). Esposito et al. have also shown that there are different meiotic protein profiles in *RAD52* and *rad52-1* cells indicating a possible type of *RAD52* control.

A second explanation for the apparent increased activity in the *rad52-2* over the *rad52-1* gene product assumes that the *RAD52* gene product is a multifunctional enzyme with different active sites for different cellular processes. All of the active sites could be abolished by the *rad52-1* mutation while the *rad52-2* gene product could have at least one functional active site. This could also account for the ranking of phenotypes if there are different active sites responsible for mitotic and meiotic recombination. The mitotic activity could also repair UV-induced DNA damage and the meiotic activity could then repair the MMS and X-ray-induced damage. Both explanations are possible but the later assumes more evidence not in hand, so the former explanation is favored.

In conclusion, this chapter has demonstrated that the *rad52-2* allele confers more recombinational activity than the *rad52-1* allele. This difference is probably because the *rad52-2* mutation is leaky. More importantly, this has allowed the ranking of various *rad52-1* cellular phenotypes according to their *RAD52* activity requirement. The leakiness of *rad52-2* can be easily tested using the *RAD52::LEU2* interuption of Resnick et al. (107). If the *rad52-2* allele is leaky then the disruption should have the mitotic phenotype of the *rad52-1* allele.

CHAPTER IV

PLASMID STABILITY DURING MITOSIS AND MEIOSIS IN YEAST DIPLOIDS DEFICIENT FOR VARIOUS RECOMBINATION AND SPORULATION FUNCTIONS

BACKGROUND

Yeast-*E. coli* Shuttle Plasmids

Plasmids have been of great value in understanding the machanisms of prokaryotic recombination (134). Most of the plasmids used have been naturally occurring extrachromosomal plasmids called episomes. Bacterial plasmids have also helped develop recombinant DNA techniques which have proven to posses far-reaching applications. With the advent of a practical yeast transformation technique, all of the advantages of plasmid systems could be applied to an easily manipulated eukaryote (54).

The most useful plasmids for yeast investigations are collectively known as shuttle plasmid. They are genetically engineered chimeric plasmids containing *E. coli* and yeast sequences. The *E. coli* sequences include an origin of replication, useful for the propagation of large numbers of plasmid molecules, and antibiotic resistance markers, often for ampicillin or tetracycline resistance. These genes allow for the genetic selection of the plasmid in *E. coli* strains. Also, within the *E. coli* sequences, there are unique restriction endonuclease sites that can be used for cloning. The yeast genes give the plasmid its shuttle quality. These genes are wild type genes that can complement auxotrophic mutations in yeast (and often in E. coli), thereby providing a selectable marker in yeast. The most commonly used yeast genes are *LEU2*, *TRP1*, *URA3*, and *HIS3* (11).

Shuttle plasmids can be further broken down into four catagories, YIp, YEp, YRp, and YCp (11). YIp plasmids require integration into chromosomal DNA through reciprocal recombination to be maintained in yeast cells (*Y*east *I*ntegrating *p*lasmid). YEp plasmids (*Y*east *E*pisomal *p*lasmid) contain the origin of replication found in the naturally occurring yeast episomal 2u plasmid, thereby allowing autonomous replication in yeast cells. YRp plasmids (*Y*east *R*eplicating *p*lasmid) are also capable of autonomous replication in yeast, but unlike the YEp plasmids, the sequences bestowing autonomous replication are found in yeast genomic DNA. Lastly, YCp plasmids (*Y*east *C*entromeric *p*lasmid) contain not only a yeast origin of replication but also a yeast centromeric sequence that conveys near chromosomal stability on these plasmids (11). Of these plasmid species, this chapter is concerned only with the YIp and YRp plasmids.

YIp Plasmids

The yeast transformation system developed by Hinnen et al. utilized a YIp plasmid containing the LEU2 gene (54). They found that markers on these plasmids are only maintained through recombination events with the chromosome. The recombination events appear to be, at least formally, single reciprocal crossovers (54). Smolik-Utlaut and Petes showed that plasmid-chromosome recombination is dependent on sequence homology since small amounts of sequence heterogeneity reduces the frequency of recombination (118).

Orr-Weaver et al. began using integrating plasmids (and later replicating plasmids) as a model system for yeast recombination (96). They transformed wild type and rad52-1 yeast cells with circular replicating plasmids or with the same plasmids linearized by cutting them at a unique restriction site. They then compared the transformation frequencies obtained from the cut and uncut plasmids and found that the cut plasmid had a much higher transformation frequency in wild type cells but not in rad52-1 cells. They concluded that circular plasmid integration is not dependent on the RAD52 gene product, but integration of gapped and linear plasmids is dependent on the RAD52 gene product (96). This was reinforced by Symington et al. when they showed that cell-free extracts of rad52-1 could promote recombination between homologous circular plasmids, but no recombination occurred if one of the plasmids was linearized (130).

Since YIp plasmids must integrate to be maintained in yeast cells they posses certain characteristics. First, the recombination dependency results in low transfromation frequencies with an average of 1 to 10 transformants/ug of DNA (11). Secondly, integrated plasmids are extremly stable with much less than 1% of the transformants losing the plasmid every cell generation in nonselective medium (11). In diploids homozygous for an integrated plasmid, the plasmid will segregate in a mendelian fashion (11). YIp plasmids have or could be used for a variety of experiments including incorporation of mutations created in vitro, fine structure mapping, and creation of duplications, inversions, and translocations (11).

YRp Plasmids

In 1979 Struhl et al. discovered two plasmid types capable of self-replication (128). These plasmids do not require recombination to be maintained in yeast cells and hence give high frequencies of transformation with as many as 5,000 to 25,000 transformants per ug of DNA (128). One of these plasmid types became known as the YEp class since they contain part of the yeast episomal 2u plasmid. The other plasmid type is the YRp class which contains yeast sequences allowing for plasmid replication (128).

The YRp plasmid created by Struhl et al. contains the yeast genomic sequence surrounding and including the

TRP1 gene (128), and was named YRp7. Although they originally could not detect YRp7 plasmids integrated in the yeast genome, they later found that YRp plasmids can integrate under selective growth conditions (123, 128). This was confirmed by Hsaio and Carbon who used a YRp plasmid containing the ARG4 region of yeast instead of the TRP1 region (59). An integrated YRp plasmid has the same stability properties as the YIp plasmids (59, 123). Unintegrated YRp plasmids are unstable in both nonselective and selective mitotic growth conditions (59, 122). After 12 generations in nonselective medium, Hsiao and Carbon found that only 0.6-21% of the cells retained the ARG4 based plasmid. This compares with 13-50% of cells grown under selective conditions (59). Warren derived a formula for calculating the rate of plasmid loss per generation (132). Application of this formula to data generated by Bloom et al. (8) for YRp7 indicates that up to 44% of the cells lose the plasmid every generation in nonselective medium. No other published data is available for YRp7. This is a phenomenal rate considering that YRp plasmids are present in high copy number with as many as 100 copies per cell (61). Warren's formula will be used later in this chapter.

Murray and Szostak investigated the behavior of YRp plasmids under selective conditions (91). By separating mother and daughter cells with a micromanipulator, they found that frequently the plasmid fails to segregate into the daughter cell. They also found that the daughter cell could usually divide once or twice without the plasmid. This nonequal segregation accounts for much of the loss of the plasmid and the slow growth rates of cells with YRp plasmids in selective medium as observed by Hsaio and Carbon (59). In addition to mitotic instability, YRp plasmids are meiotically unstable (67). Kingsman et al. found that approximately 25% of the spores from cells containing a YRp plasmid still contained the plasmid. Hicks et al. have also reported that YRp plasmids are unstable in meiosis (53).

A YRp plasmid called the TRP1 R1 circle is an exeption to the observed instability of other YRp plasmids (137). This plasmid is made up of only the TRP1 region of YRp7 and it is very stable mitotically and meiotically and it is present in as many as 200 copies per cell. Zakian and Scott attribute the stability of this plasmid to its small size (137) which may allow the plasmid to segregate more easily into the daughter cells and spores. Alternatively, the stability may have been enhanced by the absence of prokaryotic sequences.

Stinchcomb et al. determined that the yeast sequence responsible for the autonomous replication of YRp7 to be outside the TRP1 gene and they called it ARS1 for autonomous replicating sequence (123). Hsiao and Carbon found another ARS adjacent to the ARG4 gene (59). By cloning yeast genomic libraries into a YIp plasmid and then screening for plasmids that transform at high frequencies, Beach et al. and Chan and Tye found that ARSs are found about every 30-40 kilobase pairs (kb) of chromosomal DNA (5, 19). This correlates with the estimated frequency of yeast replication origins (92). For this and other reasons ARSs are thought to be yeast origins of replication (32), but this hypothesis has yet to be confirmed.

As one would expect for an origin of replication, YRp plasmids are replicated in the S phase of the cell cycle (33). By comparing the sequences of various ARSs an eleven base pair consenses sequence has been determined as being necessary for ARS function and it is A/TTTTATPuTTTA/T (13). Finally a 14 base pair sequence containing the consenses sequence is able to function as an ARS although not as efficiently (120).

Experimental Rational

Orr-Weaver et al. have also used YRp plasmids as part of an experimental system for studying yeast recombination (94, 96, 97). Their work with circular and gapped YIp and YRp plasmids in wild type and rad52-1 cells led them to develop the double-strand break model of recombination (131). Their work was done entirely with mitotic cells and it is the goal of the experiments within this chapter to exploit the circular YRp plasmid system to try to understand plasmid behavior during meiotic recombination in wild type cells and then to use this knowledge to try to gain some insight into the effects of various Rec$^-$ and Spo$^-$ mutations. The mutations used in these studies are rad50-1, rad52-1, spo11-1, and spo13-1.

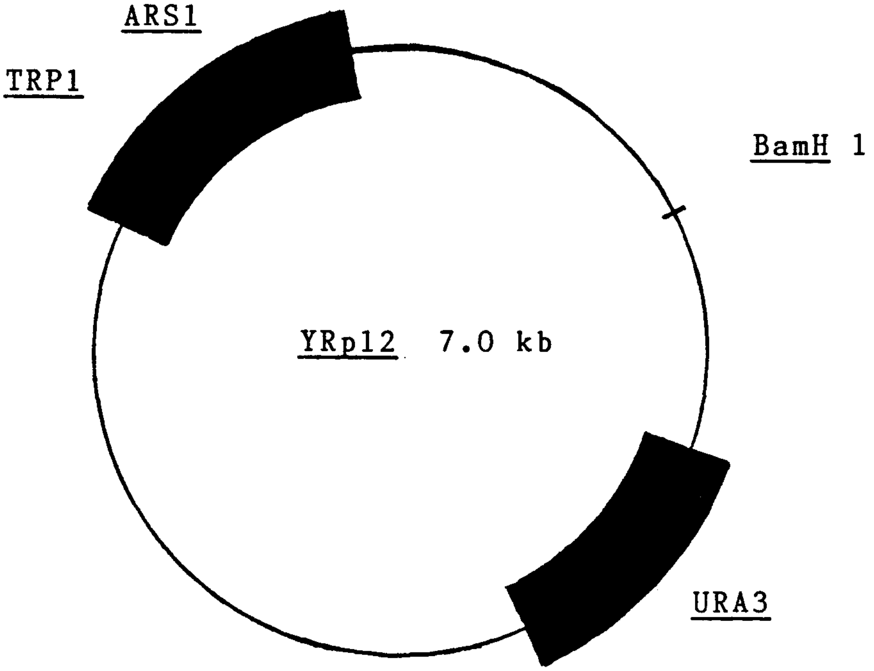
The rad50-1 mutation was originally isolated by Cox and Parry (21). It has been highly characterized and its phenotypes are as follows. It causes sensitivity to X-rays and the alkylating agent methyl methanesulfonate and is part of the double-strand break repair pathway (12, 21, 41, 51). Unlike rad52-1 cells, rad50-1 cells have elevated levels of spontaneous mitotic recombination, homothallic interconversion of mating type is normal, and there is no induction of recombination in meiosis (41, 80, 107, and Chapter II). Multiple mutant analysis using rad50-1 spo13-1, rad52-1 spo13-1, and rad50-1 rad52-1 spo13-1 diploids has shown that the RAD50 gene product acts before RAD52 in meiosis but not in mitosis (79, 81, and see Chapter II for a more thorough discussion). The phenotypes of rad52-1 cells have been discussed at length in Chapters II and III and can be found in Table 13.

The spo11-1 mutation was originally isolated by Esposito and Esposito and Esposito et al. (25, 28). It is a mutation that specifically affects meiotic but not mitotic recombination (71). Diploid cells have reduced sporulation with poor spore viability and the few asci formed are morphologically abnormal (71). The SPO11 gene appears to act after the RAD50 and before the RAD52 genes in meiotic recombination (R. E. Malone, personal communication).

The spo13-1 mutant was isolated by Klapholz and Esposito (70). This mutation abolishes the meiosis I reductional division resulting in the production of diploid two spored asci (dyads). There is no effect on mitotic or meiotic recombination (70). Because there is no meiosis I reductional division the need for chromosome pairing and recombination is formally alleviated (3, 70). Malone and Esposito and then Malone used this knowledge in their multiple mutant analyses which ordered recombination mutations in meiosis (79, 81, and see Chapter II). The double mutants rad50-1 spo13-1 and spo11-1 spo13-1 produce nonrecombinant diploid dyads in meiosis and the former diploid will be used in this study (71, 81).

In all, six diploid strains were used in the following studies; they were wild type, rad50-1, rad52-1, spo11-1, spo13-1 and rad50-1 spo13-1 diploids. These diploids were transformed with plasmid YRp12 which is YRp7 with the URA3 gene (see Figure 19) (124). Transformants were tested for mitotic plasmid stability in nonselective and selective media and they were put through meiosis. Samples were taken before and during meiosis to genetically follow the plasmid and DNA was prepared from the cells to physically follow the fate of the plasmid. For those strains that produced viable spores, asci were dissected and the spores were tested gen-

Figure 19 Plasmid YRp12


ARS1
TRP1
BamH 1
YRp12 7.0 kb
URA3

etically for the presence of the plasmid. These experiments showed that all strains demonstrated plasmid instability but the plasmid loss rates were lower than those reported by Bloom et al. (8). Interestingly , plasmid integration occurred in all strains during selective growth except the *rad52-1* diploid. This is in opposition to the published data of Orr-Weaver et al. (94, 96, 97).

MATERIALS AND METHODS

Strains

The strains used are listed in Table 20. The diploids were constructed immediately prior to use using standard genetic techniques (45). The plasmid YRp12 is 7.0 kb in size and contains the TRP1, ARS1, and URA3 yeast genes (Figure 19 (124). Selection for the plasmid was accomplished through complementation between the ura3 auxotrophic mutations in the diploids and the URA3 gene of YRp12.

Yeast Transformation

The transformation technique used was a slight modification of that of Hicks et al. (53). Cells were grown in 10 mls liquid YPD medium to a concentration of $1\text{-}2(10)^7$ cells/ml. The cultures were harvested by centrifugation and washed once in sterile deionized water and resuspended in 1 ml of SED (1 M sorbitol, 25 mM EDTA, 50 mM dithiothreitol), and incubated at 30° C for 10 minutes. The cells were then washed in 1 ml of 1 M sorbitol and resuspended in 1 ml of SCE (1 M sorbitol, 0.1 M sodium citrate, and 10 mM EDTA). to this 20 ul of glusalase (DuPont) was added and the cells were incubated at 30° C until spheroplasting was complete

Table 20 Strains and Their Genotypes

CE109	$\frac{a}{\alpha}$	$\frac{\text{RAD50}}{\text{RAD50}}$	$\frac{\text{RAD52}}{\text{RAD52}}$	$\frac{\text{SPO11}}{\text{SPO11}}$	$\frac{\text{SPO13}}{\text{SPO13}}$	$\frac{\text{ura3-1}}{\text{ura3-1}}$
CE110	$\frac{a}{\alpha}$	$\frac{\text{RAD50}}{\text{RAD50}}$	$\frac{\text{RAD52}}{\text{RAD52}}$	$\frac{\text{SPO11}}{\text{SPO11}}$	$\frac{\text{spo13-1}}{\text{spo13-1}}$	$\frac{\text{ura3-1}}{\text{ura3-1}}$
CE111	$\frac{a}{\alpha}$	$\frac{\text{RAD50}}{\text{RAD50}}$	$\frac{\text{RAD52}}{\text{RAD52}}$	$\frac{\text{spo11-1}}{\text{spo11-1}}$	$\frac{\text{SPO13}}{\text{SPO13}}$	$\frac{\text{ura3-13}}{\text{ura3-13}}$
CE112	$\frac{a}{\alpha}$	$\frac{\text{rad50-1}}{\text{rad50-1}}$	$\frac{\text{RAD52}}{\text{RAD52}}$	$\frac{\text{SPO11}}{\text{SPO11}}$	$\frac{\text{spo13-1}}{\text{spo13-1}}$	$\frac{\text{ura3-1}}{\text{ura3-1}}$
CE113	$\frac{a}{\alpha}$	$\frac{\text{rad50-1}}{\text{rad50-1}}$	$\frac{\text{RAD52}}{\text{RAD52}}$	$\frac{\text{SPO11}}{\text{SPO11}}$	$\frac{\text{SPO13}}{\text{SPO13}}$	$\frac{\text{ura3-13}}{\text{ura3-13}}$
CE114	$\frac{a}{\alpha}$	$\frac{\text{RAD50}}{\text{RAD50}}$	$\frac{\text{rad52-1}}{\text{rad52-1}}$	$\frac{\text{SPO11}}{\text{SPO11}}$	$\frac{\text{SPO13}}{\text{SPO13}}$	$\frac{\text{ura3-1}}{\text{ura3-1}}$

(30-60 minutes). The sheroplasts were centrifuged and resuspended in 5 mls of 1 M sorbitol and this was repeated twice more and the cells were resuspended in CaST (1 M sorbitol, 10 mM calcium chloride, and 10 mM TRIS). Five ug of plasmid DNA was then added and the cells were incubated at room temperature for 15 minutes at which time 1 ml of 30% polyethylene glycol was added and this mixture was incubated another 15 minutes at room temperature. The suspension was the centrifuged and the cells were resuspended in 0.25 mls of SOS (1 M sorbitol, 33% YPD liquid medium, 0.65% 10 mM calcium chloride) and incubated at 30° C for 40 minutes at which time the cells were plated on complete and uracil omission regeneration plates (1 M sorbitol, 2.5% agar, 2% glucose, 0.67% yeast nitrogen base without amino acids and bases, and necessary amino acids and bases. The plates were incubated at 30° C. After the transfomants had appearred on the URA drop regeneration plates (4-5 days) 5-10 transformants were picked to a URA-drop (Complete synthetic medium lacking uracil) master plate and the rest of the transformant colony was resuspended in 0.5 mls of 0.2 M PO_4 buffer pH 7.5.

Mitotic Stability of YRp12

The PO_4 buffer transformant suspensions were used to inoculate 5 mls YPD cultures at $1(10)^5$ cells/ml which were Incubated with shaking at 30° C. The suspensions were also serially diluted and plated on COM (complete synthetic media) and URA drop media to determine the initial fequency of cells containing the plasmid YRp12. After at least four generations of growth in YPD (nonselective) media, as determined using a hemocytometer, the cultures were harvested by centrifugation, washed twice with PO_4 buffer, resuspended in 5 mls PO_4 buffer, serially diluted, and plated as before to determine the final frequency of cells containing the plasmid. The initial and final frequencies were incorporated into Warren's rate loss formula:

Rate of plasmid loss = 1 - $(\text{Freq. final} / \text{Freq. initial})^{1/G}$

where G is the number of cell generations (132).

Premeiotic and Meiotic Cultures

The same PO_4 suspensions used above were used to inoculate 25 mls CGAU (complete synthetic medium lacking uracil and containing 0.2% glucose, 1.0% potassium acetate). The selective conditions were necessary to maintain the plasmid in as many cells as possible. The small amount of glucose was necessary for rapid initial growth but was insufficient for the cultures to reach the desired concentration of $1(10)^7$ cells/ml; the cells were then forced to use the acetate for growth which is the standard sporulation carbon source (45). When these cultures reached $1(10)^7$ cells/ml they were serially diluted and plated as before and they were also used to inoculate 750 mls CAU (like CGAU but without glucose) cultures to a concentration of $1(10)^5$ cells/ml. These cultures were the actual presporulation cultures and were grown to a cell concentration of $1(10)^7$ cells/ml at 30° C with shaking. When the cultures reached the desired concentration they were harvested by centrifugation, washed twice with sporulation medium (2% potassium acetate and necessary amino acids), resuspended in 375 mls of sporulation medium, sampled (see below) and incubated with shaking at 30° C.

Meiotic Culture Sampling

Starting with the time of inoculation, sporulation cultures were sampled. At 0, 5, 10, 15, 20, 25, and 48 hours 40 mls was removed and the cells were pelleted, washed once with 10 mls of 10mM Tris 10 mM EDTA pH 8.0, pelleted again and frozen at -70° C. A portion of the cell suspension was removed before pelleting, a portion of that was mixed 1:1 with 8% formaldehyde and the remainder was used for serial dilutions which were plated on COM and URA media as before. The formaldehyde suspension was used to visually monitor ascospore development. The frozen pellet of meiotic cells was used to prepare DNA.

DNA Preparation

The DNA preparation technique used was a modifica- of the method of Petes et al. (100). The frozen pellets were thawed and resuspended with 1 ml. of 50 mM Tris and 50 mM EDTA pH 8.0. To this 1.0 ml. equivalent of 0.5 mm diameter glass beads were added and the suspension was vortexed vigorously for 2 minutes of cooling on ice. This was repeated until > 95% of the cells and spores were disrupted as determined microspically. The beads were allowed to settle and the supernatant was removed with an automatic pipet. The beads were washed once with a small volume of 50 mM Tris, 50 mM EDTA pH 8.0 and enough of this was added to bring the total volume of supernatant to 1.0 ml. DNA was then prepared from this cell suspension by following the procedures of Maniatis et al. (83).

DNA Characterization

Sample DNA concentrations were determined and similar amounts were loaded on a 0.7% agarose gel and electrophoresed for 20-24 hours at 50 mamps constant current. Controls were YRp12 monomer, dimer, and linear (BamHI digest, BRL). The gels were stained with ethidium bromide and photographed. DNA blot analysis followed general laboratory procedures as described by Hoekstra and Malone (56). The autoradiograms were further analyzed by using a Hoefer densitometer model number GS300 with an LKB 2210 chart recorder. The areas under the resulting tracings were calculated arithmatically and were used to estimate the ratios of stable versus unstable plasmid sequences.

RESULTS

YRp12 Plasmid Stability in Mitosis

The stability of plasmid YRp12 was examined under both nonselective and selective conditions. Under nonselective conditions all diploids demonstrated plasmid instability (Tables 21, 22, and 23). The formula of Warren gives plasmid loss rate per generation and the values can range from -1.0 to 1.0 (132) with positive numbers reflecting a loss of the plasmid. Only those cultures that experienced a loss of plasmid were included in Table 23. One wild type, two spo13-1, and one rad50-1 transformant cultures experienced a net gain of plasmid during growth. The one rad50-1 transformant contained an integrated YRp12 plasmid (see below). The other transformants showing an increase in cells with plasmid may also have maintained the plasmid as an integrant but the cells of these cultures were not analyzed for this possibility. It is sufficient to know that YRp plasmids are unstable in mitosis in all of the strains tested.

The average plasmid loss rate is different from one strain to another. These differences can not be easily correlated with either the mitotic phenotype of the strain, the

Table 21 YRp Plasmid Stability Under Nonselective Conditions

Genotype	Culture #	Initial Ura^+ Freq.	Final Ura^+ Freq.	# of Gener.	Rate of Loss/Gener.
Wild Type	1	0.0223	0.0111	5.68	0.1156
	2	0.0314	0.0154	5.41	0.1234
	3	0.0195	0.0186	6.13	0.00768
	4	0.0708	0.0943	6.08	-0.0483
	5	0.0194	0.00934	6.71	0.1032
	6	0.0765	0.0216	10.24	0.1162
	7	0.0816	0.0239	9.86	0.1169
	8	0.0809	0.0264	11.06	0.0964
	9	0.0918	0.0368	10.33	0.0847
	10	0.0834	0.0257	10.22	0.1086
spo13-1 / spo13-1	1	0.1663	0.2159	11.55	-0.0228
	2	0.1216	0.1898	11.72	-0.0387
	3	0.1165	0.0992	12.78	0.0125
	4	0.1386	0.0408	12029	0.0946
spo11-1 / spo11-1	1	0.386	0.0255	8.20	0.2821
	2	0.229	0.0581	10.25	0.1252
	3	0.146	0.0513	9.21	0.1074
	4	0.215	0.0523	10.37	0.1274
	5	0.172	0.0548	9.98	0.1086
	6	0.0448	0.0105	11.10	0.1225

Table 22 YRp Plasmid Stability Under Nonselective Conditions

Genotype	Culture #	Initial Ura^+ Freq.	Final Ura^+ Freq.	# of Gener.	Rate of Loss/Gener.
spo13-1, spo13-1 rad50-1 rad50-1	1	0.113	0.0271	13.37	0.1013
	2	0.101	0.00473	12.62	0.2154
	3	0.0977	0.0038	13.32	0.2163
	4	0.154	0.0169	11.12	0.1802
	5	0.2190	0.00450	13.53	0.2496
rad50-1 rad50-1	1	0.0303	0.0022	13.56	0.1759
	2	0.0478	0.00386	12.74	0.1792
	3	0.880	1.24	10.32	-0.0338
	4	0.0331	0.00286	12.27	0.1809
	5	0.0807	0.00149	11.86	0.2858
	6	0.0435	0.00117	5.63	0.4739
	7	0.0730	0.0180	6.33	0.1984
	8	0.0664	0.0228	5.10	0.1891
	9	0.0575	0.0143	5.64	0.2186
	10	0.0600	0.0143	5.03	0.2481
rad52-1 rad52-1	1	0.238	0.120	4.70	0.1356
	2	0.359	0.117	4.15	0.2367
	3	0.132	0.070	3.75	0.1556
	4	0.171	0.00308	5.77	0.2570
	5	0.164	0.0680	4.33	0.1840
	6	0.0557	0.01127	4.57	0.2950

Table 23 Average Rate of Plasmid Loss Per Generation Under Nonselective Growth Conditions

Genotype	# of Cultures With Loss	Initial Mean Freq. Ura$^+$ (Median)	Final Mean Freq. Ura$^+$ (Median)	Mean # of Generations (Median)	Rate of Loss/Gen. (s.d.)	Rate of Loss/Gen. Median
Wild Type	9[a]	0.0465 (0.0765)	0.0194 (0.0216)	8.02	0.0802 (0.0412)	0.109
$\frac{spo13\text{-}1}{spo13\text{-}1}$	2[b]	0.1271 (0.1276)	0.0636 (0.0700)	12.53	0.0344 (0.0172)	0.054
$\frac{spo11\text{-}1}{spo11\text{-}1}$	6	0.1666 (0.1935)	0.0360 (0.0518)	9.81	0.1360 (0.0005)	0.124
$\frac{spo13\text{-}1\ rad50\text{-}1}{spo13\text{-}1\ rad50\text{-}1}$	5	0.1303 (0.1010)	0.0082 (0.0047)	8.35	0.1840 (0.0036)	0.215
$\frac{rad50\text{-}1}{rad50\text{-}1}$	9[a]	0.0672 (0.0575)	0.0053 (0.0039)	7.97	0.2260 (0.0623)	0.198
$\frac{rad52\text{-}1}{rad52\text{-}1}$	6	0.1613 (0.1675)	0.0364 (0.0690)	4.50	0.2030 (0.0017)	0.210
Wild Type*	1	0.10 (----)	0.01 (----)	4.0	0.44 (----)	-----

Averages are geometric means

Plasmid loss rates were calculated using the formula of Warren (131)

[a] One additional culture showed a net gain of cells with plasmid

[b] Two additional cultures showed a net increase of cells with plasmid

*Strain J17 transformed with YRp7, from Bloom et al. (8)

frequency of cells with plasmid originally, nor the number of generations. All of the strains, however, have much lower values than the wild type strain of Bloom et al. using the related plasmid YRp7 (8). Plus he only examined one culture. Interestingly, rad52 diploids, which have a high rate of spontaneous chromosome loss in mitosis do not affect plasmid loss markedly, although the rate is 3 fold higher than wild type. Also unusual is that the same strain with the lowest rate of loss (1/2 that of wild type) is the spo13 diploid which is a meiotic specific mutation.

During selective growth prior to meiosis there were three stages where the frequency of cells with plasmid were measured. These were the original inoculum of the 25 mls preculture, the final frequency of the preculture which also served as the inoculum of the 750 mls presporulation culture, and the 750 mls culture frequency. It would be expected that the frequency of cells with plasmid would decrease during growth as was shown by Murray and Szostak (91). Table 24 shows that the contrary is the case. All of the strains showed an increase in plasmid frequency from the start of the preculture to the start of sporulation.

A possible explanation for this phenomena may be related to the Hsiao and Carbon observation that YRp transformants with an integrated plasmid grew faster than transformants containing the free plasmid (59). Murray and Szostak showed that the reason for this is that cells with

Table 24 Cells With Plasmid Frequency Under Selective Conditions

Genotype	Ura^+ Freq. Preculture Inoculum	# of Gen.	Initial[a] Ura^+ Freq. 750 ml Cult.	# of Gen.	Final Ura^+ Freq. 750 ml Cult.
Wild type	0.155	8.36	0.724	7.31	0.960
Wild type	0.077	7.88	0.091	5.10	0.530
spo13-1	0.0325	7.59	0.027	6.94	0.095
spo13-1	0.100	6.92	0.420	4.70	0.650
spo11-1	0.044	8.43	0.182	5.72	0.512
13-50*	0.099	7.91	0.594	6.94	0.870
rad50-1	0.041	5.78	0.022	5.16	0.380
rad52-1	0.056	6.92	0.088	4.70	0.082

[a] The inoculum for the 750 ml culture was taken from the preculture; hence the initial Ura^+ frequency of the 750 ml. culture is also the final frequency for the preculture.

*13-50 is the double mutant _spo13-1_ _rad50-1_.

The preculture was grown in liquid CGAU, a complete synthetic medium containing 0.2% glucose and 1.0% potassium acetate but lacking uracil. The 750 ml premeiotic culture was grown in liquid CAU medium which is exactly like CGAU except it also lacks glucose.

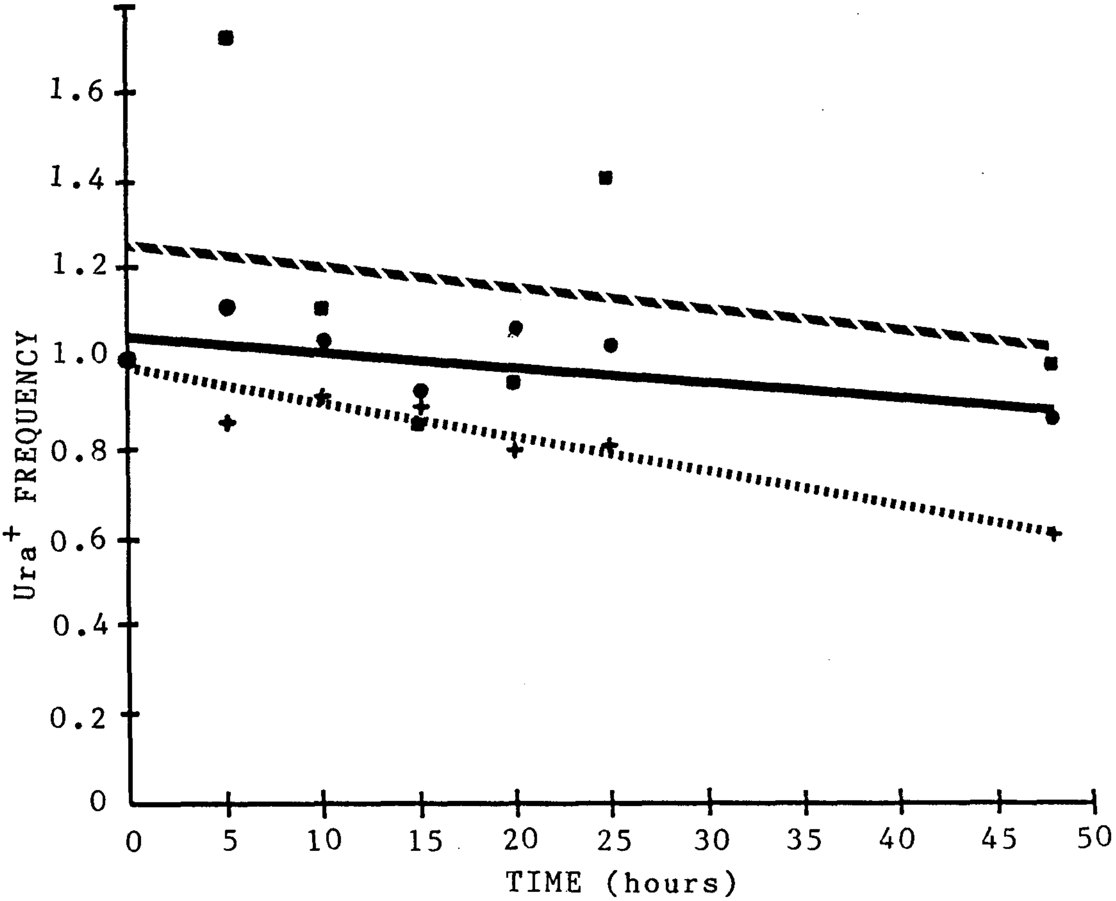
the free plasmid frequently bud off cells without plasmid (91). The plasmidless cells eventually die under selective conditions and thereby do not contribute to the growth rate. Cells with an integrated plasmid bud off cells that always contain an integrated copy of the plasmid so these cells contribute to the growth rate. In a population of cells with a mixture of cells with an integrated plasmid and cells with a free plasmid, the portion with the integrated plasmid will contribute proportionately more cells to the growth rate per division than cells with the free plasmid. This would eventually lead to an increase in the frequency of cells containing plasmid.

Meiotic Stability of YRp12

Kingsman et al. suggested that YRp plasmids are unstable in meiosis (67). By taking samples every five hours of sporulating cells it was hoped that this could be more thoroughly demonstrated. Figures 20-22 and Table 25 show that the frequency of cells with plasmids does decrease during meiosis for all strains tested. Wild type cells show the smallest frequency decline and the rad50-1 spo13-1 strain showed scattered data complicating interpretation. All other strains show observable decreases in frequency.

Since the frequencies of cells with plasmids are declining, it means that cells are actively losing the plasmid. In the case of rad50-1, spo11-1, and rad52-1 cells

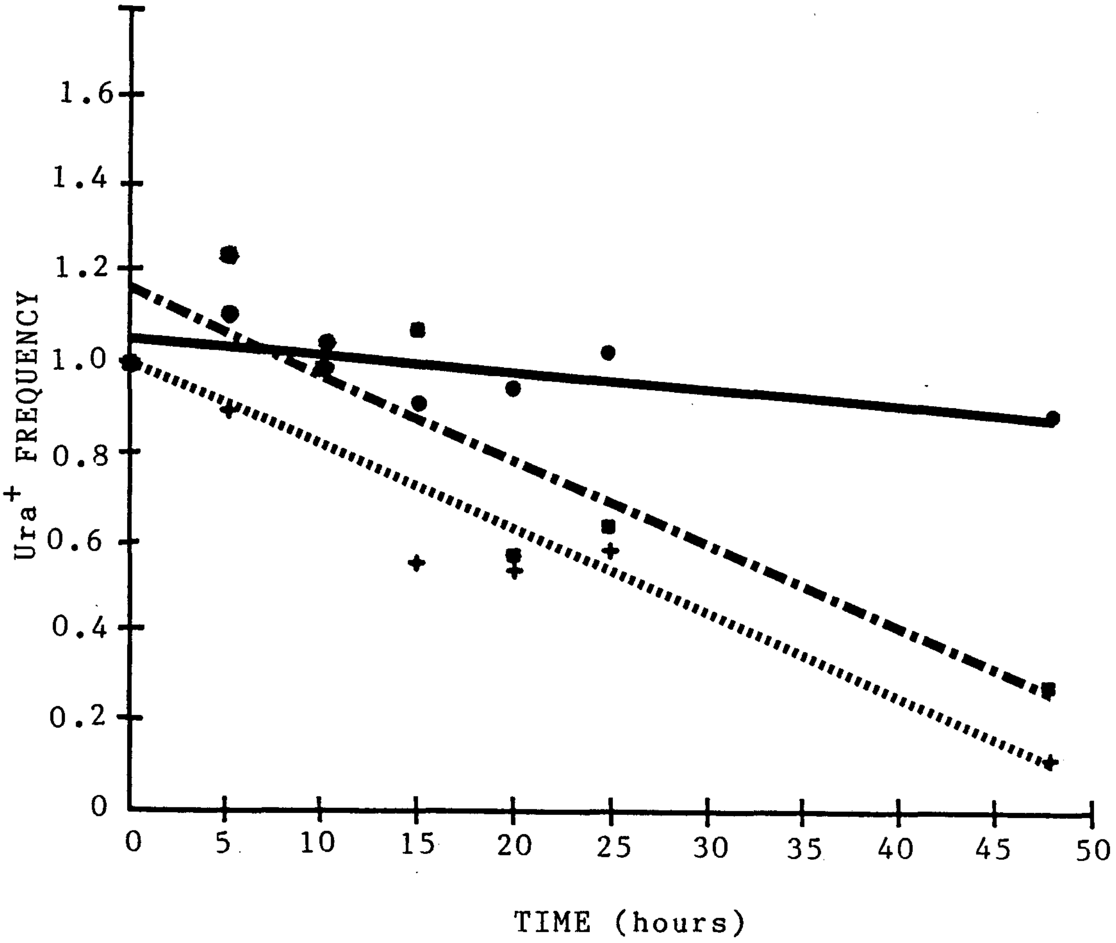
Figure 20 Plasmid Stability During Meiosis For Wild Type and spo13-1 Diploids



All actual Ura^+ frequencies were normalized to the 0 hour frequency by dividing all frequencies by the 0 hour value. ●━━━● , Wild type diploid (CE109); +┈┈┈+ and ■━ ━ ━■ , spo13-1 diploids (CE110-1 and CE110-2).

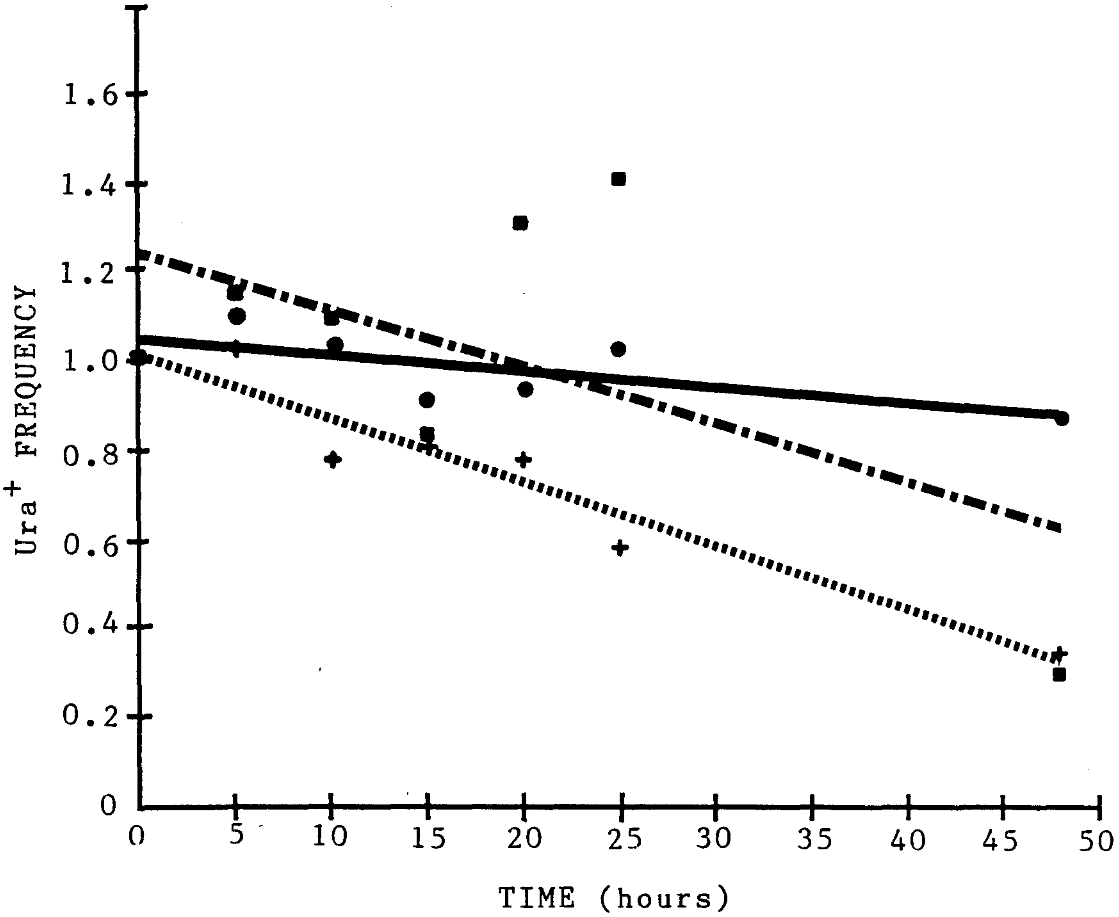
The lines in the figure were generated using the Curve Fitter least squares program (Interactive Microware Inc.) for an Apple IIe computer.

Figure 21 Plasmid Stability During Meiosis For Wild Type, spo11-1, and rad52-1 Cells



All actual Ura^+ frequencies were normalized to the 0 hour frequency by dividing all frequencies by the 0 hour value. ●——● , Wild type diploid (CE109); ■-·-·■ , spo11-1 diploid (CE111); +·······+ , rad52-1 strain (CE114). The lines in the figure were generated using the Curve Fitter least squares program (Interactive Microware Inc.) for an Apple IIe computer.

Figure 22 Plasmid Stability During Meiosis For Wild Type, spo13-1 rad50-1, and rad50-1 Cells



All actual Ura$^+$ frequencies were normalized to the 0 hour frequency by dividing all frequencies by the 0 hour value. ●━━━━━━━━● , Wild type diploid (CE109); ■ ▪ ▬ ▪ ▬ ▪ ■, spo13-1 rad50-1 diploid (CE112); +ııııııııııııııı+ , rad50-1 diploid (CE113). The lines in the figure were generated using the Curve Fitter least squares program (Interactive Microware Inc.) for an Apple IIe computer.

Table 25 Stability of the Ura^+ Phenotype in Meiosis: Slopes for the Lines in Figures 20-22

Genotype	Figure	Slope	CoC*
Wild type	20, 21, & 22	-0.0044	0.937
spo13-1a	20	-0.013	0.551
spo13-1b	20	-0.014	0.964
spo11-1	21	-0.019	0.944
rad52-1	21	-0.019	0.887
spo13-1 rad50-1	22	-0.0049	0.255
rad50-1	22	-0.0078	0.964

The lines in Figures 20-22 were generated using the Curve Fitter least squares program (Interactive Microware Inc.) for an Apple IIe computer.

*CoC = Coefficient of Correlation for the fitted line generated by the Curve Fitter program.

that experience cell mortality during meiosis, cells are losing the plasmid faster than the cells are dying. With this information it was decided to analyze those cells that retained the plasmid during meiosis. Colonies formed on URA drop plates at the meiotic time points were replica plated to YPD plates two times in succession and then to URA-drop plate. This procedure can detect colonies of free plasmid and colonies that may contain some cells with the URA3 gene of the plasmid stablized either through integration into the chromosome or through a gene conversion event with the genomic ura3 mutation. Colonies in which the Ura^+ characteristic is only carried on the unstable free plasmid will lose the plasmid during nonselective growth and then will not grow on the URA-drop plate (59).

The results of these tests are presented in Table 26. As can be seen, wild type, spo11-1, and spo13-1 colonies have a stabilized Ura^+ phenotype indicating that at least some of the cells within the colony have had a recombination event with a homologous chromosomal loci. These tests also revealed that none of the rad52-1 cells have stabilized the plasmid. By examining Table 26, it can be seen that greater than 90% of the $Rad52^+$ colonies with a Ura^+ phenotype are stable Ura^+ colonies. However, the overall frequency of Ura^+ cells declines rapidly during meiosis (see discussion).

The Ura^+ frequency decline (Figures 20-22) and the

Table 26 Fraction of Cells With A Stable Ura^{+} Phenotype During Premeiotic and Meiotic Conditions

	Fraction of Stable Ura^{+} Cells							
Genotype	Precul Inoc	Premei Inoc	0 hour	5 hour	10 hour	15 hour	20 hour	25 hour
Wild type	6/77	40/59	119/128	131/149	85/98	105/111	138/146	126/131
(%)	(8)	(68)	(93)	(88)	(87)	(95)	(95)	(96)
spo13-1	33/144	27/35	90/96	116/129	98/102	67/72	115/125	87/94
(%)	(23)	(77)	(94)	(90)	(96)	(93)	(92)	(93)
spo11-1	6/104	85/104	103/104	98/104	100/104	99/104	101/104	103/104
(%)	(6)	(82)	(99)	(94)	(96)	(95)	(97)	(99)
rad52-1	0/104	0/65	0/104	0/104	0/104	0/104	N.D.	0/104
(%)	(0)	(0)	(0)	(0)	(0)	(0)	----	(0)

Preculture was grown in CGAU (synthetic complete medium with 0.2% glucose, 1.0% potassium acetate lacking uracil).

Premeiotic culture was inoculated from the preculture and grown in CUA (like CGAU except it also lacks glucose).

For the wild type and *spo13-1* strains, Ura-drop plates were used to determine the Ura^{+} frequency at a given time point were replica plated 2X to YPD and then once to Ura-drop plates.

For the *spo11-1* and *rad52-1* strains, 104 Ura^{+} colonies from a given time point were picked to YPD and replica plated once to YPD and then to a Ura-drop plate.

actual loss of the plasmid cannot be due to nonrandon segregation of the plasmid during meiosis since the nuclear bodies formed during meiotic divisions remain together as one plating unit. In wild type and spo13-1 cells, which produce viable spores, this means that if all of the plasmid molecules remained in one nuclear body, the result would be that there would still be one plating unit would appear to have the plasmid. Possible explanations are that the plasmid is either being degraded, or the plasmid is being lost outside of the segregating nuclear bodies such that the plasmid resides in the interspore space of the ascus.

Of the strains used only the wild type, spo13-1, and spo13-1 rad50-1 strains produced viable spores. Asci from YRp12 transformants of these strains were dissected on YPD plates and in one case also on URA-drop plates. Two wild type strains were used, CE109 and K65-3D and full four spored asci were dissected. The spo13-1 and spo13-1 rad50-1 (CE110 and CE112) produce two spore asci containing diploid spores. Table 27 summarizes the Ura^+:Ura^- segregation of spores and the results show that 9.7-17.1% of the wild type spores retained the Ura^+ phenotype. This is in good agreement with the results of Kingsman et al. who found no more than 25% of wild type cells retaining the phenotype of the selectable marker of the plasmid they used (67). In addition, the spores of the spo13-1 and spo13-1 rad50-1 strains retained the Ura^+ phenotype to a similar degree

Table 27 Meiotic Segregation of YRp12; Plasmid$^+$:Plasmid$^-$ Segregation

	4:0	3:1	2:2	1:3	0:4	Total +:-
K65-3D	1	0	1	0	4	6:18
CE109	0	0	0	0	4	0:16

	3:0	2:1	1:2	0:3	
K65-3D	0	0	0	3	0:9
CE109	1	0	0	2	3:6

	2:0	1:1	0:2	
K65-3D	0	0	1	0:2
CE109	0	0	3	0:6
CE110	1	3	20	5:43
CE112	3	0	9	6:18
CE112*	3	1	8	7:17

	1:0	0:1	
CE110	0	11	0:11
CE112	0	5	0:5
CE112*	0	4	0:4

	+:-	%+
K65-3D	6:29	17.1
CE109	3:28	9.7
CE110	5:54	8.5
CE112	6:29	17.1
CE112*	7:28	20.0

CE112* was disected on a URA-drop plate

K65-3D is a wild type, homothallic strain carrying the *ura3-1* allele.

CE109 is wild type

CE110 is *spo13-1*

CE112 is *spo13-1* *rad50-1*

as the wild type strains.

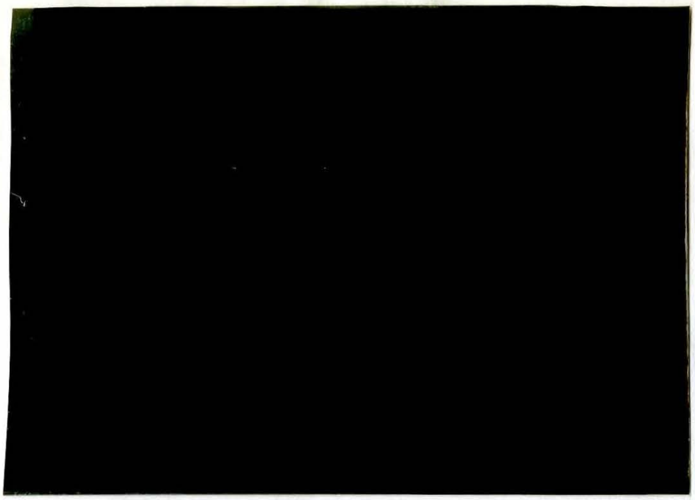
The spores containing plasmids from strain CE112 were replica plated to YPD twice and then to URA-drop plates to determine if the Ura$^+$ phenotype was stable or unstable. All six 2:0 asci from strain CE112 (rad50-1 spo13-1) contained a stable Ura$^+$ phenotype so it seems likely that the 4:0, 3:0, and other 2:0 asci from the other strains also contained the stabilized Ura$^+$ phenotype.

Meiotic DNA Analysis

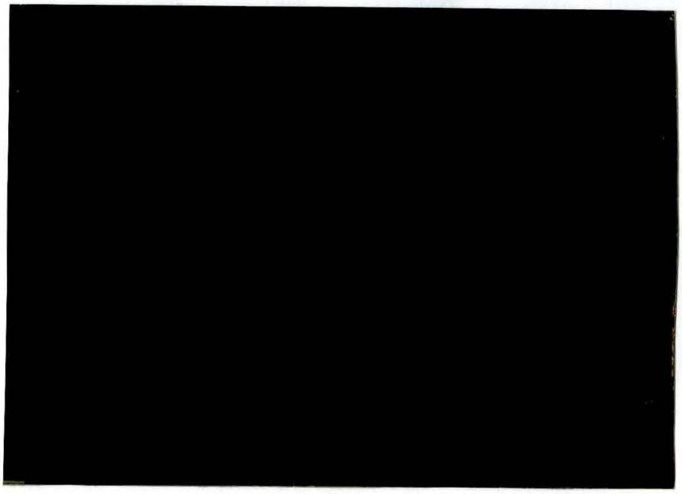
Premeiotic and meiotic DNA was prepared and examined as described. Figure 23 shows a typical ethidium bromide stained gel before and after the transfer of DNA to nitrocellulose. It shows that the DNA was sheared into random size pieces resulting in the smears in the sample wells (lanes 5-20). Pictures of the autoradiograms are presented in Figures 24-27. Figure 24 is a control gel. The gel contains controls for background hybridization between yeast DNA and the pBR322 probe (lane 10), detection of integrated plasmids (lanes 12-18), YRp12 plasmid size (lanes 1-3), and for DNA isolation (lanes 6-9). The pBR32 probe did not hybridize to any yeast sequences (lane 10) but it did hybridize to yeast DNA containing an integrated plasmid and it hybridized to the entire DNA smear as would be expected if the DNA was sheared into random sizes (lanes 12-18). The DNA solation controls (lanes 6-9) were included to determine if

Figure 23 Typical DNA Transfer Control

a)

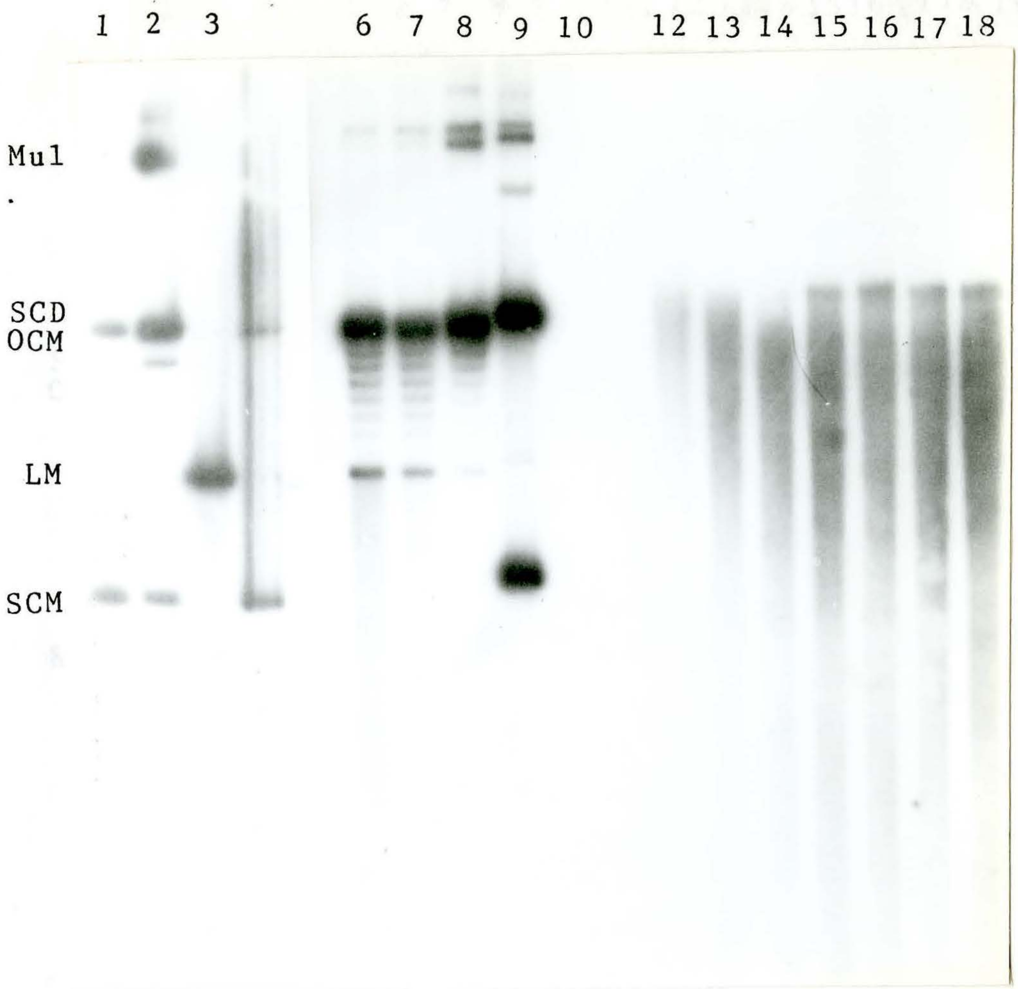


b)



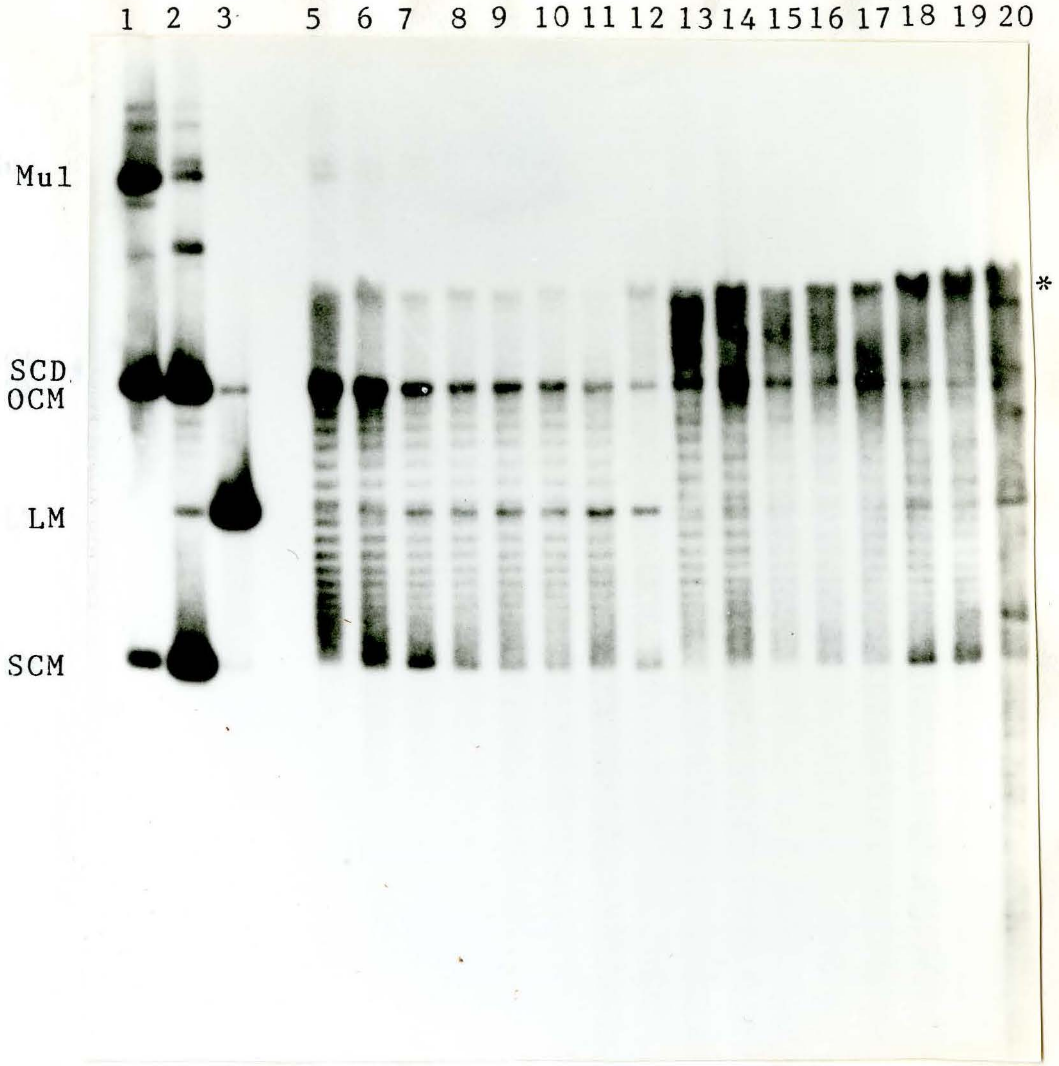
a) After separating DNA samples on 0.7% agarose horizontal slab gels, the gels were stained with a 0.5 ug/ml solution of ethidium bromide and then photographed. The DNA was then transferred from the gel to a nitrocellulose membrane as described.
b) Following transfer, the gel was restained and photographed to check for any untransferred DNA remaining in the gel.

Figure 24 Meiotic DNA Controls



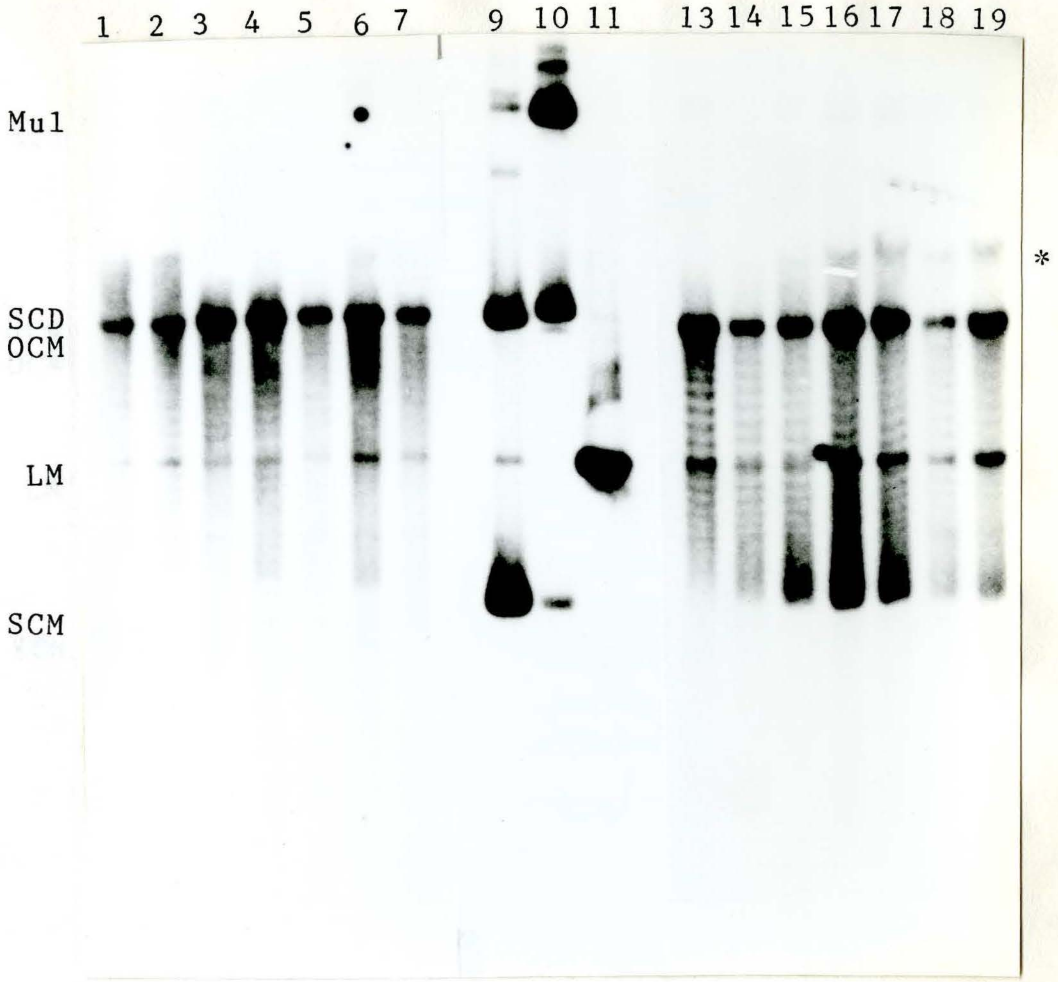
This is an autoradiogram of various control DNAs probed with ^{32}P-pBR322. Lanes 1-3, YRp12 monomer, dimer, and linear respectively. Lanes 6-9 bead beating controls; 6 YRp12 added before beating, 7 YRp12 added during beating, 8 YRp12 added after beating, 9 YRp12 added just prior to loading DNA on gel. Lane 10 is yeast DNA without YRp12. Lanes 12-18 contain meiotic yeast DNA with an integrated YRp12 plasmid. OCM = Open Circle Monomer. SCM = Super Coiled Monomer. SCD = Super Coiled Dimer. Mul = Multimers. LM = Linear Monomer.

Figure 25 Meiotic DNA From Wild Type and spo13-1 Strains



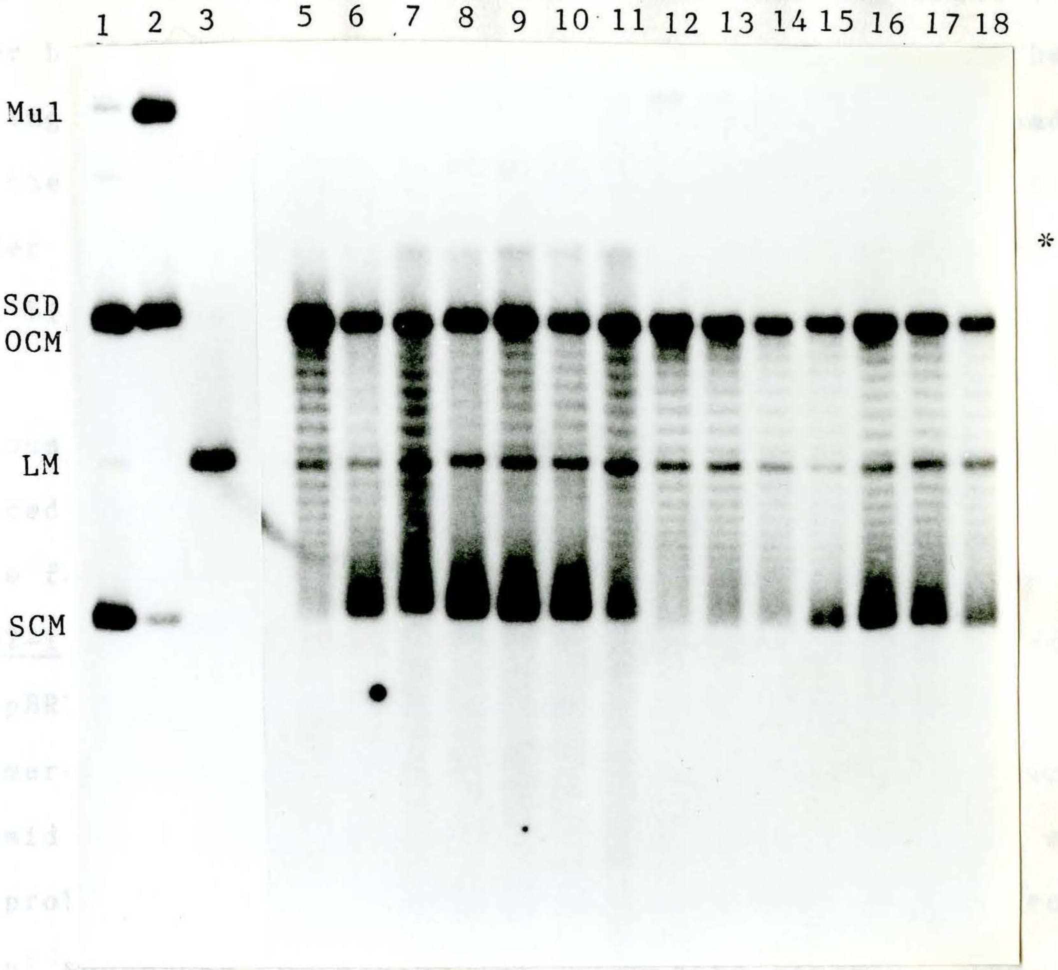
This is an autoradiogram of meiotic DNA from wild type and spo13-1 strains probed with ^{32}P-pBR322. Lanes 1-3 contain YRp12 dimer, monomer, and linear respec- tively. Lanes 5-12 contain spo13-1 DNA from 0, 5, 10, 15, 20, 25, 48 hours into meiosis and stationary mitotic cells. Lanes 13-20 contain wild type DNA form 0, 5, 10, 15, 20, 25, 48 hours into meiosis and stationary mitotic cells. OCM = Open Circle Monomer. SCM = Super Coiled Monomer. SCD = Super Coiled Dimer. Mul = Multimers. LM = Linear Monomer. * = Position of highest molecular weight yeast DNA.

Figure 26 Meiotic DNA From *rad50-1 spo13-1* and *rad50-1* Cells



This is an autoradiogram of meiotic DNA from *rad50-1 spo13-1* and *rad50-1* cells. Lanes 1-7 contains DNA from *rad50-1 spo13-1* cells at 0, 5, 10, 15, 20, 25, and 48 hours into meiosis. Lanes 9-11 have YRp12 monomer, dimer, and linear. Lanes 13-19 contains DNA from *rad50-1* cells at 0, 5, 10, 15, 20, 25, and 48 hours into meiosis. OCM = Open Circle Monomer. SCM = Super Coiled Monomer. SCD = Super Coiled Dimer. Mul = Multimers. LM= Linear Monomer. * = Position of highest molecular weight yeast DNA.

Figure 27 Meiotic DNA From *spoll-1* and *rad52-1* Cells



This is an autoradiogram of meiotic DNA from *spoll-1* and *rad52-1* cells. Lanes 1-3 contains YRp12 monomer, dimer, and linear. Lanes 5-11 contain DNA from *spoll-1* at 0, 5, 10, 15, 20, 25, and 48 hours into meiosis. Lanes 12-19 contains DNA from *rad52-1* cells at 0, 5, 10, 15, 20, 25, and 48 hours into meiosis. OCM = Open Circle Monomer. SCM = Super Coiled Monomer. SCD = Super Coiled Dimer. Mul = Multimers. LM = Linear Monomer. * = Position of highest molecular weight yeast DNA.

there were any artifacts introduced during the disruption of the cells and spores by the bead beating method. Plasmid was added to control cells either before bead beating (lane 6), after half of the bead beating (lane 7), after complete bead beating (lane 8), and to control cell DNA just before loading the sample in the gel. It appears that the supercoiling ladder seen in lanes 6-8 is caused by a yeast cellular enzyme liberated during bead beating.

Figures 25-27 picture the meiotic samples of the various experimental strains. Two items should be readily noticed from these figures. First, all diploids show evidence for integration of the YRp12 plasmid except for the rad52-1 diploid. For all strains except the rad52-1 strain the pBR322 probe hybridizes to areas above the open circle monomer-supercoiled circular dimer plasmid band (these two plasmid forms appeared to comigrate in these procedures) so the probe must be hybridized to high molecular weight chromosomal sequences containing the integrated plasmid. The fact that there is no evidence for plasmid integration in the rad52-1 strain is contrary to the results of Orr-Weaver et al. (94, 96). Second, there is no evidence for formaation of higher order plasmid multimers during meiosis. A possible explanation for this is that multimers are formed but are then efficiently converted back to monomers. This is in accord with the observation of Stinchcomb et al. that Yrp trimers are readily converted to monomers in mitotic cells (124).

In order to fix a limit of detection for stabilized Ura^+ cells, the autoradiograms of the 0, 5, 15, and 25 hour meiotic samples of each strain were analyzed using a densitometer. The densitometer tracings included all of the free plasmid regions as well as the region above the open circle monomer band where any stabilized plasmid signals could be measured without interference from the free plasmid forms. The areas under the tracings correspond to the amount of plasmid sequences in each sample. The free plasmid and stabilized plasmid regions were measured separately and compared (Tables 28 and 29). Table 28 contains the raw data obtained from the densitometry tracings and it is readily seen that only the rad52-1 strain failed to show significant levels of stabilized plasmid sequences. Table 29 contains the ratios of unstable free plasmid to stabilized plasmid and demonstates that the rad52-1 strain has 100 times less stabilized plasmid than the wild type strain and 9-20 times less than the other recombination deficient strains tested (spo11-1, rad50-1, and rad50-1 spo13-1). This supports the contention that The ARS-plasmid YRp12 does not integrate in rad52-1 strains.

Table 28 Relative amount of integrated plasmid and free plasmid sequences in meiotic samples

	Integrated plasmid/Free plasmid			
Genotype	0 hours	5 hours	15 hours	25 hours
Wild type	$\frac{225.2}{332.2}$	$\frac{150.4}{304.2}$	$\frac{111.8}{129.2}$	$\frac{126.2}{217.4}$
spo13-1	$\frac{83.2}{372.4}$	$\frac{72.6}{349.2}$	$\frac{23.0}{218.2}$	$\frac{16.8}{155.6}$
spo11-1	$\frac{33.2}{328.4}$	$\frac{11.3}{253.7}$	$\frac{12.2}{302.6}$	$\frac{9.7}{283.6}$
rad50-1	$\frac{38.6}{323.2}$	$\frac{10.2}{222.6}$	$\frac{31.2}{504.8}$	$\frac{24.0}{184.4}$
rad50-1 spo13-1	$\frac{60.4}{191.6}$	$\frac{82.0}{218.2}$	$\frac{19.2}{376.4}$	$\frac{42.6}{401.8}$
rad52-1	$\frac{<1}{199.6}$	$\frac{<1}{244.8}$	$\frac{<1}{128.0}$	$\frac{<1}{271.5}$

Densitometry tracings were generated for the meiotic samples using the autoradiograms of each strain Figures 25-27). The numbers above reflect the area under the tracing curve corresponding to the integrated or free plasmid regions and the units of each value is cm^2. Since the densitometer had to be zeroed for each individual tracing, the values above can- not be directly compared to each other.

Table 29 Relative frequency of integrated plasmid in meiotic samples

Genotype	0 hr.	5 hrs.	15 hrs.	25 hrs.	Ave. 5-25	$\frac{\text{Ave.}}{\text{Ave. WT}}$
Wild type	0.68	0.50	0.86	0.58	0.61	1.00
spo13-1	0.22	0.21	0.11	0.11	0.13	0.21
spo11-1	0.10	0.044	0.040	0.034	0.039	0.064
rad50-1	0.12	0.046	0.062	0.13	0.066	0.11
50-13	0.32	0.38	0.051	0.11	0.095	0.15
rad52-1	<0.005	<0.0041	<0.0078	<0.0037	<0.0046	<0.0076

The relative frequency of integrated plasmid was determined using the following equation:

$$\text{Freq. of integrated plasmid} = \frac{\text{Area of integrated plasmid region}}{\text{Area of free plasmid region}}.$$

The frequency values for the rad52-1 strain are maximum values since the area of integrated plasmid in each case is less than 1.

DISCUSSION

The experiments in this chapter have examined the effects of recombination and meiosis deficient mutations on the mitotic and meiotic stability of a YRp plasmid. The results indicate that the plasmid YRp12 is not stable in mitotic cells under nonselective conditions, and that the plasmid's selectable phenotype (Ura^+) can be stabilized through an integration or gene conversion event with the homologous chromosomal region under selective conditions. Also, stable Ura^+ cells contribute more cells to the culture's growth rate under selective conditions leading to an increase in the frequency of cells with plasmid. This is because cells with the unstable free plasmid frequently bud off daughter cells devoid of plasmid (and are Ura^-). These cells do not add to the culture's growth rate, but stable Ura^+ cells should always bud off Ura^+ daughter cells and these cells will contribute to the culture's growth rate. In a culture containing stable and unstable Ura^+ cells, subsequent stablization events add to this effect.

Until proven otherwise, it should be assumed that the rate of integration is constant for nonselective and selective conditions. Under nonselective conditions, the proportion of cells with free plasmid declines for two reasons. First, stable Ura$^+$ cells produce stable Ura$^+$ daughters; unstable Ura$^+$ cells frequently bud off Ura$^-$ cells. Second, some cells with free plasmid undergo a stabilization event. For these reasons, even under nonselective conditions, the proportion of cells with free plasmid should decline and the fraction of cells with a stabilized plasmid should increase. This can be easily tested, in the future, by sampling cultures throughout mitotic growth in nonselective medium.

It is very important to note that the experiments performed here did not show any physical evidence for plasmid integration in rad52-1 cells. Only the indirect evidence that the Ura$^+$ frequency increased slightly in rad52-1 cells in mitosis under selective conditions (Table 24) suggests that some stabilization was taking place. This should have been detected with the hybridization procedures if the stabilization was the result of an integration event. Densitometer tracings of the autoradiograms derived from the hybridization procedures indicate that the rad52-1 strain contained 10-100 times less integrated plasmid than any of the other strains examined (Table 29). Although it is most likely that the failure to detect integration is due to the lack of recombination functions, other explanations are possible. One such explanation is that the YRp12 plasmid is more stable in a cell devoid of mitotic recombination. This seems unlikely since the rad52-1 strain exhibited a higher rate of plasmid loss in mitosis than the wild type strain (Table 23). Also, the error in the measurement of Ura$^+$ frequency under selective conditions and during meiosis were large since the experiments were complex and could only be performed once, and this could be responsible for the observed increase in frequency. Ideally, more selective growth experiments with rad52-1 should be performed in the future.

The meiotic instability of YRp plasmids (Figures 20-22 and Table 25) may be due to a meiotic process that actually works to rid the developing nuclear bodies of the plasmid. This 'active' loss may occur by the sequestering of the plasmid molecules outside of the nuclear bodies but within the interspore region of the ascus. Plasmids could then still be detected by hybridization since they would be liberated from the ascus by the bead beating procedure. Another possible mechanism for plasmid loss involves the degradation of the molecules by cellular enzymes. The presence of the linear form of YRp12 in meiotic cells may support this idea unless they were formed as a direct result of the bead beating procedure. The bead beating procedure was employed because of the resistance of the spore wall to enzymatic degradation.

Although the overall meiotic Ura$^+$ frequency is decreasing as meiosis progresses (Figures 20-22 and Table 25) the proportion of stable Ura$^+$ cells remains fairly constant with a range of 88% to 96% for wild type, 90% to 96% for the spo13-1 strain, and 94% to 99% for the spo11-1 strain (Table 26). This suggests that unstable and stable Ura$^+$ cells are losing the plasmid with equal probability. It is presumed that the unstable Ura$^+$ cells become Ura$^-$ by the loss of the plasmid; it is not known how the stable Ura$^+$ cells become Ura$^-$. It is hard to understand how an integrated plasmid could excise at such high frequencies. Furthermore once excised, the cell should become an unstable Ura$^-$. In order for a stable Ura$^+$ cell to lose the plasmid it first must become a cell with the unstable free plasmid; the stably incorporated URA3 gene from the plasmid must be excised from its chromosomal location. Once excised, the free plasmid could be 'actively' lost as discussed above.

The question of whether plasmids are recombinationally active in meiosis has never been addressed. If the stabily incorporated URA3 plasmid gene can be excised from its chromosomal site, then free plasmids should also be able to recombine with the chromosome as well as with themselves. However, there was no evidence of plasmid multimer formation. Plasmids may not be able to initiate recombination between themselves. This indicates there are either specific recombination initiation sites or initiation structures. If this is the case then if an initiation site can be cloned into a YRp plasmid, multimeric plasmid forms may be formed. The putative hot spots for yeast recombination are candidates for recombination initiation sites and could test the prediction. The HIS2 region of yeast is such a hotspot and has been cloned into a YRp plasmid (R. Malone, personal communication). Preliminary results using this HIS2-containing plasmid indicate that multimers can be formed in meiosis if a recombination initiation site (hotspot) is present (data not shown).

These experiments have shown that YRp plasmid systems have some problems that must be solved before they can be used as models for meiotic recombination. Improvements in spore DNA isolation will remove a major problem concerning the artifacts the bead beating procedure may be introducing. Once this problem is solved other problems such as the location of the unstable free plasmid within the ascus, the mechanics of active plasmid loss during meiosis, and plasmid recombination may become more clear.

Clearly, the results of this investigation show that there is no evidence that YRp plasmids can integrate into homologous chromosomal regions in rad52-1 strains although it has been reported that YIp plasmid can integrate in rad52-1 strains (94, 96). Also there is evidence that selective growth conditions select for cells with the plasmid selectable marker stabily incorporated in the chromosome in recombination proficient strains. Finally, YRp plasmids are unstable in meiosis but the mechanism behind the loss is unclear.

CHAPTER V

CONCLUSIONS

The results of the experiments within this thesis were designed to shed more light on the mechanisms of yeast recombination. Because the RAD52 gene is so centrally involved in recombination and recombination repair, it served as the focal point for all of the experiments (41, 51, 95). Specifically, the question asked in Chapter II was whether or not viable recombinants could be recovered from rad52 meiotic cells using a return to mitotic growth procedure similar to that of Sherman and Roman (116). The experiments of Chapter III stemmed from an observation of Chapter II that rad52-2 diploids appeared to allow significant levels of spontaneous mitotic recombination unlike rad52-1 diploids. Chapter IV explored the possibility of using replicating plasmids as part of a model system for analyzing meiotic recombination in strains containing mutations affecting recombination or meiosis. A brief description of the results of each chapter as well as discussions dealing with their significance follows.

The mitotic and meiotic recombination deficient mutations rad52-1 and rad52-2 were analyzed and it was shown that some meiotic recombination can be initiated and viable recombinants can be recovered by using a return to mitotic growth. This was contradictory to previously published results (44, 101). It is also very strong evidence that the RAD52 gene product is required late in meiotic recombination. However, the overall level of recombination in rad52 mutant strains is still very low when compared to wild type strains. Because RAD52 is required for the repair of double-strand breaks (41) and because gapped and linear plasmids cannot integrate in rad52-1 cells, the highly reduced levels of meiotic recombination in these cells has led to speculation that meiotic recombination occurs through a double-strand break mechanism (131). Two hypotheses can be generated to account for the experimental observations. The low levels of meiotic recombination observed may be the result of a secondary, Meselson and Radding type mechanism for recombination which occurs in addition to or instead of the double-strand break pathway in rad52 strains. Alternatively, it may simply reflect the mitotic resolution of events initiated in meiosis by a double-strand break pathway.

The Meselson and Radding model for recombination and the double-strand break repair model have many features in common (86, 131). Initiation in the Meselson and Radding model requires a single-strand nick and the DSB model requires a double-strand break. However, there is no a priori reason that the DSB system cannot be initiated by creating a double-strand break at an existing single-strand nick. Both models require strand invasion followed by DNA polymerase mediated strand displacement. Both models allow for branch migration, heteroduplex formation, Holliday structure formation, endonucleolytic cleavage for resolution, and mismatch repair could occur through the same mechanism in each model. The two models can use many of the same enzymes so they could conceivably be able to coexist within the same organism. This hypothesis should be amenable to incorporating the results presented in this thesis and it is.

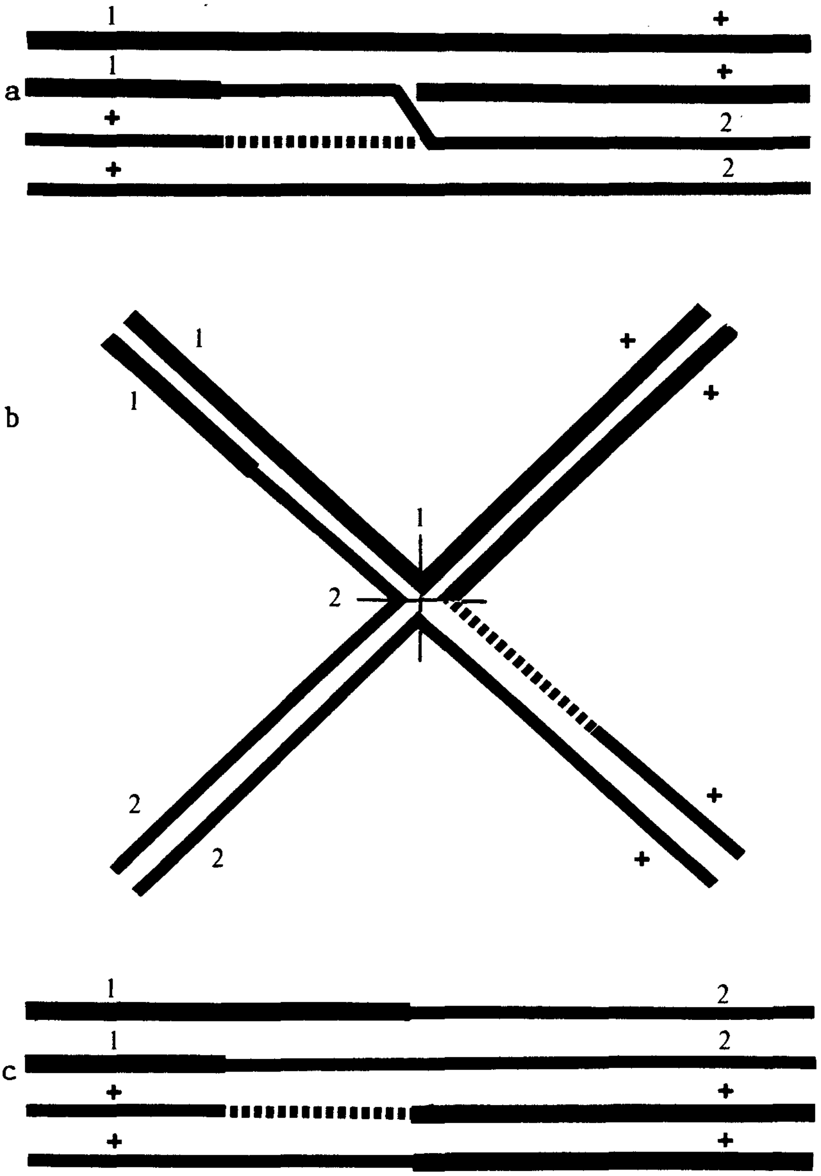
Meiotic recombination is initiated by a single strand nick that is usually recognized by a single-strand endonuclease that creates the double-strand break necessary for DSB recombination. Chow and Resnick have evidence that such an enzyme is absent in rad52-1 cells (20) and in its absence then recombination can occur through the Meselson and Radding model. From this point on recombination follows either model up until the point of mismatch repair. Recently, Hastings has proposed that mismatch repair can occur by the DSB model for recombination or by an excision repair mechanism (50). If this is the case then mismatch repair cannot occur by the DSB pathway in rad52-1 cells. Unless the excision repair pathway can compensate for the loss of the DSB system in mismatch repair, there should be a decrease in the level of gene conversion events in both mitosis and meiosis in rad52 cells. This is because gene conversion requires mismatch repair to be detected as recombinants in the Meselson and Radding model of recombination (86). However, certain reciprocal crossover events do not require mismatch repair to produce detectable recombinants (Figure 28). Therefore, by eliminating the DSB pathway by using rad52 cells, gene conversion events could be more effected than reciprocal crossover events.

There is some indirect evidence that this may be true. First, there are many types of reciprocal recombination that are RAD52 independent (95). Second, Resnick et al. have demonstrated that the recombinants formed in meiosis in rad52 cells are incomplete and disappear in nonselective mitotic medium. Their disappearance can be explained in the context of Meselson and Radding recombination. If strand invasion and branch migration establish the position of the cross strand as being between the two opposing heteroalleles then the possibility arises that a wild type sense strand capable of transcribing a functional gene product could exist (Figure 28). It is then necessary to postulate that by plating a cell with this recombination intermediate on medium requiring transcription through the intermediate, the intermediate is locked in position until it is resolved. In nonselective medium further branch migration may eliminate the wild type sense strand and the recombinant would disappear. This type of recombination does not require

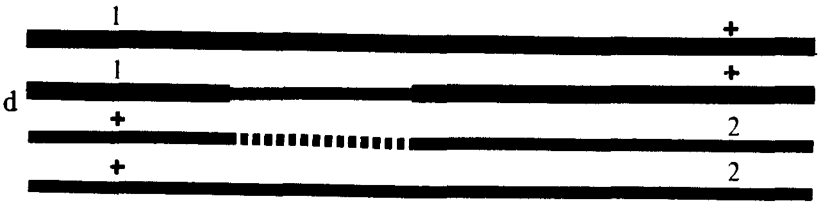
Figure 28 The Production of Prototrophs Through a Reciprocal Crossover Event Between Two Heteroalleles

In diploids carrying the rad52-1 mutation and hence double-strand-break recombination, recombination can be initiated through a Meselson and Radding mechanism in a region of DNA between two heterolleles, 1 and 2 as shown in a). Isomerization of the recombination intermediate produces the strucure in b). Cleavage of this structure in orientation 1 yields the reciprocal crossover configuration in c) which has a wild type duplex and a doubly mutant duplex containing both heteroalleles. Cleavage of the structure in b) in orientation 2 yields the nonreciprocal exchange configuration in d) which has the parental arrangemant of heteroalleles.

Figure 28 The Production of Prototrophs Through a Reciprocal Crossover Event Between Two Heteroalleles


a
1
+
1
+
+
2
+
2
b
1
1
+
+
1
2
2
2
+
+
c
1
2
1
2
+
+
+
+

Reciprocal Crossover



Non-Reciprocal Crossover

double-strand breaks and should be *RAD52* independent.

This hypothesis does not, however, account for the observed meiotic lethality of *rad52* cells. Resnick et al have found that *rad52-1* strains accumulate single-strand breaks in meiotic cells and no double-strand breaks are observed (105). Also, the frequency of these breaks corresponds with the number of recombinational events in meiosis. All of these single-strand breaks could initiate recombination in a Meselson and Radding fashion nonenzymatically (86). It then must be assumed that in the absence of the *RAD52* gene product, and the DSB pathway, there are insufficient levels of recombination enzymes to resolve all of the recombination intermediates through the Meselson and Radding recombination model and the chromosomes remain intertwined. Subsequent meiotic divisions would then create aneuploid spores which would be inviable (3).

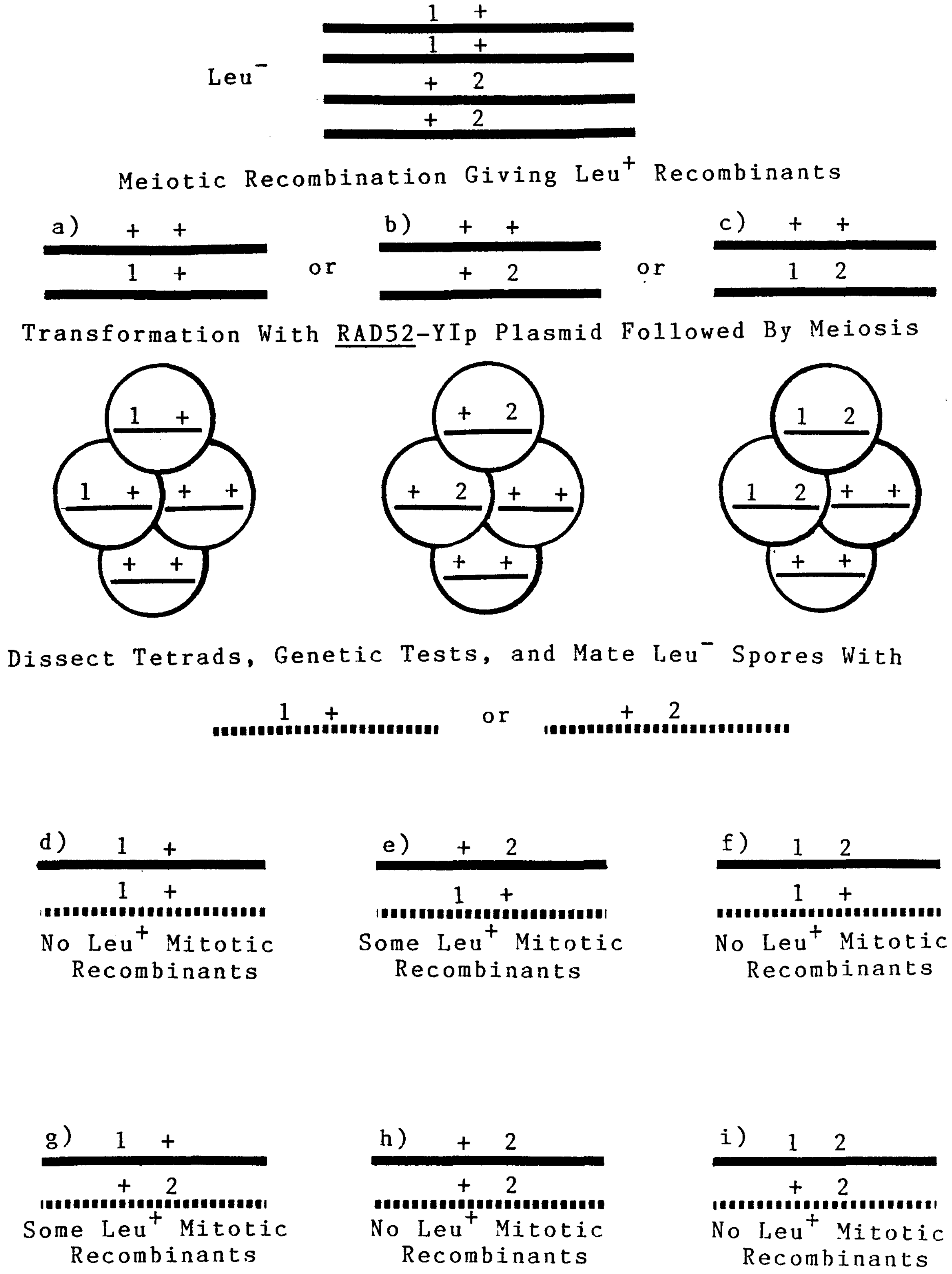
As previously mentioned, *rad52-1* cells lack a single-strand endonuclease, but the sequence of the *RAD52* gene is not long enough to code for the nuclease indicating that it is under the control of the *RAD52* gene (1, 105). This endonuclease could be responsible for the creation of the double-strand break necessary for DSB recombination by acting on the single-strand breaks that are present even in the absence of *RAD52* (20, 131). Therefore, by postulating that the *RAD52* gene is responsible for the control of DSB recombination initiation allows for the presence of two competing recombination mechanisms as well as the meiotic phenotypes of rad52-1 cells.

A testable prediction is produced by the hypothesis presented above. If the incomplete recombination observed in meiosis in rad52-1 cells is due to Meselson and Radding crossing over then both heteroalleles would be present on one of the chromatids involved (86, 109). By following a modification of the procedure of Roman (109) it should be possible to detect this chromatid. The rad52-1 meiotic recombinants produced in Chapter II are diploid in nature but they cannot be sporulated to analyze the genetic configuration of the chromatids. This problem can be alleviated if the recombinants are transformed with a RAD52 containing integrating plasmid and then by selecting for Mms^r transformants. These transformants are capable of undergoing a successful meiosis and will produce viable haploid spores. Figure 29 outlines this experiment. If the original recombinant had been produced by a crossover event between the two heteralleles, of the four spores produced by the RAD52 transformant two spores would contain one or the other heteroallele, one spore would be wild type and one spore would be doubly mutant for both heteroalles. If the recombinant had been formed by gene conversion and mismatch repair, no doubly mutant spore would be produced (109). Therefore, the presence of a spore containing both heteroalleles is indicative of a crossover produced recombinant (109). In previous

Figure 29 Detection of Doubly Mutant DNA Duplexes

After meiotic recombinants have been detected in a rad52-1 strain using a return to mitotic growth procedure (116 and Chapter II) the chromosomes involved segregate in one of three ways if the recombination event was a reciprocal crossover between the heteroalleles a), b), and c). These recombinants are then transformed with a RAD52 containing YIp plasmid which gives the cells the ability to successfully proceed through meiosis. The spores produced in meiosis are dissected, grown and then mated with strains containing only one of the two heteroalleles involved in the original meiotic recombination event. These diploids are allowed to grow mitotically d), e), f), g), h), and i). If the diploid has a duplex containing both heteroalleles as the result of a meiotic crossover event between the heteroalleles, it cannot recombine with its homologue from the tester strain so as to form prototrophic recombinants f) and i).

Figure 29 Detection of Doubly Mutant DNA Duplexes


1 +
1 +
Leu⁻
+ 2
+ 2
Meiotic Recombination Giving Leu⁺ Recombinants
a) + +
1 +
or
b) + +
+ 2
or
c) + +
1 2
Transformation With RAD52-YIp Plasmid Followed By Meiosis
1 +
1 +
+ +
+ +
+ 2
+ 2
+ +
+ +
1 2
1 2
+ +
+ +
Dissect Tetrads, Genetic Tests, and Mate Leu⁻ Spores With
1 +
or
+ 2
d) 1 +
1 +
No Leu⁺ Mitotic Recombinants
e) + 2
1 +
Some Leu⁺ Mitotic Recombinants
f) 1 2
1 +
No Leu⁺ Mitotic Recombinants
g) 1 +
+ 2
Some Leu⁺ Mitotic Recombinants
h) + 2
+ 2
No Leu⁺ Mitotic Recombinants
i) 1 2
+ 2
No Leu⁺ Mitotic Recombinants

experiments using wild type cells virtually all meiotic and mitotic recombination between heteroalleles that produced a wild type recombinant was due to gene conversion and not crossing over (109). If the proposed hypothesis is correct then the opposite should be the case.

It was demonstrated in Chapter III that the rad52-2 mutation is not as detrimental to yeast cells as the rad52-1 mutation. Spontaneous mitotic recombination is not affected by the rad52-2 mutation and other processes affected by the rad52-1 mutation are less affected by the rad52-2 mutation. This suggests that these different processes require different levels of RAD52 activity. In fact, the various tested processes can be ranked in order of their RAD52 activity requirement with spontaneous mitotic recombination < UV resistance < interconversion of mating type < MMS resistance and meiotic recombination. The effects of the rad52-2 mutation can be explained by accepting the hypothesis that it is a leaky mutation and its gene product retains some fraction of the wild type gene product's activity.

The experiments of Chapter IV used a replicating plasmid to analyze recombination and meiosis deficient mutations. As in Chapter II some of the results were contradictory to published reports. First, the plasmid loss rates for YRp12 were lower than those published for another replicating plasmid, YRp7 (8). And secondly, there was absolutely no evidence supporting the observations of Orr-Weaver et al. that circular YRp plasmids can integrate in rad52-1 cells (94, 96). Densitometry tracings of the autoradiograms of the meiotic DNA from each transformed strain revealed that integrated, or stabilized, plasmids could be detected easily in all strains except the rad52-1 strain. There is at least 100 times more stabilized plasmid in the wild type cell and up to 20 times more in the other recombination deficeint strains than there is in the rad52-1 strain. It is possible that because only one rad52-1 transformant was physically examined that plasmid integration could occur in another transformant. However, the strong selection pressure exerted upon the selectively growing rad52-1 transformant should have been sufficient to find some physical evidence for plasmid integration. In light of the phenotypic differences between rad52-1 and rad52-2 it would be interesting to examine the effects of rad52-2 on plasmid stability.

One possible reason why there was no evidence for plasmid integration in rad52-1 cells stems from the presence of the ARS sequence itself. Its presence eliminates the need for the plasmid to integrate into the chromosome in order to be replicated and maintained in the transformed cell. Orr-Weaver et al. claim that integrating plasmids (YIp) can integrate in rad52-1 cells (96). This RAD52-independent recombination event is probably detected only because of the tremendous selection pressure of YIp transformations. Although this selection pressure is absent in YRp transformations, growth of the transformant in selective medium also selects for integration events as was shown in Chapter IV. The fact that no stabilized YRp sequences were detected in _rad52-1_ cells suggests that this later selection pressure is much less than the former selection pressure for integration.

If ARS regions really are yeast origins of replication then a necessary consequence is the production of single-strand nicks. Some of these nicks may not be closed because they have a specific sequence or position and serve as a focus for chromosomal pairing and recombination. These would be called recombination initiation sites and might also be recombination hot spots. However, if ARS sequences do lead to recombination initiation sites there should have been some evidence for plasmid-plasmid recombination in wild type cells. There was no evidence for the ability of YRp12 plasmids to form higher order multimers. This indicates that hot spots can be far removed from ARS sites and there are no hot spots present on the YRp12 plasmid. On the other hand, Stinchcomb et al. showed that YRp7 trimers were readily converted to monomers in wild type cells (124) and this could prevent detection of any multimers that might be formed. Preliminary experiments performed with a plasmid containing a putative hotspot that is associated with an ARS indicates that some multimers can be formed again suggesting that the YRp12 plasmid does not contain a recombination initiation site (data not shown). Finally, if ARSs do lead to single-strand breaks that can be processed into double-strand breaks for recombination by *RAD52*, one would expect single-strand breaks to accumulate in *rad52-1* cells during meiosis and their frequency would correlate with the number of recombination events in meiosis. This is exactly what Resnick et al. found to be the case (104).

In conclusion, this thesis has shown that a) meiotic recombination can be initiated in $Rad52^-$ cells; b) spontaneous mitotic recombination appears to require the least amount of *RAD52* activity of other *RAD52* dependent events; c) YRp plasmids are unstable in mitosis and meiosis in all strains examined, and the loss of plasmid in meiosis may be an active loss; and d) YRp plasmids do not integrate in *rad52-1* cells but will in the other strains tested. This thesis has also raised questions concerning the possibility of there being two recombination pathways present in yeast meiotic cells, and if ARSs may be indirectly linked to recombination through its role in replication. And some experiments have been proposed that can test certain predictions derived from these experiments and hypotheses.

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The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

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