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A

Recombinational Repair of Double-Strand Breaks in the Yeast *Saccharomyces cerevisiae*. The role of *RAD51*, *RAD54*, *RAD55*, and *RAD57*.

by

Louis Roccanova

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1999

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Abstract

Recombinational Repair of Double Strand Breaks in the Yeast *Saccharomyces cerevisiae*.

The role of *RAD51*, *RAD54*, *RAD55*, and *RAD57*.

by

Louis Roccanova

Advisor: Professor Wilma Saffran

Double-strand breaks in chromosomal DNA are lethal to cells. They are repaired primarily by homologous recombination in the yeast *Saccharomyces cerevisiae*. The double-strand break repair model has been proposed as a mechanism for this repair (Szostak et. al. 1983 Cell 33, 26-36). Many of the proteins involved are proposed to make up a multimer referred to as the 'recombinosome'. Strains mutated in genes of the *RAD52* epistasis group have been shown to be sensitive to double-strand damage. Due to various interactions with each other, their products are proposed to be part of the recombinosome.

This is a study of double-strand break repair of plasmids in the yeast *Saccharomyces cerevisiae*. We have analyzed double-strand break induced gene conversion and crossover recombination between plasmids and chromosomes. Experiments were conducted in wild type and recombinational repair deficient strains, *rad51*, *rad54*, *rad55*, and *rad57*.

Plasmids were constructed which carry the *TRP1* gene, and one of five nonfunctional alleles of the *HIS3* gene. These *his3* genes contain frameshift mutations made by the insertion of a restriction site marker at different locations. The plasmids were linearized and transformed into wild type and recombinational repair deficient strains. Colonies

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containing repaired plasmids were examined for recombination by selective plating and PCR analysis. Double strand breaks were found to induce both gene conversion and plasmid integration. The gene products of *RAD51* and *RAD54* were shown to be essential in recombinational repair while *RAD55* and *RAD57* were shown to play an auxiliary role. In the wild type strain, conversion occurred in both directions from uncut to linearized DNA and from linearized to uncut DNA. Conversion tract lengths were at least 13 base pairs, and as many as 457 base pairs. In repair events in recombinational repair deficient strains, we observed complete marker recovery in the absence of gene conversion. This suggests the existence of a second repair pathway for the repair of double strand breaks. The patterns of gene conversion leading to His^- colonies were consistant with the predictions of the double-strand break repair model.

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Introduction

The genetic material of a cell must be maintained in order for the cell to survive. DNA repair mechanisms evolved in living organisms more than 1.4 billion years ago before the split from prokaryotes to eukaryotes (Purves et. al. 1995). Evidence to support this can be seen in the conserved homology between the RecA protein of the bacteria *Escherichia coli*, proteins of the *RAD52* epistasis group in the yeast *Saccharomyces cerevisiae* (Friedberg et al 1995) and a homologous mammalian *RAD51* gene (Benson et al 1994). The human Rad51 homologue shares 83% homology with the *S. cerevisiae* Rad51 protein and 55% homology to the *E. coli* RecA protein (reviewed in Friedberg et al 1995).

The viability of a cell is in constant jeopardy. Mechanisms of DNA repair are necessary to maintain the fidelity of the genetic material. There are many mechanisms by which genetic material can become damaged. The process of respiration produces oxygen radicals that can react with DNA. Based on urinary excretion of DNA adducts, oxidative damage to DNA is produced at an estimated rate of ten thousand hits per cell per day in human beings (Cathcart et al 1984). This is known as endogenous damage. Oxidants are a major contributing factor to aging and diseases such as cancer (Ames et al 1993). There are a myriad of agents in the environment that can cause exogenous damage. These include chemical and physical mutagens and radiation.

DNA repair

DNA damage can occur in either the single strand form, where damage occurs to only one of the two strands of the double helix or double strand form, where damage occurs to both strands. Damage to both strands can occur as crosslinks or double strand breaks. A

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class of carcinogens known as clastogens causes chromosomal breaks. If breaks are not rejoined properly, loss of portions of chromosomes or rearrangements can occur (reviewed in Wood 1996).

When single strand DNA damage occurs, the cell can excise a portion of the damaged strand and use the undamaged strand of the double helix as a template to repair the damaged strand. This is known as excision repair. In situations when there is damage to both strands, such as a double strand break, the cell must use another molecule of DNA as a template, which possesses a sequence homologous to the damaged molecule for nonmutagenic repair. Recombination events have been shown to be dependent upon homology between two molecules (Silberman & Kupiec, 1994).

Double-strand breaks can also be repaired by a direct DNA end-joining pathway (Lieber et. al. 1997, Troelstra & Jaspers 1994). One possible alternative pathway involves the Ku protein which is also involved in V(D)J recombination in the mammalian immune system (Chu 1996). It may also be involved in the rejoining of broken chromosomal ends for DNA repair. It protects DNA from radiation, and has been identified in both yeast and mammals (Lieber et. al. 1997, Troelstra & Jaspers 1994).

According to the double-strand-break repair model for recombination (Szostak et al 1983), when double strand damage occurs, the damaged site is excised from both strands (figure 1). The 5' end of each double strand is resected to create 3' overhangs (Sun et. al., 1989, 1991; Sugawara & Haber, 1992) approximately six hundred base pairs in length (reviewed in Stahl 1996). These free ends then invade the homologous sequence on another DNA molecule. Products of the *RAD52* epistasis group will align each of the

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single strands on the damaged molecule with its complementary sequence on the undamaged molecule. A DNA polymerase will then synthesize new DNA according to the undamaged template. The two molecules may then separate and the DNA is ligated. This is referred to as gap filling. However, they may not separate at this point. The regions of exchange of alignment between the two molecules are known as Holliday junctions (Holliday 1964, 1968). These junctions may travel down the molecules of DNA and exchange more material between the two molecules. This is referred to as branch migration. It occurs when complementary sequences on each molecule disassociate and then associate with the complementary sequence on the second molecule. Resolvase will then cut the DNA so the strands can separate. This can result in each molecule retaining its sequences flanking the break site or an exchange of flanking sequences between the two molecules. If the sequences of the two strands in heteroduplex regions are not identical, the disparity is known as a mismatch. Mismatches can occur if there is a disparity between the invading single strand and the complementary strand, or if a Holliday junction travels beyond a region of complete homology. A process known as mismatch repair will repair an alteration of the DNA structure due to such a mismatch. The genetic material on one strand will be excised and resynthesized according to the sequence of the opposing strand. This can result in the transfer of genetic information from one strand to the other. The mutation may be lost if the mismatch is resolved to the original sequence, or a permanent mutation may result from the acquired sequence.

Since the original inception of the Double-Strand-Break Repair Model (Szostak et al 1983) modifications have been proposed. These mostly deal with the initial event leading

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to recombination and the final resolution of the Holliday junctions. The two ends of a double strand break may invade a homologous strand independently (Gilbertson and Stahl 1996). This is a reversible invasion, which becomes irreversible with the formation of the joint molecule made up of the two DNA strands (Stahl 1996). If resection of each end of the double strand break is asymmetric, conversion may occur on one side of the double strand break but not the other (reviewed in Stahl 1996). Noncrossover events may occur if resolution of the joint molecule is catalyzed by topoisomerase instead of cleavage (reviewed in Stahl 1996). Holliday junctions may slide to avoid mismatches (reviewed in Stahl 1996). A noncrossover may result if resolvase acts at only one of the two Holliday junctions and it is left unsealed while the other Holliday junction migrates to meet it before the two molecules separate (reviewed in Stahl 1996). Gilbertson & Stahl (1996) have provided evidence that a topoisomerase can resolve the heteroduplex double-strand break repair intermediate. According to the synthesis dependent strand annealing model (SSDA) (Paques et. al. 1998), DNA that is newly synthesized does not remain based paired to the template, but are displaced. This allows them to anneal. The total number of repeats in the recipient site may not have the same number of copies as the donor because alignment of complementary strands that are newly synthesized can occur in different registers.

Double-strand breaks do not only occur by DNA damaging agents. They can also be induced in meiotic recombination (Szostak et. al. 1983).

DNA repair genes, and the functions of their products

The *RAD52* epistasis group is involved in double strand break repair and consists of several known genes, *RAD50-59*, *MRE2*, *MRE11*, *XRS2* and *RFA1* (reviewed in

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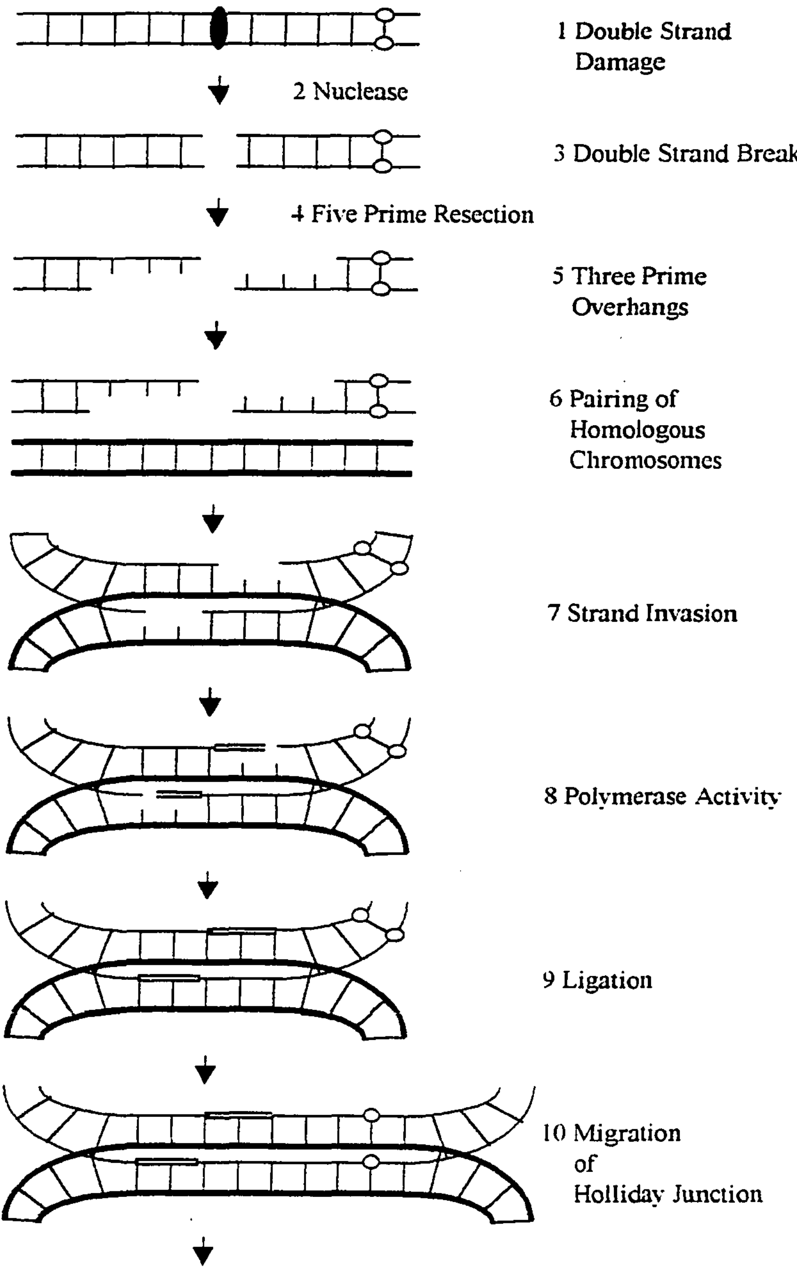
Double-Strand Break Model of Repair



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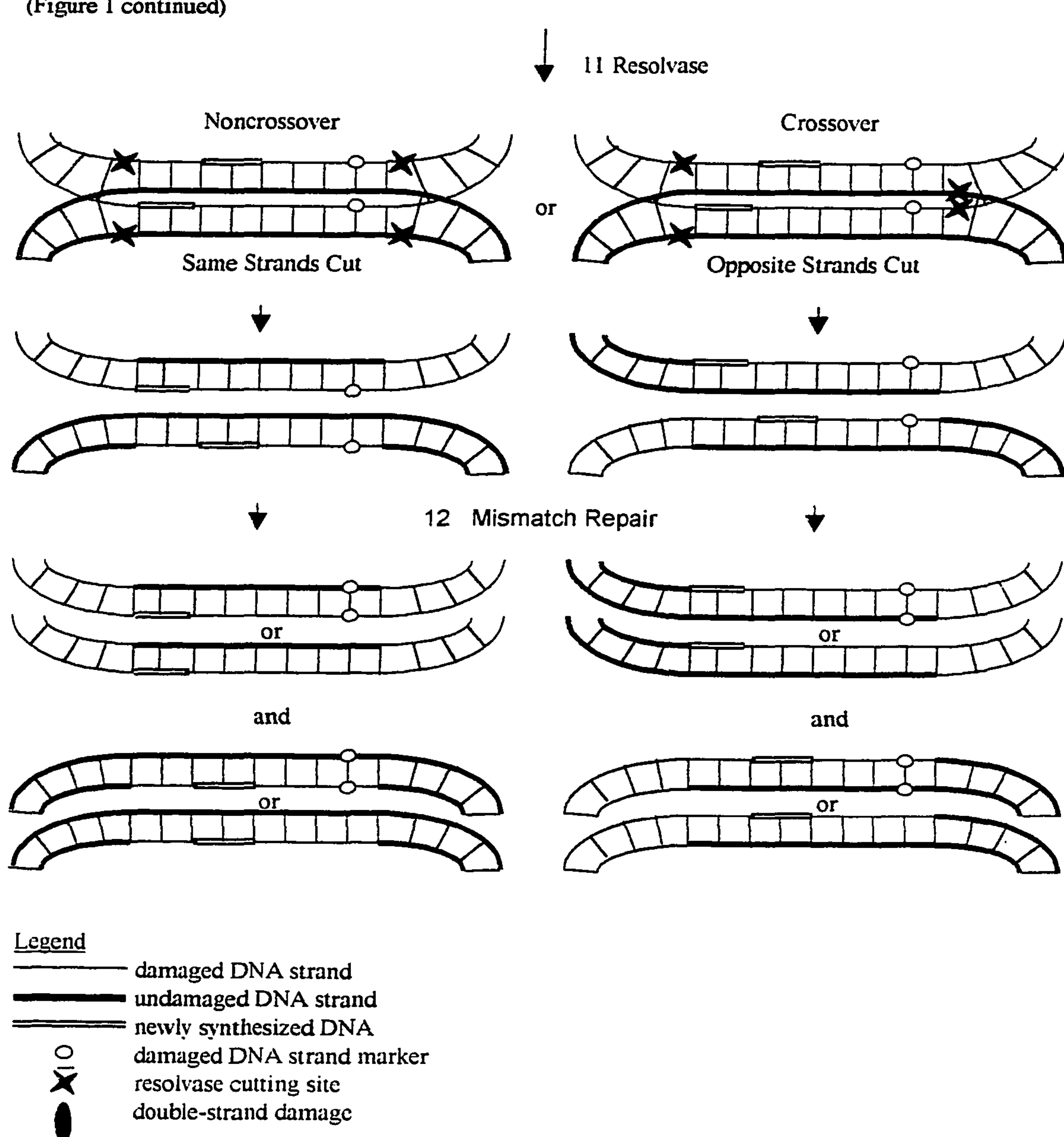
(Figure 1 continued)



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Friedberg 1995 and Wood 1996). *RAD51, RAD52, RAD55, RAD57,* and *RFA1* have been postulated to be part of a recombinosome (figure 2) involved in the repair of double strand breaks (Hays et. al. 1995). *RFA1* encodes the large subunit of a heterotrimer known as replication protein A (RPA) (Firmenich 1995). This is a single strand DNA binding protein that interacts with the *RAD52* gene product (Firmenich 1995, Hays 1998, Shinohara 1998). The *RAD52* gene product is a single strand DNA binding protein that can anneal homologous single strand DNA to a duplex (reviewed in Shinohara 1995, Mortensen 1996, Shinohara 1998). It interacts with the *RAD51* gene product (Donovan et. al. 1994, Hays et. al. 1995), and modulates the catalytic activities of *RAD51* (Shen et al 1996). *RAD51, RAD55* and *RAD57* have sequence homology to RecA (Lovett1994), and *RAD51* is a functional homologue of RecA (reviewed in Friedberg et al 1995). Aided by RPA, *RAD51* can perform homologous pairing and low levels of strand exchange activity (Sung 1994). It forms helical filaments with single strand DNA (Ogawa et al 1993) and interacts with the *RAD55* gene product (Hays et. al. 1995).

The *RAD55* gene product aids the recombinosome by facilitating strand invasion into donor sequences, which would otherwise be inaccessible (Sugawara et al 1995). The *RAD55* gene product also interacts with the *RAD57* gene product and the two may help to stabilize the recombinosome at low temperatures (Hays et. al. 1995). Yeast Rad55 and Rad57 were shown to form a stable heterodimer and stimulate strand exchange when included with Rad51 and RPA (Sung 1997). *RAD54* has homology to helicases (reviewed in Friedberg et al 1995). It has also been shown to be involved in meiotic and mitotic recombination in *Saccharomyces cerevisiae* (reviewed in Friedberg et al 1995). *RAD54*

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has been shown to have a direct interaction with *RAD51* and is postulated to be part of the recombinosome (Jiang et al 1996).

Using genetic, molecular and biochemical criteria, Clever et. al. (1997) demonstrated that the Rad51 and Rad54 proteins interact in vivo. Using the yeast two hybrid system, Rad51 and Rad54 proteins were shown to have a direct association (Jiang, 1996). Human HsRad54 was shown to interact with human Rad51 recombinase expressed in *Escherichia coli* (Golub, 1997). Petukhova et. al. (1998) have shown that the Rad54 protein has double-strand DNA-dependent ATPase activity, interacts with Rad51, and strongly stimulates pairing between homologous single-strand and double-strand molecules of DNA when added to Rad51.

RAD51, *RAD54*, *RAD55*, and *RAD57* are required to facilitate strand invasion during HO endonuclease-induced mating-type (MAT) switching into inaccessible donor sequences (Sugawara et. al., 1995). Hays et. al. (1995) have shown that *RAD51*, *RAD52*, and *RAD54* are essential in recombination repair, while *RAD55* and *RAD57* may act in an accessory role.

The *E. coli* RecA protein performs all these functions by itself, although there is a single strand binding protein that can facilitate filament formation but inhibits the initial nucleation event (reviewed in Friedberg, 1995). Since *RAD51*, *RAD55*, and *RAD57*, all share some homology with RecA (reviewed in Friedberg 1995), these proteins may have evolved from the original RecA protein.

The importance of the study of DNA repair

Continued study of the recombinational DNA repair pathways is important to society a

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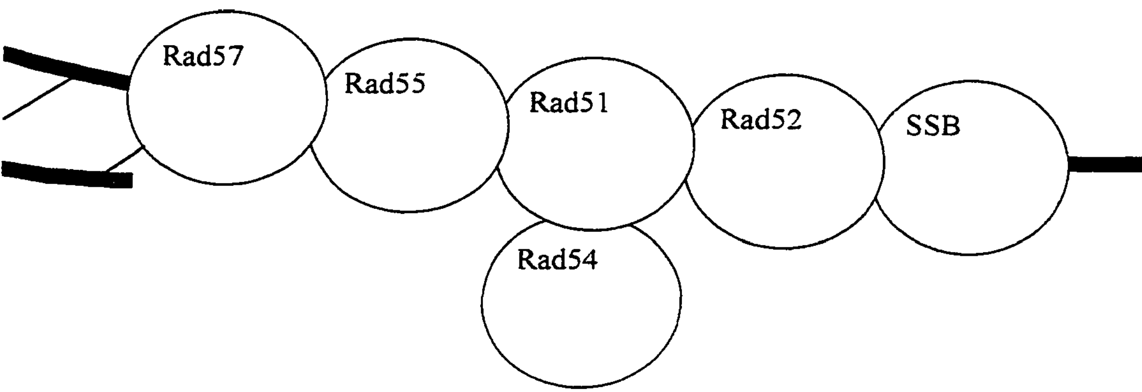
Figure 2
The Recombinosome



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because of its relevance to human disease and aging. The large numbers of mutations commonly reported in human cancers cannot be accounted for by the rate of spontaneous mutation found in human cancer cells. This supports the contention that cancer cells are genetically unstable and possess a mutator phenotype (Loeb & Christians 1996). Abnormal chromosome/DNA repair can produce an unstable genome, which leaves genetic alterations relevant to the development of cancer to occur (Au 1993, Bohr 1995). The hazard of a particular lesion is dependent on whether it is repaired and the probability that it leads to a mutation during cell division (Ames et al 1992). Specific mutations have been connected to a wide range of human cancers, and the accumulation of mutations may be the most significant cause of aging (Ames 1993).

Mismatch repair following double-strand-break recombination can be a critical step in carcinogenesis. In cases of tumor suppressor genes such as retinoblastoma which exhibit recessive loss of function, both alleles of a gene must become altered in order to produce deleterious phenotype (Lasko & Cavenee 1991). If a formally functional, damaged allele is repaired and converted to the sequence of a nonfunctional allele, the cell can become cancerous. This can also occur by loss of heterozygosity by mitotic crossover. Autosomal recessive diseases that are caused by defects in DNA repair processes include xeroderma pigmentosum, Cockayne's syndrome, ataxia telangiectasia, Fanconi's anemia, Bloom's syndrome and trichothiodystrophy (reviewed in Friedberg et al 1995). Postreplicative repair is also a damage tolerance pathway because it can not directly repair damage (Shinohara 1995).

Hereditary nonpolyposis colon cancer accounts for fifteen percent of the 20,000 cases

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of colon cancer that occur in the United States each year (Marx 1994). It has been linked to defects in mismatch repair and transcription coupled repair (Mellon 1996). Of the women who inherit mutated forms of *BRCA1* or *BRCA2* (breast cancer susceptibility genes 1 & 2), up to 80% will develop breast cancer (Marx 1997). *BRCA1* and Rad51 containing complexes interact with damaged, replicating DNA (Scully et. al. 1997a). *BRCA1* and *RAD51* were found to coimmunoprecipitate. Both were associated with developing synaptonemal complexes in meiotic cells. This suggests a role for *BRCA1* in recombination (Scully et. al. 1997b). The *BRCA2* protein binds to the Rad51 repair protein and plays a critical role in enabling cells to repair their damaged DNA (Sharon et al 1997, Scully et al 1997). In in vitro experiments using the yeast two hybrid system, *BRCA2* was confirmed to interact with *RAD51*, and this binding may be a critical mechanism by which *BRCA2* causes suppression of the abnormal proliferation of mammary cells (Katagiri, 1998, Mizuta 1997, Wong 1997). Ionizing radiation was shown to stimulate phosphorylation of Rad51 by *c-abl*. This inhibits the binding of Rad51 to DNA and its role in strand exchange reactions (Yuan, 1998). *c-abl* on chromosome 9 is the proto-oncogene known to cause myelogenous leukemia when joined to the bcr gene on chromosome 22 by translocation. This upregulates the transcription of c-able which causes the proliferation of cells in the bone marrow. Fanconi's anemia is a disease in which there are elevated levels of spontaneous chromosomal breakage and hypersensitivity to DNA crosslinking agents (reviewed in Friedberg et al 1995). Spontaneous chromosomal breaks and rearrangements are characteristic of Bloom's syndrome (reviewed in Friedberg et al 1995).This is the type of damage that is repaired by the *RAD52* epistasis group.

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Ataxia telangiectasia cells have been shown to be defective in circularizing transfected linear plasmids (reviewed in Friedberg et al 1995). There is potential for repair deficiencies to affect humans during early development. Work in the mouse has demonstrated that exposure to environmental mutagens during early developmental stages leads to high frequencies of malformations as well as death (Dellarco 1993). Damage to germ cells during spermatogenesis could lead to mutations in the embryo and defects in the fetus if it is not repaired (Frega et al 1991). The cells that matter the most for cancer are the stem cells, which will not be discarded (Ames et al 1992).

The study of recombinational repair is also relevant to evolution. Leigh (1987) argues that recombination increases the likelihood of simultaneous fixation of new favorable mutations and combines the different advantageous mutations from previous generations into a single offspring. It also increases the genetic variance in the offspring of an individual by producing new gene combinations. An individual who produces more varied offspring is more likely to produce a better competitor for a particular niche and a litter, which contains a variety of offspring to ensure progeny in an environment that is not static. This means that recombination itself is selected for because individuals with greater recombination rates will be better represented in successive generations than those whose recombination rates are lower. Recombination not only increases the spread of favorable mutations, more importantly it can eliminate unfavorable mutations. Recombination also ensures that alleles are selected for based on their contribution to the fitness of an individual and not on linkage to another allele that is favorable. Thus we see high levels of recombination in many populations. Leigh calculates that facilitating the fixation of

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favorable mutations can increase the fitness of a population by up to 0.14 percent per year.

In the following study, we further test the functions of the recombination repair genes *RAD51*, *RAD54*, *RAD55*, and *RAD57*, the Double-strand Break Repair Model and the hypothesized structure of the recombinosome. We studied repair of double-strand breaks placed in plasmids. We measured recombination as gene conversion at various distances from break sites and as crossing over. We did experiments in both repair-proficient and recombinational repair-deficient stains. The *rad51* and *rad54* strains were found to be completely deficient in gene conversion and integration while the *rad55* and *rad57* strains were found to be partially deficient. This supports the hypothesized structure for the recombinosome.

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Methods

Strains

The *Saccharomyces cerevisiae* DNA repair proficient strain WS93 is MAT∝ *leu2-3,112 trp1-1 his3(304x) ade2-1 ura3-1 can1-100* (Donna Luisi, personal communication). It was made by two step gene replacement of strain W303 (Rothstein, 1983) with the *his3(304x)* allele which contains an *XbaI* site at position 304 in the coding region. The DNA recombination repair deficient strains 304-51∝, 304-54∝, 304-55∝,and 304-57∝ are MAT∝ *LEU2 ade2-1 can1-100 leu2-3,112 trp1-1 ura3-1 his3* 304X (Xuefei Shen, personal communication). These strains are isogenic to W303. They are deficient in the *RAD51*, *RAD54*, *RAD55*, and *RAD57* genes respectively. All strains are mutated in the *HIS3* gene by the addition of an *XbaI* restriction site at position 304 in the coding region.

Construction and Treatment of Plasmids

The *TRP1* gene and autonomous replication sequence (ARS) from pUC18-*TRP1* were ligated into the pIBI25 plasmid at an *EcoRI* site in the polylinker. This plasmid was transformed into *E. coli* LL308 cells and a small scale preparation of plasmid DNA (Sambrook et al 1989) was used to screen for plasmids with the insert. A large scale preparation followed (Sambrook et al 1989). These were digested with *BstXI* to check for single insertion. They were then linearized with *BamHI* for further ligation.

The mutant *his3* alleles 75, 207, 304, 395, and 622 were digested from pUC18 *his3* plasmids with *BamHI*. *BamHI* does not cut within the coding region of the *HIS3* gene and cuts only once in the pIBI25 ARS *TRP1* plasmid within the polylinker. The *his3* fragments were electroeluted (Sambrook et al 1989), and ligated into the pIBI25 ARS *TRP1* plasmid at the polylinker. These

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Table 1

Table of Strains

Escherichia coli

Strain	Genotype
LL308	*F lac 2b/306/187X 306F lac ib-pro/(lac pro) SupE thi-bfenalA$^-$ recA$^-$*

Saccharomyces cerevisiae

Strain	Genotype
WS93	MAT∝ *leu2-3,112 trp1-1 his3(304) ade2-1 ura3-1 can1-100*
W303	MAT∝ *leu2-3,112 trp1-1 ade2-1 ura3-1 can1-100*
304-51∝	MAT∝ *leu2-3,112 trp1-1 his3(304) ade2-1 ura3-1 can1-100 rad51::LEU2*
304-54∝	MAT∝ *leu2-3,112 trp1-1 his3(304) ade2-1 ura3-1 can1-100 rad54::LEU2*
304-55∝	MAT∝ *leu2-3,112 trp1-1 his3(304) ade2-1 ura3-1 can1-100 rad55::LEU2*
304-57∝	MAT∝ *leu2-3,112 trp1-1 his3(304) ade2-1 ura3-1 can1-100 rad57::LEU2*

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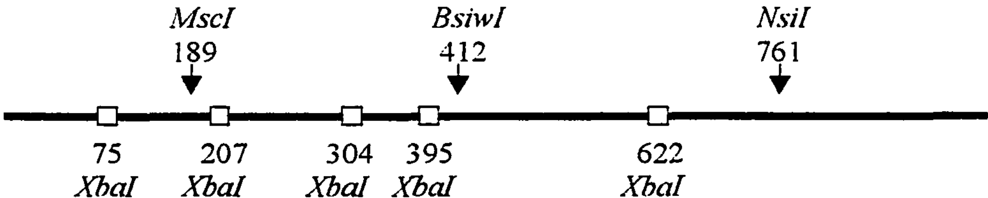
Figure 3
his3 gene

Restriction Site Damage



Restriction Site Markers

Legend
□ *XbaI* restriction site marker

↓ restriction enzyme induced double-strand break cutting site

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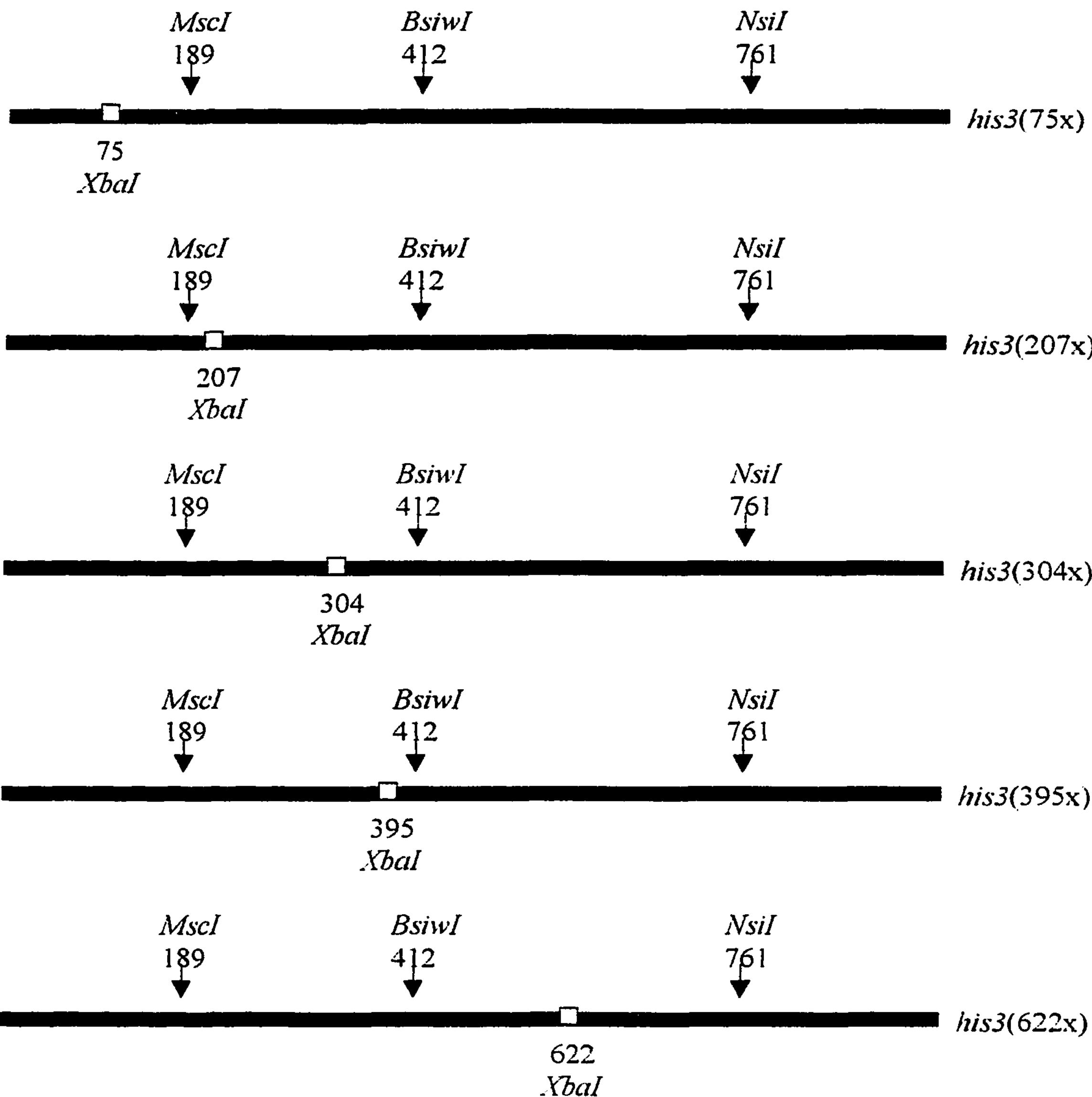
Figure 4
His⁻ Construction with Restriction Site Markers



Legend
□ *XbaI* restriction site marker
↓ restriction enzyme induced double-strand break site

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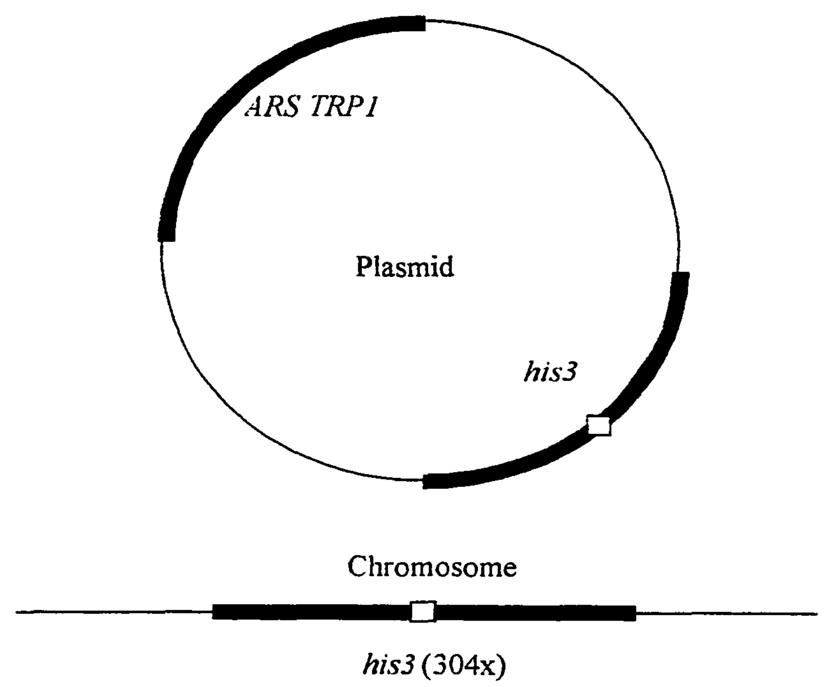
Figure 5
The *his3* , *TRPI* plasmid and the *his3* chromosome



Legend
□ *XbaI* restriction site marker

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were transformed into *E. coli* LL308 cells (Sambrook et al 1989). A small scale DNA plasmid preparation was used to screen for fragment insertion. These were digested with *XbaI* and *XhoI* to check the orientation of the *his3* gene. Large scale preparations again followed.

Each mutant was linearized with *MscI*, *BswiI* and *NsiI* separately. The optical density was measured with a spectrophometer and the samples were run on agarose gel electropheresis to further assess the concentration of DNA.

Yeast Transformation

Yeast culture media were prepared according to the method of Sherman et al (1986). Yeast spheroplasts were prepared from wild type and recombinational repair deficient yeast strains according to a variation of the methods of Beggs (1978) using 1 ug plasmid DNA and no carrier DNA.

Ten milliliter cultures were inoculated and grown in YPD medium at thirty degrees Celsius to an optical density of 0.5 to 1.0. These were then inoculated into three 500 ml flask and grown overnight. For wild type strains, the volumes of 0.10 ml, 0.30 ml and 0.50 ml were used. For recombinational repair deficient strains, the volumes of 0.25 ml, 0.50 ml, 1.00 ml and 2.00 ml were used. These were grown to an optical density of approximately 0.25 at a 600 nanometer wavelength.

The cells were pelleted at 2000 rpm for five minutes in two 50 ml tubes in a clinical centrifuge. The cells in one tube were washed by resuspending and vortexing in ten milliliters of sterile water. They were then combined with the cells in the second tube which were then resuspended by vortexing. They were centrifuged again at 2000 rpm for five minutes. The cells were resuspended by vortexing and incubated for ten minutes at 30 degrees Celsius in 10 ml of 1 M sorbitol, 0.4 ml

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of 0.5 M EDTA(pH 8) and 10 µl of 2-mercaptoethanol. The mercaptoethanol acts as a reducing agent to loosen the outer layer so Glusalase will be more effective. The cells were then centrifuged at 2000 rpm for five minutes and washed twice in 10 ml of 1M sorbitol. They were incubated at 30 degrees Celsius for thirty minutes with slow shaking in 10 ml of sorbitol-citrate-EDTA containing 100 µl Glusulase. Spheroplasting was confirmed by adding 50 µl of cells to 0.1 ml of 5% SDS-0.5M sorbitol in a glass test tube. A clear suspension confirmed that spheroplasting was complete. If the suspension remained turbid, incubation with Glusalase was continued for another thirty minutes. When spheroplasting was complete, the cells were centrifuged for five minutes at 1500 rpm. They were then resuspended gently with a pipet and washed three times in 10 ml of 1 M sorbitol. After washing, the pellet was suspended in 4 ml of 1 M sorbitol. 0.4 ml of 0.1 M Tris-0.1 M calcium chloride was then added, and the suspension was kept on ice. 0.2 ml of spheroplast solution was added to 1 ug of DNA for twenty samples in 17 x 100 mm polyethelene culture tubes. They were mixed gently and incubated at room temperature for 15 minutes. 2 ml of 20% PEG-10 mM $CaCl_2$-10 mM Tris was added and the mixtures were incubated at room temperature for another twenty minutes. They were then centrufuged at 1500 rpm for 10 minutes.

Speroplasts were suspended in 0.5 ml of SOS. 250 µl were added to 10 ml of SD-his top agar and plated onto SD-his + sorbitol media and 5 µl were added to 10 ml of SD-trp top agar and plated onto SD-trp + sorbitol media.

Analysis of Transformants

Phenotypic analysis was performed by transferring transformed colonies to SD-trp (Sherman et al 1986) plates to check for transformation and repair. They were replicated to -His plates to

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check for gene conversion to His$^-$. They were replica plated on YPD nonselective plates two times to dilute the plasmid. They were then replated on -Trp plates to check for integration, and on -His plates to check for integration accompanied by gene conversion.

Colonies were prepared for polymerase chain reaction (PCR) analysis by incubation in a solution of 2.5 mg/ml zymolase in 1.2M sorbitol and 100mM sodium phosphate (pH 7.4) (Ling et al 1995) for fifteen minutes, This was followed by vortexing for ten minutes with glass beads, centrifugation for ten minutes, and extraction in an equal volume of phenol/chloroform.

PCR analysis was performed using the Perkin Elmer DNA Thermal Cycler 480. Reactions were performed in 2.5 mM magnesium, 200 uM each dNTP, 0.25 uM of each primer (a: 5'TCC ACC TAG CGG ATG ACT CT 3', b: 3'CAC CAC TAT CCA CCG TTC AC 5'), and 10 ng template DNA. Amplification was performed using two cycles. Reaction mixtures were denatured at 94 degrees Celsius for one minute. Primer annealing and extension was achieved in a single step at 60 degrees Celsius for two minutes. Segments were not extended, but remained fixed. This was done for thirty cycles.

Nucleotides were purchased from Pharmacia and Perkin Elmer. Taq polymerase was purchased from Perkin Elmer and Promega. Restriction endonucleases were purchased from New England Biolabs and Gibco BRL. Primers were purchased from Life Technologies.

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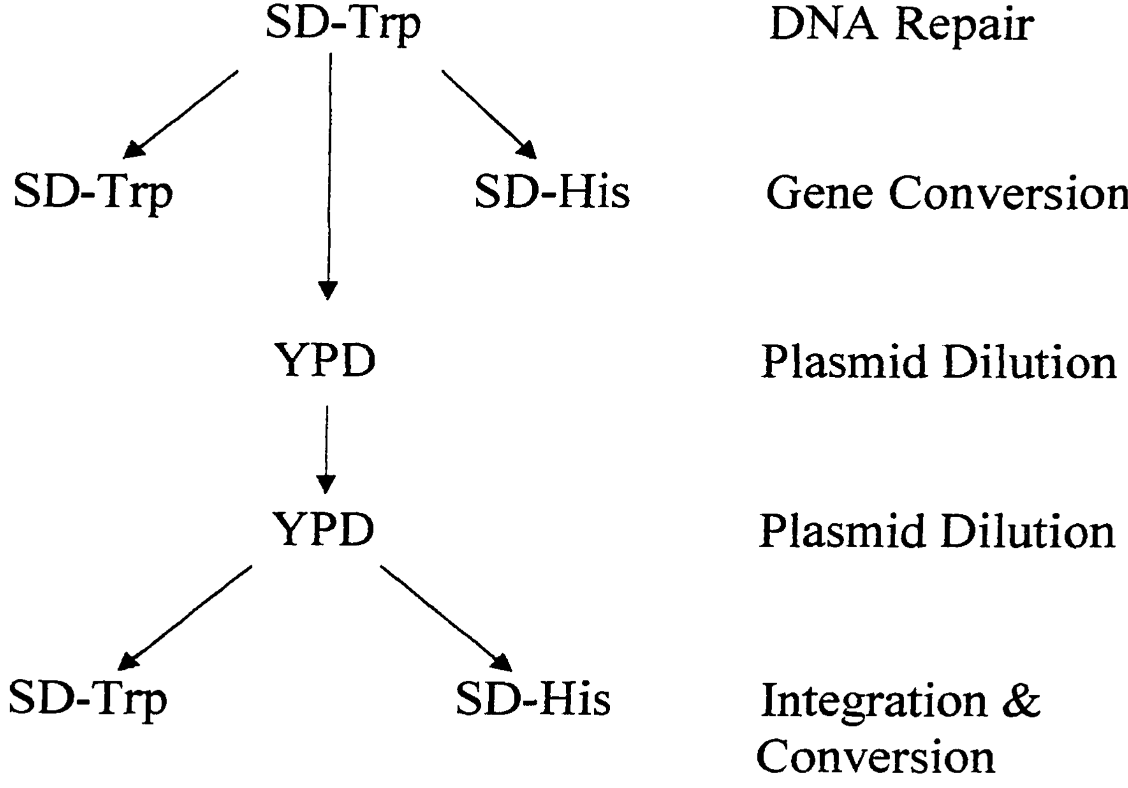
Figure 6

Flow Chart of Genetic Analysis



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Results

Experimental Design

In order to study conversion tract lengths and direction of conversion during double-strand break recombination repair, we placed restriction site markers at various locations in plasmid *his3* genes. These genes were then digested with restriction enzymes to produce double-strand breaks at various distances from the restriction site markers. We then transformed linearized or uncut plasmids into a haploid strain of the yeast *Saccharomyces cerevisiae* which contained a restriction site marker at a specific location in a chromosomal *his3* gene. This strain was constructed by two step gene replacement (Donna Luisi, personal communication) of strain W303 (Rothstein, !983) with a mutant *his3* allele. This allele contains an *XbaI* restriction site added to position 304 in the coding region. The plasmids were also transformed into recombinational repair deficient stains.

XbaI restriction site markers were placed at sites 75, 207, 304, 395, or 622 within the *HIS3* gene (figures 3 & 4). These alleles were ligated into a plasmid that carries a functional *TRP1* gene (figure 5).

The plasmids were transformed into haploid strains of yeast that carry a nonfunctional *trp1* allele and a nonfunctional *his3* allele containing an *XbaI* restriction site at position 304 in the coding region (figure 5). The *his3* allele was created by digesting the wildtype *HIS3* with a restriction enzyme, adding *XbaI* linkers, and recircularizing the plasmid with ligase. Four strains are each deficient in a gene of the *RAD52* epistasis group that is involved in recombinational repair. They include *rad51*, *rad54*, *rad55*, and *rad57*. The remaining strain carries the wild type alleles for the *RAD52* epistasis group.

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The transfected cells were plated on tryptophan omission medium to select for transformants in which the plasmid was repaired. They were also plated on histidine omission medium to select for transformants in which gene conversion to the *HIS3* allele took place (figure 8).

Experimental samples were labeled with a number corresponding to the position of the restriction site marker placed in the *his3* gene on the plasmid, and a letter refering to the enzyme with which the plasmid was linearized. For example, sample 75M has an *XbaI* restriction site marker placed at position 75 in the coding region of the *his3* gene, and was linearized by digestion with the restriction enzyme *MscI*.

Repair of plasmid double-strand breaks

TRP1 his3 plasmids were linearized by restriction digestion. Linearized and circular plasmids were transformed into wild type, *rad51*, *rad54*, *rad55*, and *rad57* strains. The ratio of linearized to circular Trp^+ transformants for each strain is compared in figure 7.

Figure 7 and table 2 represent the proportion of transformations with digested plasmids to those with undigested plasmids for Rad^- and Rad^- strains. This graph shows that there is a higher transformation efficiency with uncut DNA than with linearized DNA since the ratio of linearized to uncut DNA is always less than one. The wild type and recombinational repair-deficient strains are compared to see to what extent each of the *RAD51*, *RAD54*, *RAD55*, and *RAD57* gene products are involved in double-strand break repair. The wild type shows approximately 75% repair while the recombinational repair-deficient strains range from approximately 45% to 55%. Comparison between *RAD* and *rad51*, *rad54*, *rad55*, and *rad57* yielded chi-square values that are all significant at the

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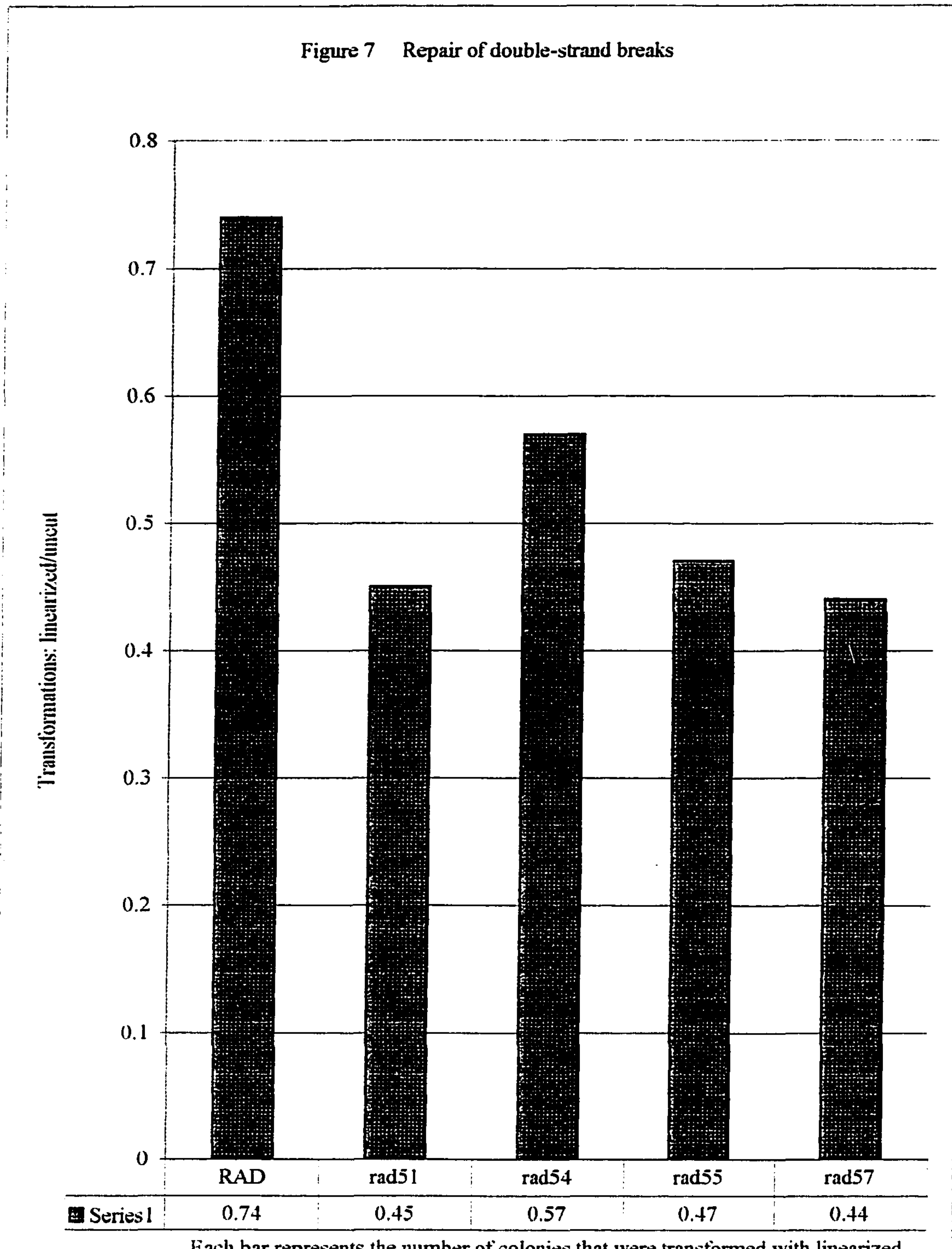
Figure 7 Repair of double-strand breaks



	RAD	rad51	rad54	rad55	rad57
Series1	0.74	0.45	0.57	0.47	0.44

Each bar represents the number of colonies that were transformed with linearized plasmids divided by the number of colonies that were transformed with uncut plasmids. Results of the five *his3* alleles and three restriction enzymes are combined for each.

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Table 2
Transformations
linearized versus uncut

experiment	Trp⁻	linearized	linearized/3	uncut	linearized/3 uncut
1	*RAD*	185890	61963	89430	0.69
2	*RAD*	4036	1345	2969	0.45
3	*RAD*	11919	3973	6483	0.61
4	*RAD*	14732	4910	4080	1.2
	average				0.74
1	*rad51*	134	45	183	0.25
2	*rad51*	830	277	656	0.42
3	*rad51*	842	281	417	0.67
	average				0.45
1	*rad54*	21	7	15	0.47
2	*rad54*	5186	1729	2536	0.68
	average				0.57
1	*rad55*	209	70	181	0.38
2	*rad55*	173	58	104	0.55
	average				0.47
1	*rad57*	2615	872	1904	0.46
2	*rad57*	1118	373	897	0.41
	average				0.44

The number of transformations listed under the column labeled 'damaged', is the result of three experiments each with a different restriction enzyme. In order to compare the transformations with linearized plasmids to those with nonlinearized plasmids, these figures are divided by three in the following column.

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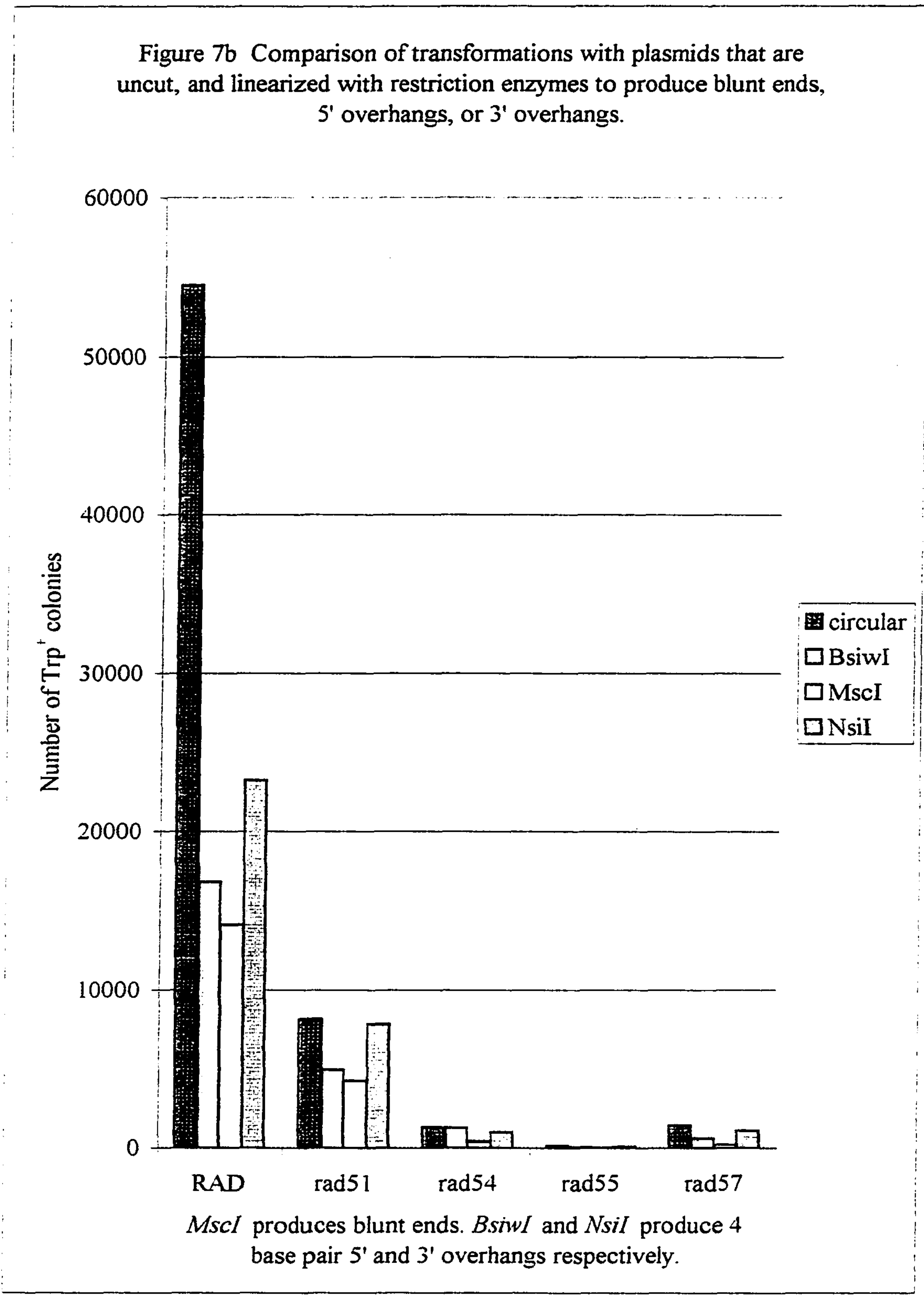
Figure 7b Comparison of transformations with plasmids that are uncut, and linearized with restriction enzymes to produce blunt ends, 5' overhangs, or 3' overhangs.



MscI produces blunt ends. *BsiwI* and *NsiI* produce 4 base pair 5' and 3' overhangs respectively.

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0.001 level. This shows that the products of each of these genes are involved in such repair. Although the ratio is lower in repair deficient strains, there is still considerable double-strand break repair. This suggests that there is another mechanism of double-strand break repair that is independent of the *RAD52* epistasis group, such as an end joining pathway, since each of the recombinational repair-deficient strains shows a significant amount of repair. This repair could be the result of a DNA end joining pathway. The Ku proteins are involved in such a pathway in yeast, and in V(D)J joining in mammalian immune systems (Chu 1996, Lieber et. al. 1997, Troelstra & Jaspers 1994). They are produced by the *HDF1* gene, and when this gene and *RAD52* are both mutated in yeast, repair is negligible (Siede et. al. 1996).

In these experiments, plasmids linearized with *MscI* to produce blunt ends resulted in less repair than those linearized with *BsiwI* or *NsiI* which produce four base pair, five prime and three prime overhangs respectively (figure 7b).

Double-strand breaks induce gene conversion to His$^+$

Yeast cells transfected with linearized or uncut plasmid DNA were plated onto histidine omission medium to measure the frequency of conversion to His$^-$. The frequency of conversion can be expressed by the ratio of His$^-$ colonies to Trp$^-$ colonies. The number of His$^-$ colonies per 1000 Trp$^-$ transformants is given in Table 3. This ratio is increased by double-strand breaks in repair proficient yeast. The ratio of His$^-$/1000 Trp$^-$ colonies is increased from 89 for uncut plasmid to 264 for plasmids cut by restriction endoucleases in *RAD* yeast cells.

We can quantitate the effect of introducing a double-strand break on gene conversion

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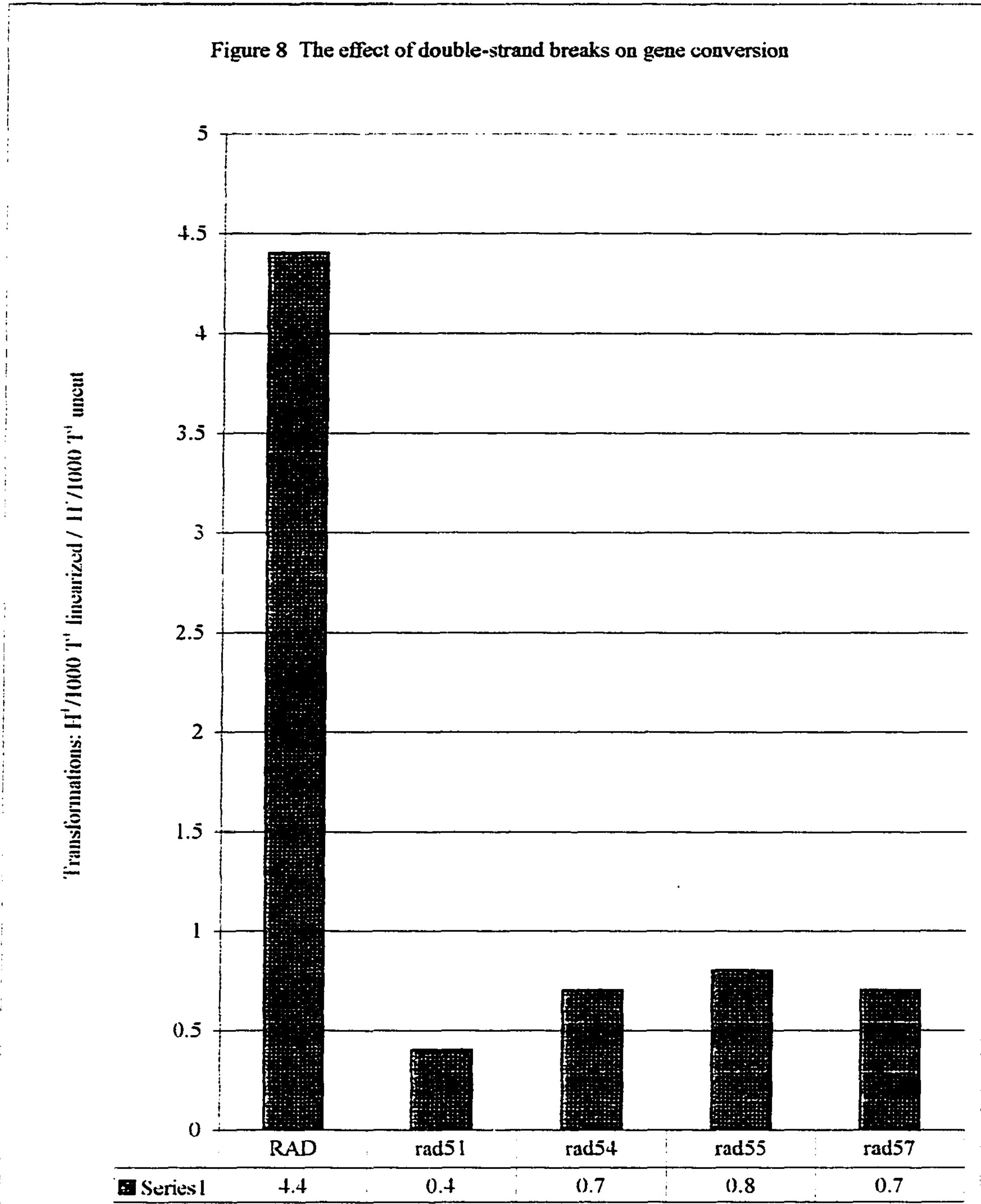
Figure 8 The effect of double-strand breaks on gene conversion



Each bar represents the number of His$^-$/1000 Trp$^-$ colonies transformed with linearized plasmids divided by the number of His$^-$/1000 Trp$^-$ colonies with uncut plasmids. Results of the five *his3* alleles and three restriction enzymes are combined for each.

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Table 3

Conversion to His^-

	RAD	*rad51*	*rad54*	*rad55*	*rad57*
linearized	263.6	83.2	490.4	441.6	19.9
uncut	88.9	174.1	844.2	664.8	55.4

These figures represent the ratio of His^-/Trp^- colonies.
The number of His^- colonies was divided by the number
of Trp^- colonies and multiplied by 1000 for each experiment.
The replicate experiments were then averaged.
The five *his3* alleles and three restricition enzymes are combined
for each strain.

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by dividing the ratio of His$^-$/1000Trp$^-$ for linearized plasmids by the ratio of His$^+$/1000Trp$^+$ for circular plasmids. Figure 8 compares this proportion in Rad$^+$ and Rad$^-$ yeast strains. It represents the double-strand break induced conversion for repair-proficient and repair-deficient strains. This ratio is significantly lower in all Rad$^-$ strains studied than in repair-proficient yeast cells. The chi-square values comparing *RAD* to *rad51*, *rad54*, *rad55*, and *rad57*, are all significant at the 0.001 level of probability.

This graph also demonstrates that double-strand breaks stimulate gene conversion in the *RAD* strain because the ratio of His$^-$/1000Trp$^-$ with linearized DNA to His$^-$/1000Trp$^-$ with uncut DNA is greater than one. In contrast double-strand breaks have not stimulated gene conversion in Rad$^-$ strains in our experiments.

I also assayed conversion to His$^-$ by carrying out analysis by replica plating of Trp$^-$ colonies appearing after five days of growth on trp omission medium. These measurements gave consistantly higher frequencies of His$^-$ colonies per Trp$^-$ transformant than colonies grown on his omission plates immediately after transfection. This suggests that appearance of His$^-$ colonies increases over several days, or that histidine is required for gene conversion. All subsequent measurements are therefore based on the genetic analysis of Trp$^-$ transformants.

Genetic analysis

Trp$^-$ colonies containing repaired plasmids were replica plated to histidine omission medium to check for transformants with repaired plasmids in which conversion to the *HIS3* allele took place. They were also tested for plasmid integration. These plasmids do not have centromeres and so are unstable in the absence of selection. The repaired

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colonies were then replica plated to nonselective media (YPD) twice to dilute out the plasmid. They were then replicated to plates lacking tryptophan to detect integration, and to plates lacking histidine to analyze conversion and integration together. (See Figure 6, Flow Chart of Genetic Analysis.)

If a transformed colony was unstable His$^-$, then the plasmid was converted by the chromosome allele. If a transformed colony was stable His$^+$, then either the chromosome was converted to *HIS3* by the plasmid, or an integration event took place since the *ARS* containing plasmids are unstable and are lost during growth on nonselective media.

Type I and Type II Arrangements

Some of the plasmid restriction site markers were placed in a location on the opposite side of the double strand break from which the chromosomal marker was placed. Other plasmid restriction site markers were placed so that they were between the double-strand break and the chromosome marker (figure 9). Both setups are classed as Type I arrangements. Other markers were placed so that the chromosomal marker would be between the plasmid marker and the double-strand break (figure 9). This is known as a Type II arrangement. The Double-Strand Break Repair Model predicts that a Type I arrangement can be repaired to give a His$^-$ phenotype with continuous conversion tracts, while a Type II arrangement requires discontinuous conversion tracts to yield His$^-$ phenotype (figures 10 & 11). Since continuous conversion tracts occur much more frequently than discontinuous tracts (Borts & Haber 1989, Sweetser et. al. 1994, Yi-shen et.al. 1996), we would therefore expect to see more transformants with a His$^-$ phenotype in Type I arrangements then in Type II arrangements. Our data support this model.

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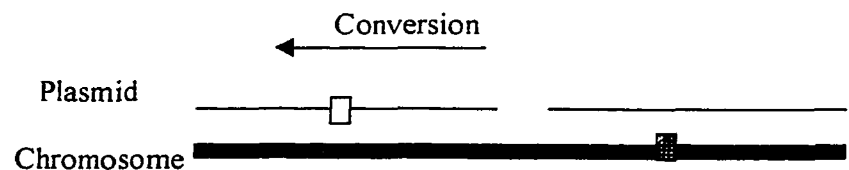
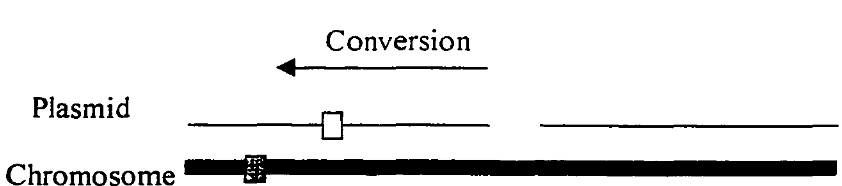
Figure 9

Gene Conversion

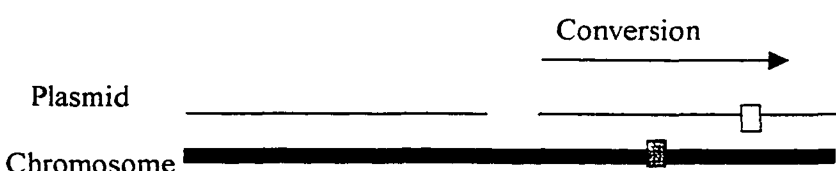
Type I



or



Type II



Legend
□ plasmid marker
▦ chromosome marker

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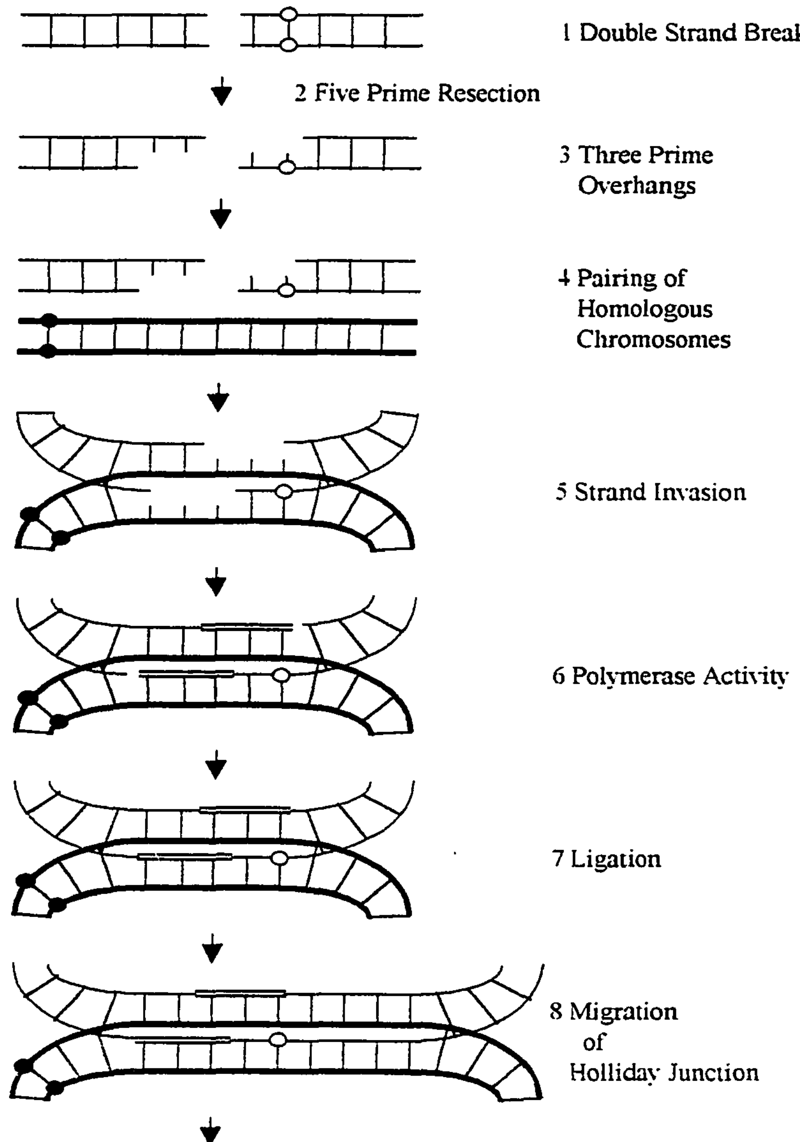
Figure 10

Type I Arrangement



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(Figure 10 continued)

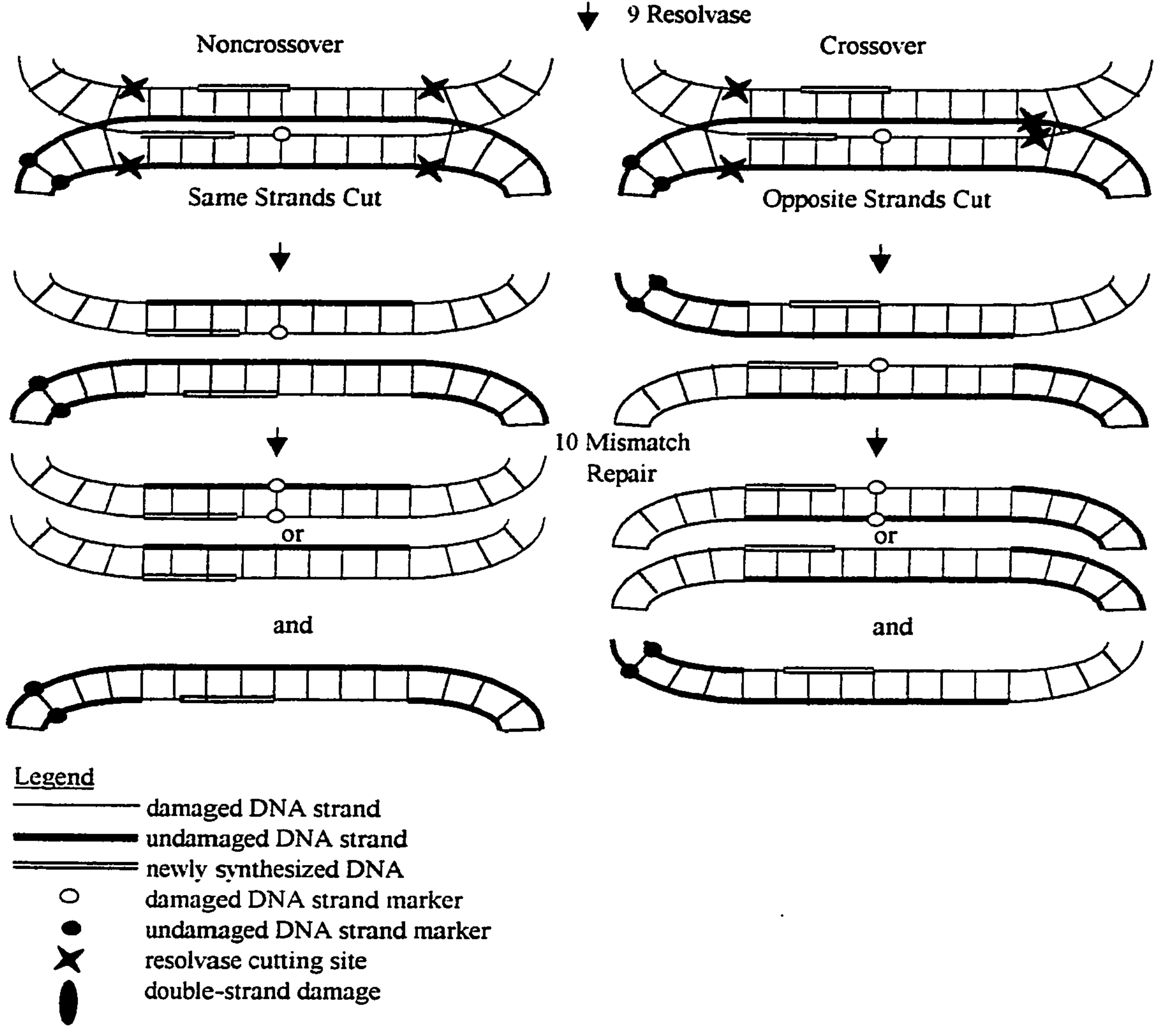

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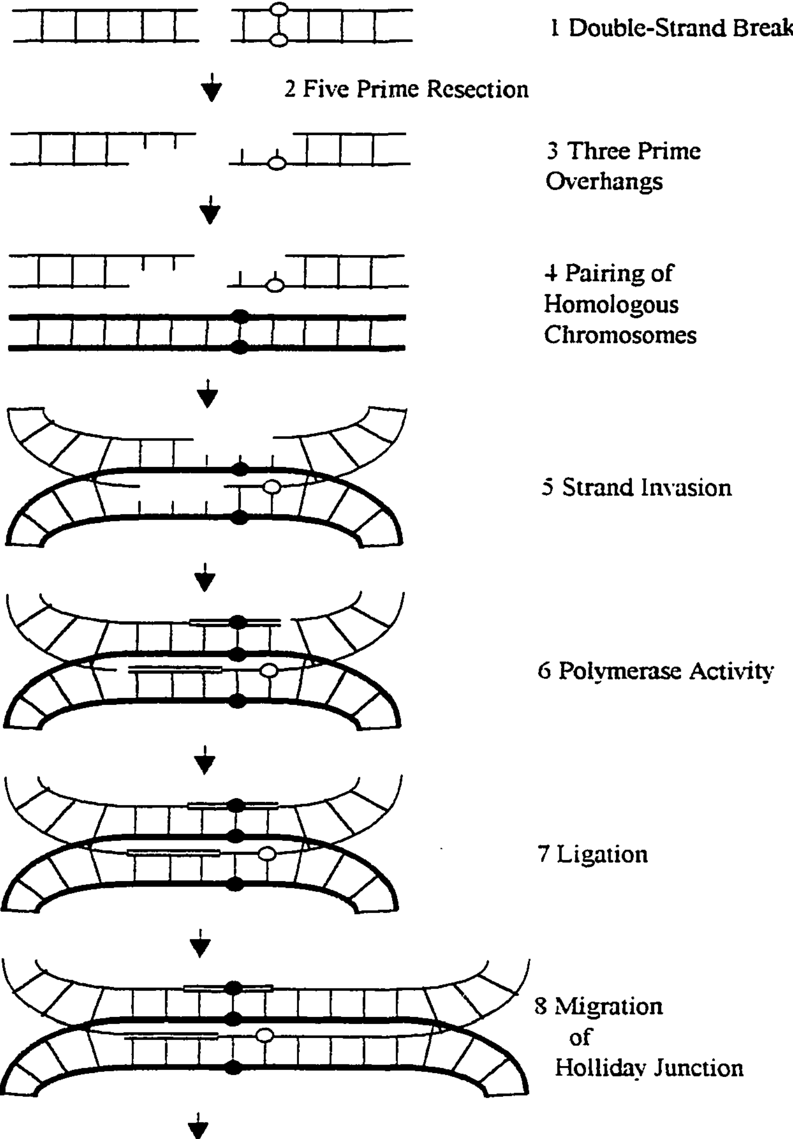
Figure 11

Type II Arrangement



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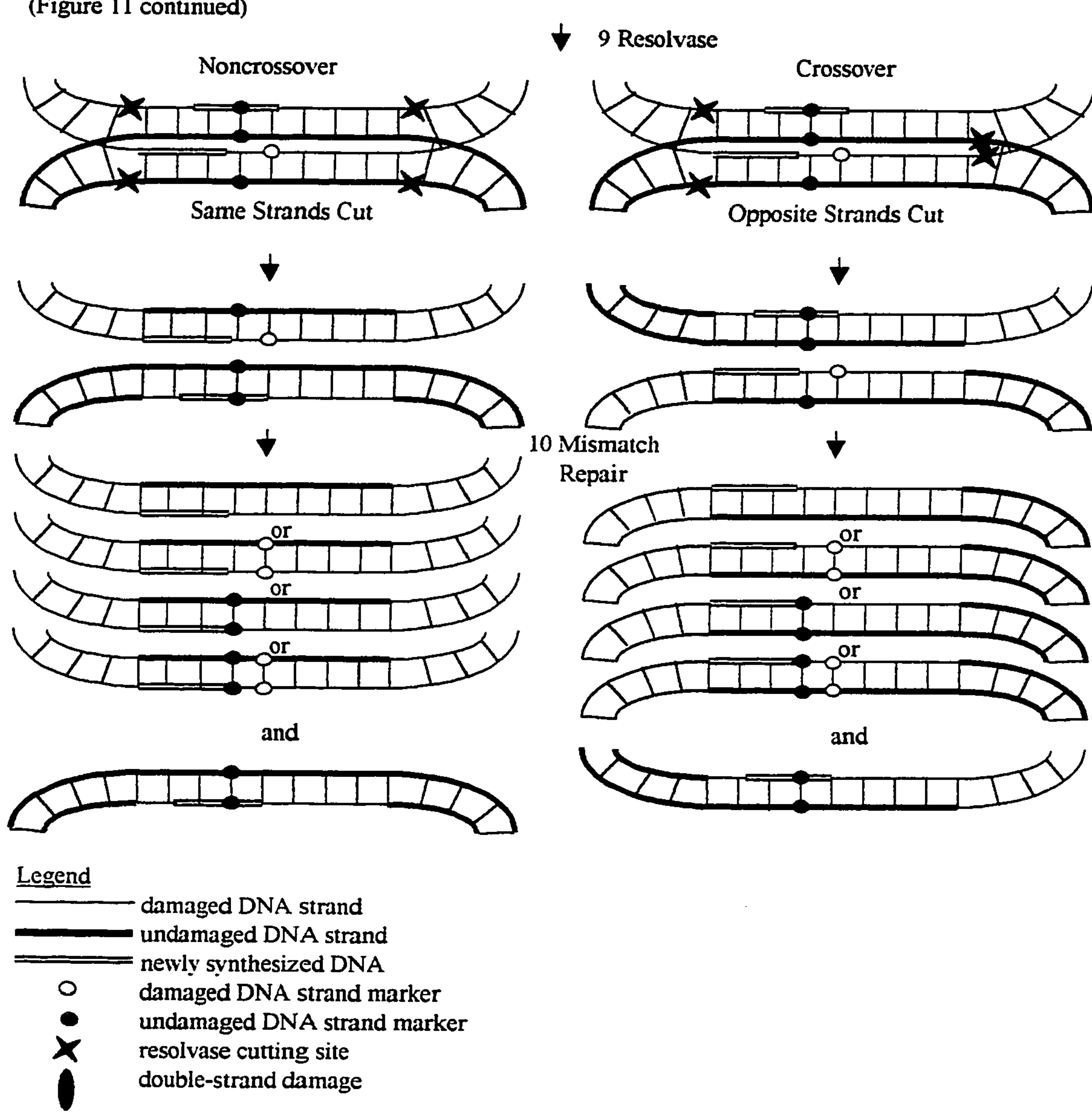
(Figure 11 continued)



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In addition, in two of our samples, 75MscI and 622BsiWI, a His$^-$ phenotype may occur by integration unaccompanied by a conversion event (figure 12). When the double strand break occurs between the plasmid and chromosome markers, the marker-free portion of the chromosome allele may become attached to the marker-free portion of the plasmid allele while the marked, mutant portion of the chromosome allele is attached to the marked portion of the plasmid allele. This will yield a functional *HIS3* allele and a nonfunctional allele, which contains both the plasmid and chromosome markers. The resulting strain will have a His$^-$, Trp$^+$ phenotype.

Since markers were placed at various sites in the *his3* gene which varied in distance from the double strand breaks, screening on histidine omission plates was also able to assess conversion tract lengths. Plasmids were digested with several restriction enzymes. Each cut at a different location in the *his3* gene. This multiplied the number of distances that could be assessed (table 4). Conversion tract lengths ranged from 13 base pairs to 457 base pairs.

Conversion to His$^+$ is higher in Type I arrangements

We compared the induction of His$^-$ colonies by double-strand breaks in Type I and Type II arrangements. Figure 13 (table 5) represents the proportion of transformed colonies that were converted to His$^-$. It compares Type I and Type II samples for each strain, and compares each Rad$^-$ strain to the wild type.

There is more gene conversion in Type I arrangements than in Type II arrangements in the *RAD* strain. The chi squared value at one degree of freedom for a comparison between Type I and Type II gene conversion is significant at the 0.001 level. This is predicted by

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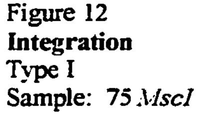
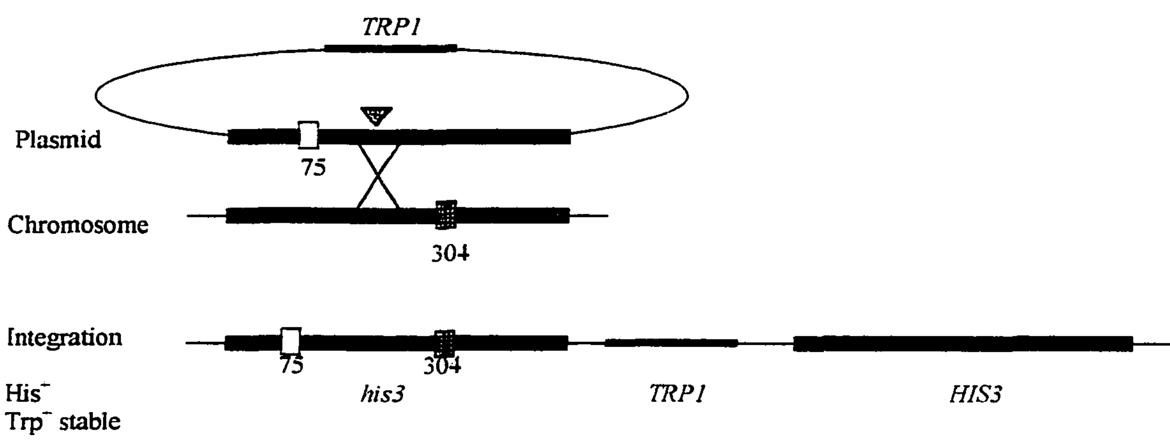
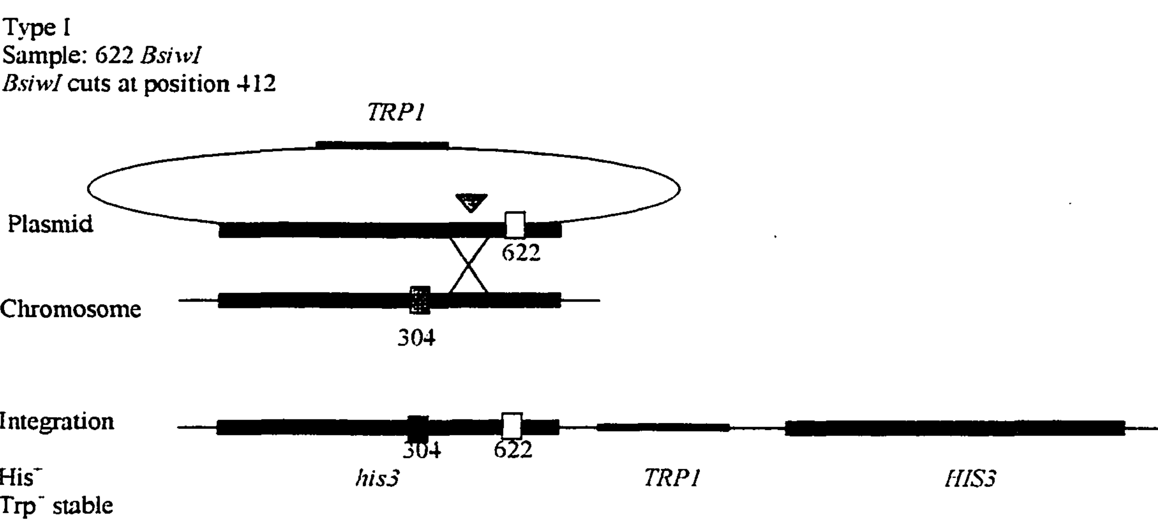


Figure 12
Integration
Type I
Sample: 75 *MscI*
MscI cuts at position 189



Type I
Sample: 622 *BsiwI*
BsiwI cuts at position 412



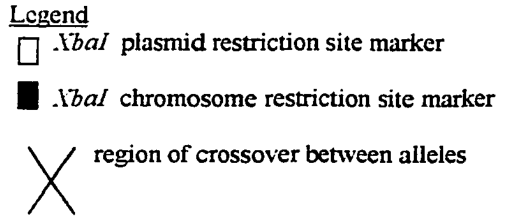


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Table 4
<u>Possible His⁻ Conversions</u>

The sample number refers to the position within the *his3* at which the restriction marker was placed. The sample letter refers to the restriction enzyme that was used to linearize the plasmid. 'B' refers to *BsiWI*. 'N' refers to *NsiI*. 'M' refers to *MscI*.

Chromosome to Plasmid Conversion

Sample	Repair Type	Distance from Damage Site (bp)	Location
395B	I	13	upstream
622N	I	143	upstream
395N	I	366	upstream
207M	I	18	downstream
75M	I	114	downstream
622B	I	210	downstream

Plasmid to Chromosome Conversion

Sample	Repair Type	Distance from Damage Site (bp)	Location
75M	I	115	upstream
622B	I	104	upstream
75B	II	104	upstream
207B	II	104	upstream
207N	II	437	upstream
75N	II	457	upstream
395M	II	115	downstream
622M	II	115	downstream

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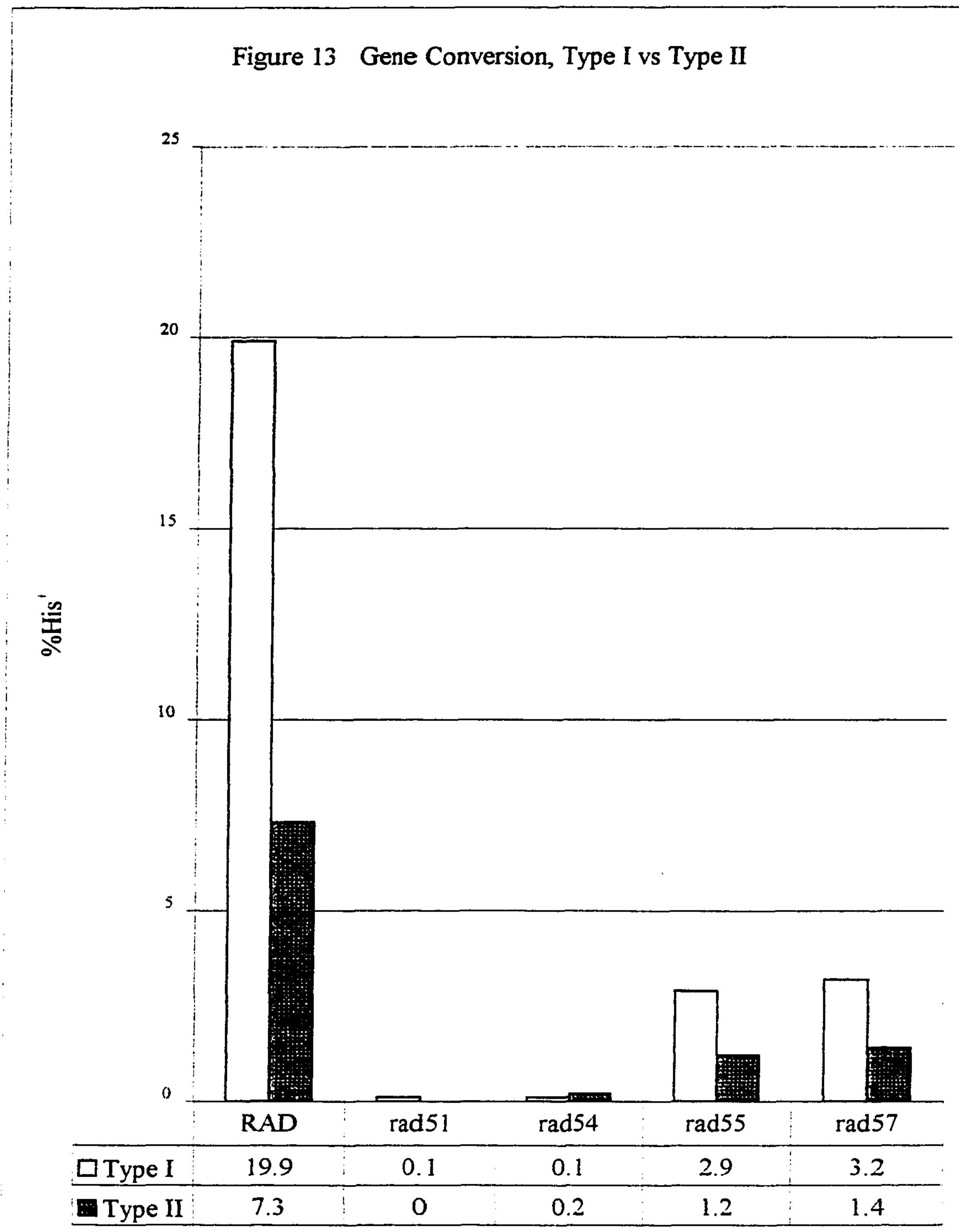
Figure 13 Gene Conversion, Type I vs Type II



	RAD	rad51	rad54	rad55	rad57
□ Type I	19.9	0.1	0.1	2.9	3.2
■ Type II	7.3	0	0.2	1.2	1.4

This graph compares the number of colonies that have resulted in a His⁻ phenotype after transformation with Type I arrangments to those that have been transformed with Type II arrangements.

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Table 5 Gene Converson Experiments

This table show the number of colonies that are His$^+$, and the total number of colonies for each experiment.

strain	*RAD*		*rad51*		*rad54*		*rad55*		*rad57*	
Type I	H$^+$	total	H$^+$	total	H$^+$	total	H$^+$	total	H$^+$	total
395b,366U	16	199	0	131	0	147	1	103	2	196
622n,143U	16	197	0	185	0	190	1	100	2	190
395n,13U	47	199	0	125	0	145	6	117	5	147
207m,18D	4	99	0	142	1	73	0	184	0	157
75m,114D	62	199	0	87	0	131	2	73	18	134
622b,210D	73	200	1	122	0	145	9	69	4	143
total	218	1093	1	792	1	831	19	646	31	967
Type II										
75n,457U	13	200	0	188	0	147	0	63	3	147
207n,437U	4	200	0	158	0	199	0	141	0	194
207b,104U	12	195	0	128	0	194	1	90	1	109
75b,104U	11	200	0	147	0	195	0	96	1	177
622m,115D	20	199	0	44	1	123	1	37	0	27
395m,115D	27	200	0	22	0	103	4	49	6	119
total	87	1194	0	687	1	961	6	476	11	773

Each sample is labeled with a number that represents the marker position on a *his3* allele. This is followed by a letter representing the restriction enzyme used to make a double-strand break, a number representing the distance between the double-strand break and the marker, and a letter representing the direction of the marker upstream(U) or downstream(D) of the break.

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the double-strand break repair model because discontinuous conversion tracts are required for the formation of His⁻ cells in Type II samples. Therefore, these results support the double-strand break repair model.

Gene conversion occurred in the *rad55* and *rad57* strains but at a lower level than in *RAD* cells. The decreased conversions in *rad55* and *rad57* cells are both significantly different from *RAD* cells at the 0.001 level by chi-square analysis. A comparison between Type I *rad55* versus Type I *rad57* yielded a chi-square value that is not significant at the 0.05 level. The same comparison for Type II yielded a non-significant chi-square value at the 0.05 level as well. This tells us that there is not a significant difference between the effect on gene conversion between the two strains. A deletion of either gene function yields the same effect. This suggests that their gene products function together in double-strand break repair. We also see more conversion in Type I samples than Type II samples in both the *rad55* and *rad57* strains. It is significantly higher for *rad57*, at the 0.02 level, but the difference between Types I and II is not significant for *rad55*. The *rad51* and *rad54* samples did not show gene conversion. These results indicate that the *rad51* and *rad54* gene products are both essential in double-strand break repair while the *rad55* and *rad57* gene products serve an accessory function.

Double-strand break-induced plasmid integration

The proportion of integrated colonies was studied by observing a stable Trp⁻ phenotype. Trp⁻ colonies were plated twice on nonselective media to dilute out the plasmid (figure 6). They were then plated on media lacking tryptophan to select for integration. Figure 14 compares integration in Type I and Type II arragements for repair-

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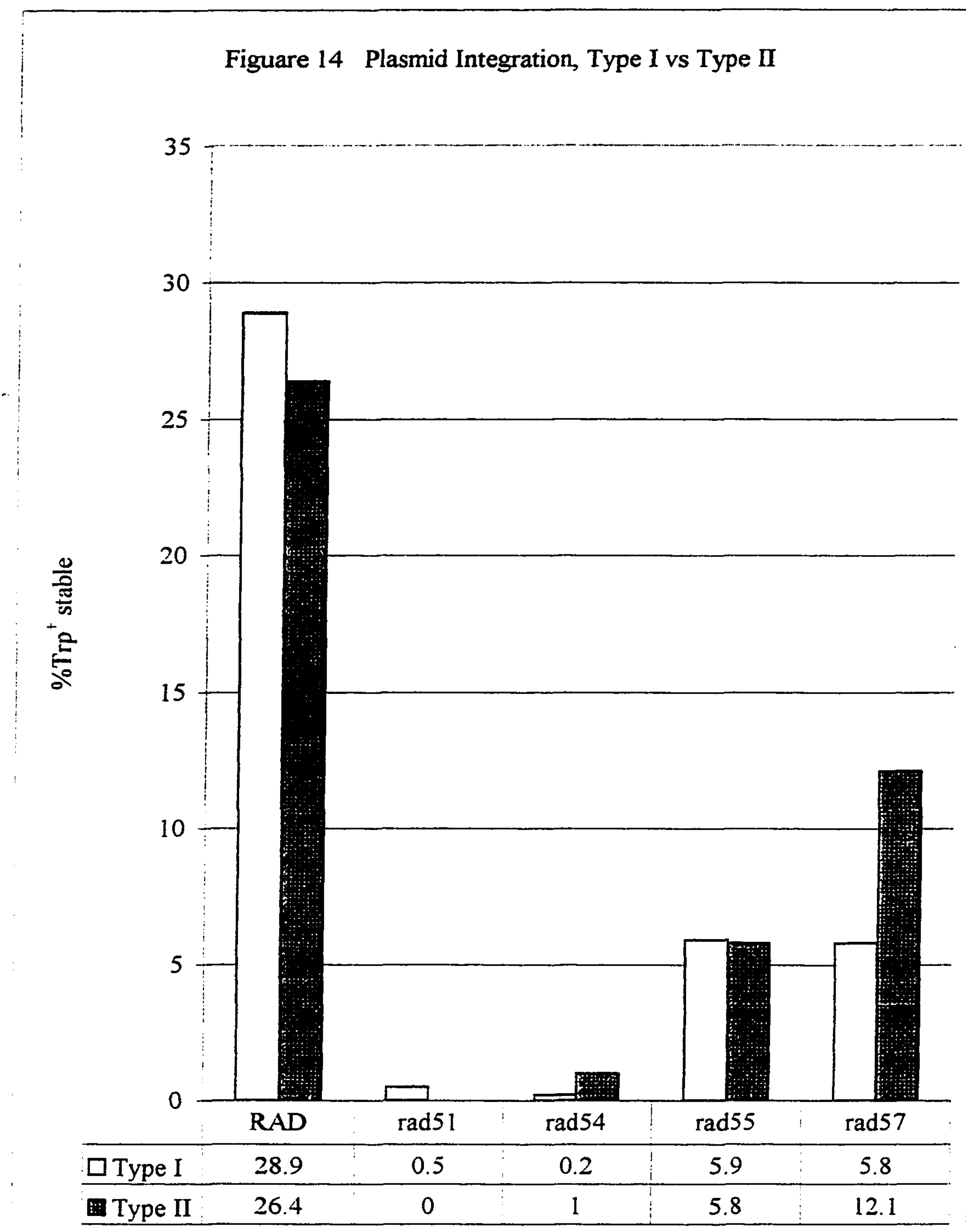

	RAD	rad51	rad54	rad55	rad57
□ Type I	28.9	0.5	0.2	5.9	5.8
▩ Type II	26.4	0	1	5.8	12.1

This graph compares the number of colonies that have resulted in a Trp^- stable phenotype after transformaton with Type I arrangements to colonies transformed with Type II arrangements.

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Table 6 Integration Experiments

This table shows the number of transformed colonies that are Trp^+ stable, and the total number of colonies for each experiment.

strain	*RAD*		*rad51*		*rad54*		*rad55*		*rad57*	
Type I	T^+s	total	T^+s	total	T^+s	total	T^+s	total	T^+s	total
395b,366U	71	199	0	131	0	147	4	103	14	196
622n,143U	39	197	0	185	0	190	2	100	3	190
395n,13U	51	199	3	125	1	145	15	117	5	147
207m,18D	0	99	0	142	1	73	0	184	0	157
75m,114D	70	199	0	87	0	131	6	73	25	134
622b,210D	85	200	1	122	0	145	11	69	9	143
total	316	1093	4	792	2	831	38	646	56	967
Type II										
75n,457U	43	200	0	188	0	147	3	63	14	147
207n,437U	23	200	1	158	1	199	3	141	0	194
207b,104U	64	195	2	128	0	194	8	90	8	109
75b,104U	43	200	0	147	0	195	4	96	13	177
622m,115D	73	199	0	44	2	123	4	37	6	27
395m,115D	69	200	2	22	2	103	11	49	56	119
total	315	1194	5	687	5	961	33	476	97	773

Each sample is labeled with a number that represents the marker position on a *his3* allele. This is followed by a letter representing the restriction enzyme used to make a double-strand break, a number representing the distance between the double-strand break and the marker, and a letter representing the direction of the marker upstream(U) or downstream(D) of the break.

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proficient and repair-deficient yeast strains.

We do not expect the positioning of the markers to affect integration and so there should not be a difference in the frequency of plasmid integration between Type I and Type II arrangements. As predicted the difference between Types I and II is small. The chi-square value for a comparison between Type I and Type II arrangements for the *RAD* strain at one degree of freedom is not significant at the 0.05 level.

Integration occurred in both the *rad55* and *rad57* strains, but at a lower rate than the *RAD* strain. The difference was significant at the 0.001 level for both *rad55* and *rad57* cells, by chi-square analysis. Their gene products are therefore involved in double-strand break repair-induced crossover recombination. As with *RAD* cells, there was no significant difference between Type I and Type II arrangements in the *rad55* strain. The *rad51* and *rad54* samples did not show integration.

Gene conversion is associated with plasmid integration

The double-strand break repair model postulates that resolution of the recombination intermediate leads to both integration and gene conversion. In alternative models, such as yeast mating type switching, integration and conversion are not so tightly linked.

In order to study the relationship between gene conversion and integration, we compared the incidence of %His$^-$ colonies (conversion) and the %Trp$^-$ stable colonies (integration). We also compared the proportion of gene conversion in non-integrated plasmids (His$^-$ unstable, Trp$^-$ unstable / Trp$^-$ unstable) to the proportion of gene converson in integrated plasmids (His$^-$ stable, Trp$^-$ stable / Trp$^-$ stable). Figure 15 and table 7 compare the proportion of colonies with a converted plasmid to the proportion of

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colonies with an integrated plasmid in the *RAD* and repair-deficient strains. Table 7b compares the proportion of colonies with gene conversion in non-integrated plasmids to those with integrated plasmids in *RAD* and repair-deficient strains. Type I and Type II samples are compared for each of the five strains in both sets of comparisons.

The ratio of His$^-$ conversions to plasmid integrations in *RAD* cells is higher for Type I than Type II arrangements, as predicted by the double-strand break repair model (figure 15). This difference is significant at the 0.001 level of probability.

The conversion to integration ratios for the *rad55* and *rad57* strains are lower than the same ratio for the *RAD* strain (table 7). The differences are significant for *rad57* Type I and Type II arrangements at the 0.001 and 0.01 levels of probability respectively. They are not significant at the 0.05 level of probability for the *rad55* strain. This may be because the *rad55* experiments did not yield enough of colonies to calculate significance.

The incidence of conversion for integrated plasmids is greater than the incidence of conversion for extrachromosomal plasmids at the 0.001 level of probability for samples with Type I and Type II arrangements. This can be seen when comparing the samples %His$^-$ unstable, Trp$^-$ unstable to the samples %His$^-$ stable, Trp$^-$ stable (table 7b). Type I samples 75M and 622B can yield His$^-$ stable colonies upon integration without gene conversion. When these two samples are eliminated from the chi-square analysis, the incidence of conversion for integrated plasmids remains greater than the incidence of conversion for extrachromosomal plasmids at the 0.01 level of probability. This is also seen in the *rad55* and *rad57* samples with Type I arrangements at the 0.001 level of probability. For Type II arrangements it is seen at the 0.01 and 0.05 levels of probability

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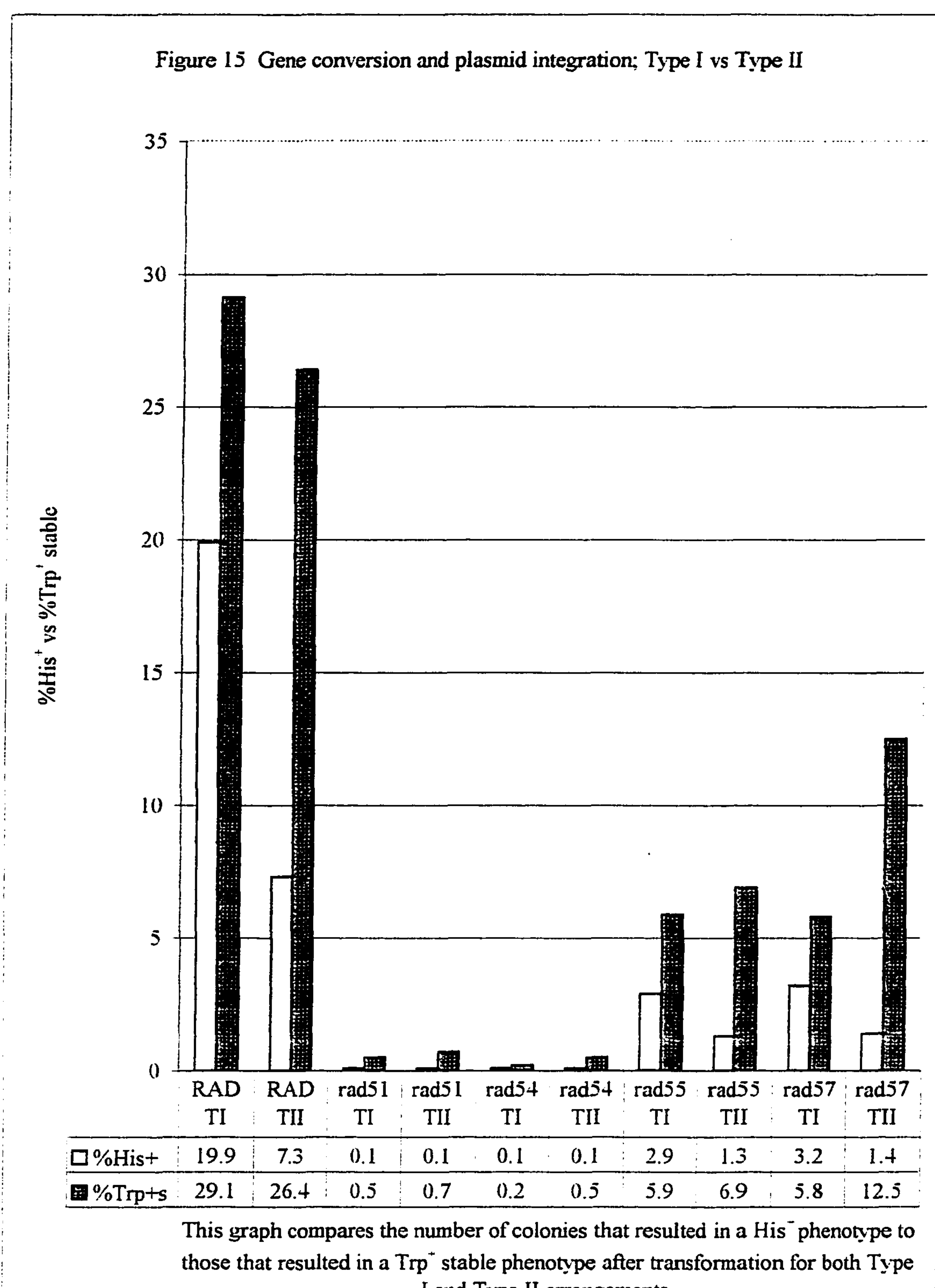

	RAD TI	RAD TII	rad51 TI	rad51 TII	rad54 TI	rad54 TII	rad55 TI	rad55 TII	rad57 TI	rad57 TII
□ %His+	19.9	7.3	0.1	0.1	0.1	0.1	2.9	1.3	3.2	1.4
■ %Trp+s	29.1	26.4	0.5	0.7	0.2	0.5	5.9	6.9	5.8	12.5

Figure 15 Gene conversion and plasmid integration; Type I vs Type II

This graph compares the number of colonies that resulted in a His⁻ phenotype to those that resulted in a Trp⁺ stable phenotype after transformation for both Type I and Type II arrangements.

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Table 7
Comparison of gene conversion and integration in repair-deficient strains
%His$^+$ vs %Trp^+_s TI vs TII

strain	%His$^-$ Rad$^-$/Rad$^-$	%Trp^-_s Rad$^-$/Rad$^-$	%His$^-$ Rad$^-$/Rad$^-$ / %Trp^-_s Rad$^-$/Rad$^-$
rad51 TI	0.005	0.017	0.29
rad51 TII	0.014	0.027	0.52
rad54 TI	0.005	0.006	0.83
rad54 TII	0.014	0.019	0.74
rad55 TI	0.146	0.203	0.72
rad55 TII	0.178	0.261	0.68
rad57 TI	0.161	0.199	0.81
rad57 TII	0.192	0.473	0.41

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Table 7b
Comparison of gene conversion in integrated and non-integrated plasmids

strain	$\frac{His^-_u, Trp^-_u}{Trp^-_u}$	$\frac{His^-_s, Trp^-_s}{Trp^+_s}$
RAD TI	0.049	0.43
RAD TII	0.01	0.16
rad51 TI	0	0.25
rad51 TII	0	0.2
rad54 TI	0	0
rad54 TII	0	0
rad55 TI	0.0016	0.39
rad55 TII	0	0.15
rad57 TI	0.001	0.43
rad57 TII	0	0.1

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respectively.

Comparison of chromosomal gene conversion upstream versus downstream of a double-strand break

We compared the frequency of chromosomal gene conversion upstream and downstream of a double-strand break. See figure 16 for a comparison of *his3* alleles and digestion sites. Two samples that have a chromosomal restriction site marker placed 104 bp upstream from a double-strand break site were compared to two samples containing a chromosomal restriction site marker 115 bp downstream. These were Type II arrangements. Figure 17 compares the proportion of colonies with a converted gene out of two hundred plated colonies for each sample. Type II upstream and downstream samples are compared for each strain. Figure 17b shows the individual samples used to make figure 17. There is a close correlation between each individual sample and the combined results.

There is more conversion downstream than upstream in *RAD*, *rad55*, and *rad57* yeast strains. The chi-square value for a comparison between upstream and downstream for the *RAD* strain at one degree of freedom is significant at the 0.01 level of probability.

The difference between upstream and downstream conversion was significant at the 0.001 level and more pronounced in *RAD55*- and *RAD57*-deficient cells than in repair-proficient yeast. We did not find a significant dependence in conversion on distance from a double-strand break site (figure 17c).

Both plasmid and chromosome are donors in gene conversion

Colonies from transformants in which both gene conversion and integration had taken place were analyzed by PCR. Figure 18 diagrams the different size fragments

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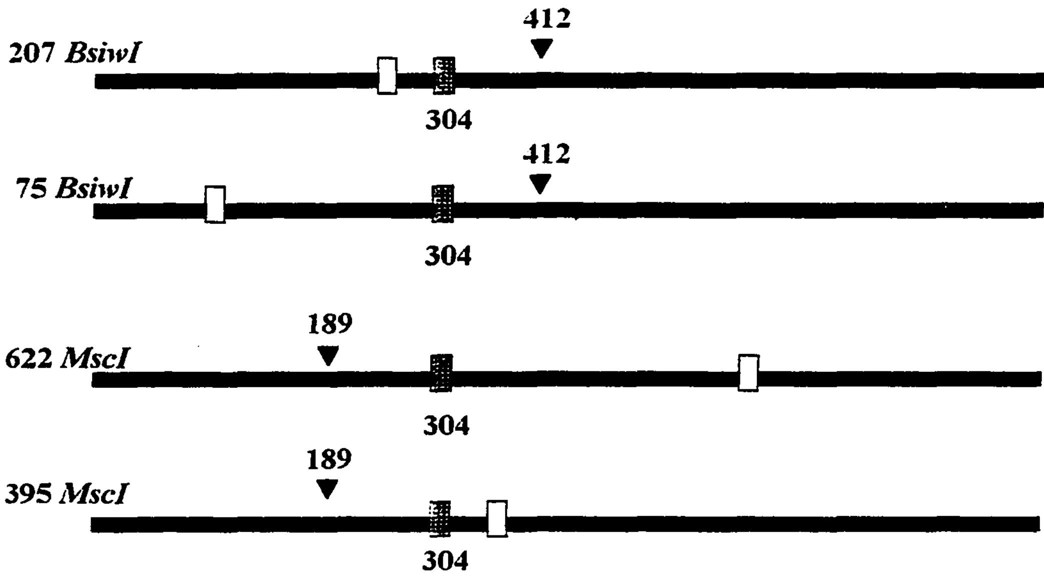
A comparison of Type II *his3* alleles



Legend

XbaI plasmid restriction site marker

XbaI chromosome restriction site marker

▼ restriction enzyme induced double-strand break site

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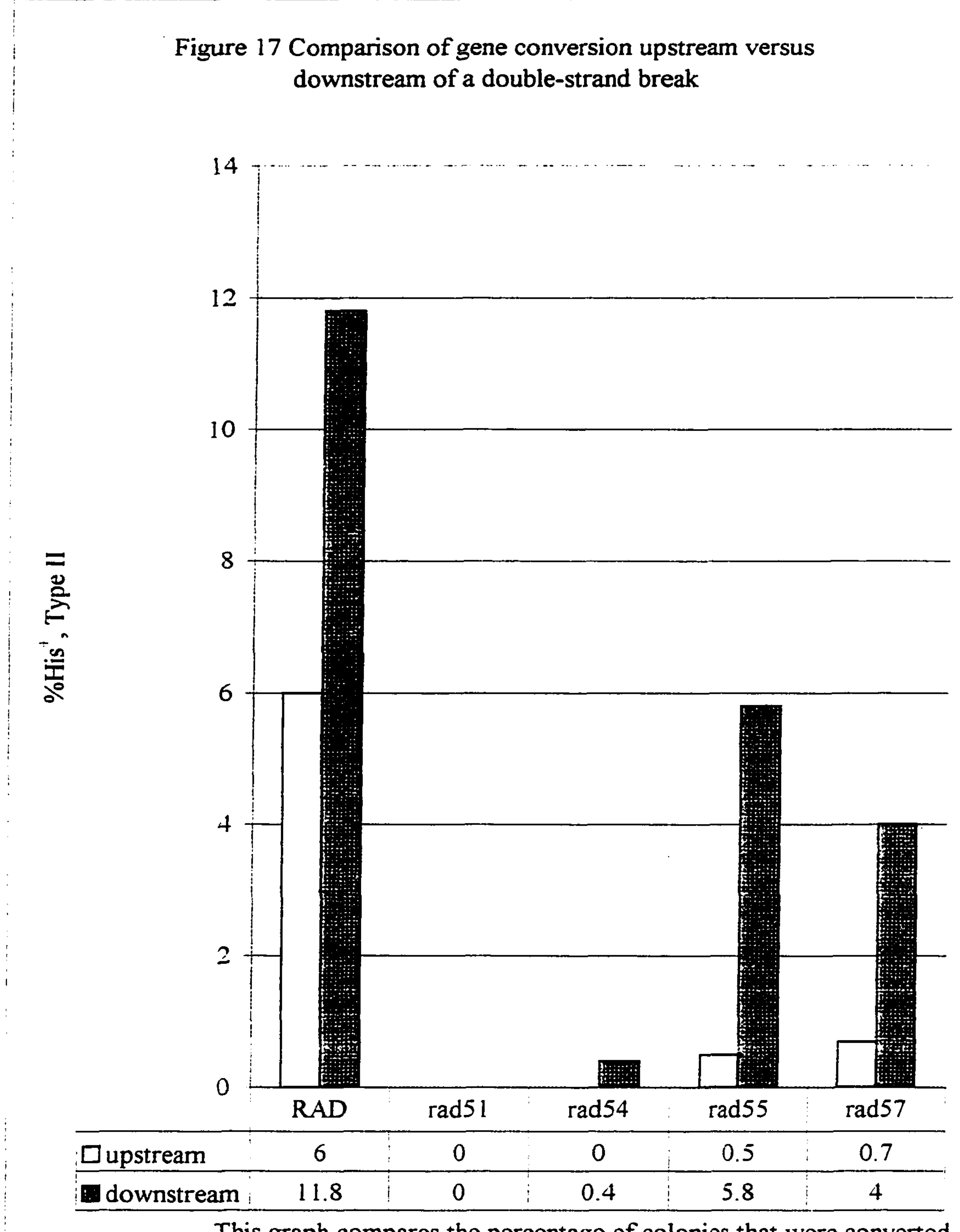
Figure 17 Comparison of gene conversion upstream versus downstream of a double-strand break



	RAD	rad51	rad54	rad55	rad57
□ upstream	6	0	0	0.5	0.7
■ downstream	11.8	0	0.4	5.8	4

This graph compares the percentage of colonies that were converted to a His⁻ phenotype after transformation with Type II arrangements upstream from a double-strand break to those converted downstream.

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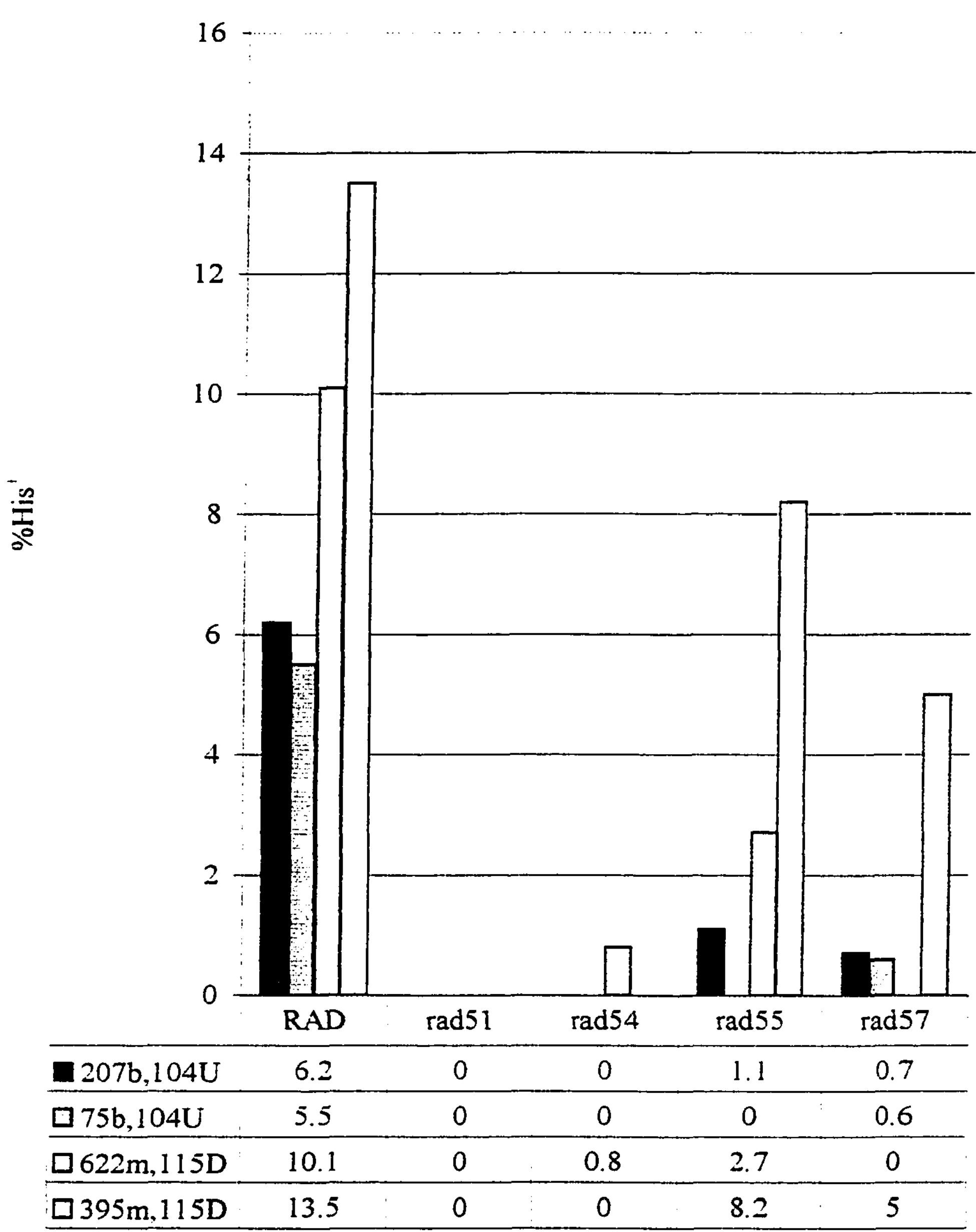
Figure 17b Comparison of gene conversion upstream versus downstream of a double-strand break



	RAD	rad51	rad54	rad55	rad57
■ 207b,104U	6.2	0	0	1.1	0.7
□ 75b,104U	5.5	0	0	0	0.6
□ 622m,115D	10.1	0	0.8	2.7	0
□ 395m,115D	13.5	0	0	8.2	5

This graph compares the number of colonies that were converted to a His⁻ phenotype after transformation with Type II arrangements upstream from a double-strand break to those converted downstream.

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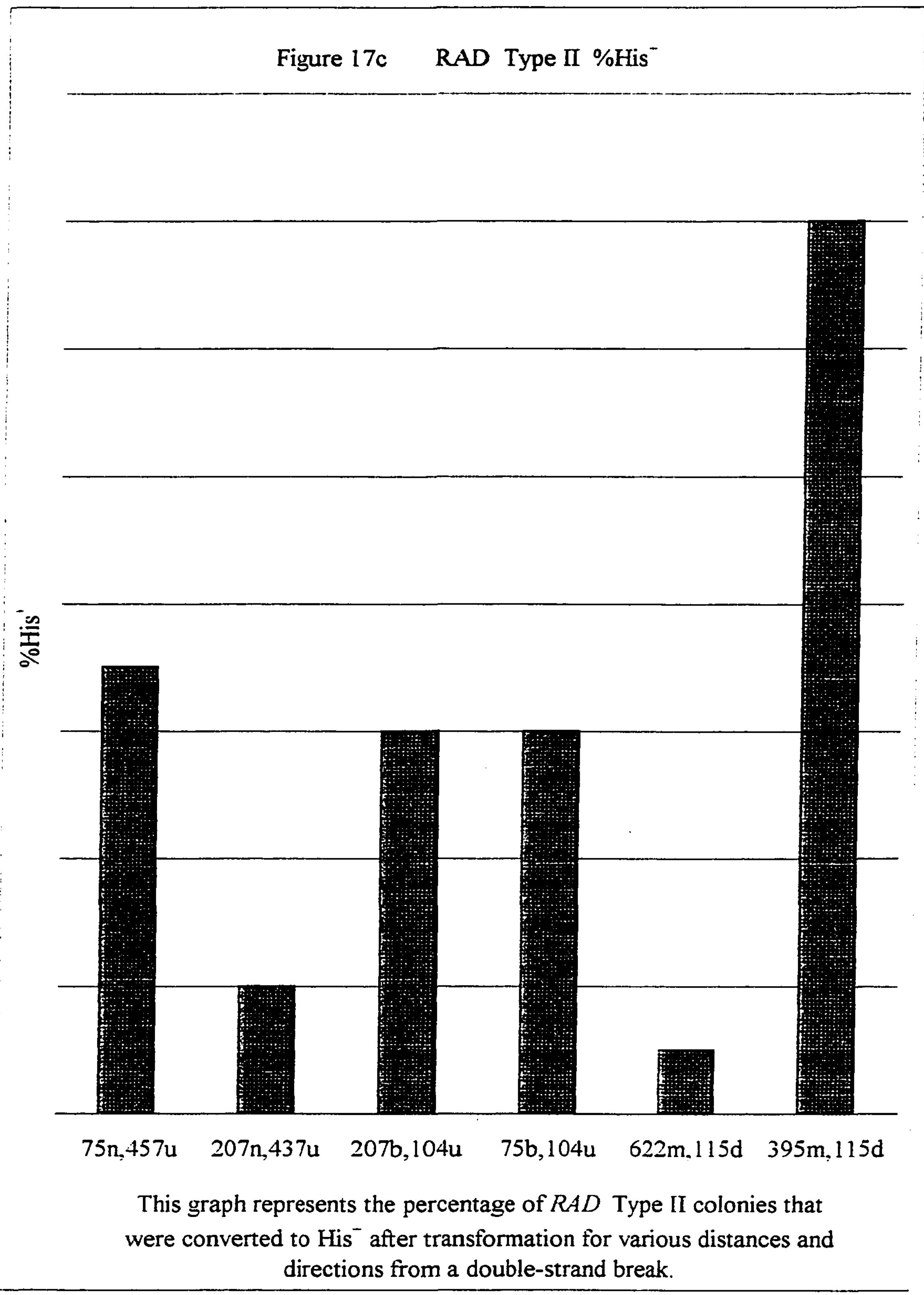
Figure 17c RAD Type II %His⁻



This graph represents the percentage of *RAD* Type II colonies that were converted to His⁻ after transformation for various distances and directions from a double-strand break.

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Table 8

Comparison of gene conversion upstream versus downstream of a double-strand break for samples with Type II arrangements

		upstream	downstream
		75b	395m
Strain		207b	622m
RAD	H^-	23	47
	total	395	399
rad51	H^+	0	0
	total	275	66
rad54	H^-	0	1
	total	389	226
rad55	H^-	1	5
	total	186	86
rad57	H^-	2	6
	total	286	146

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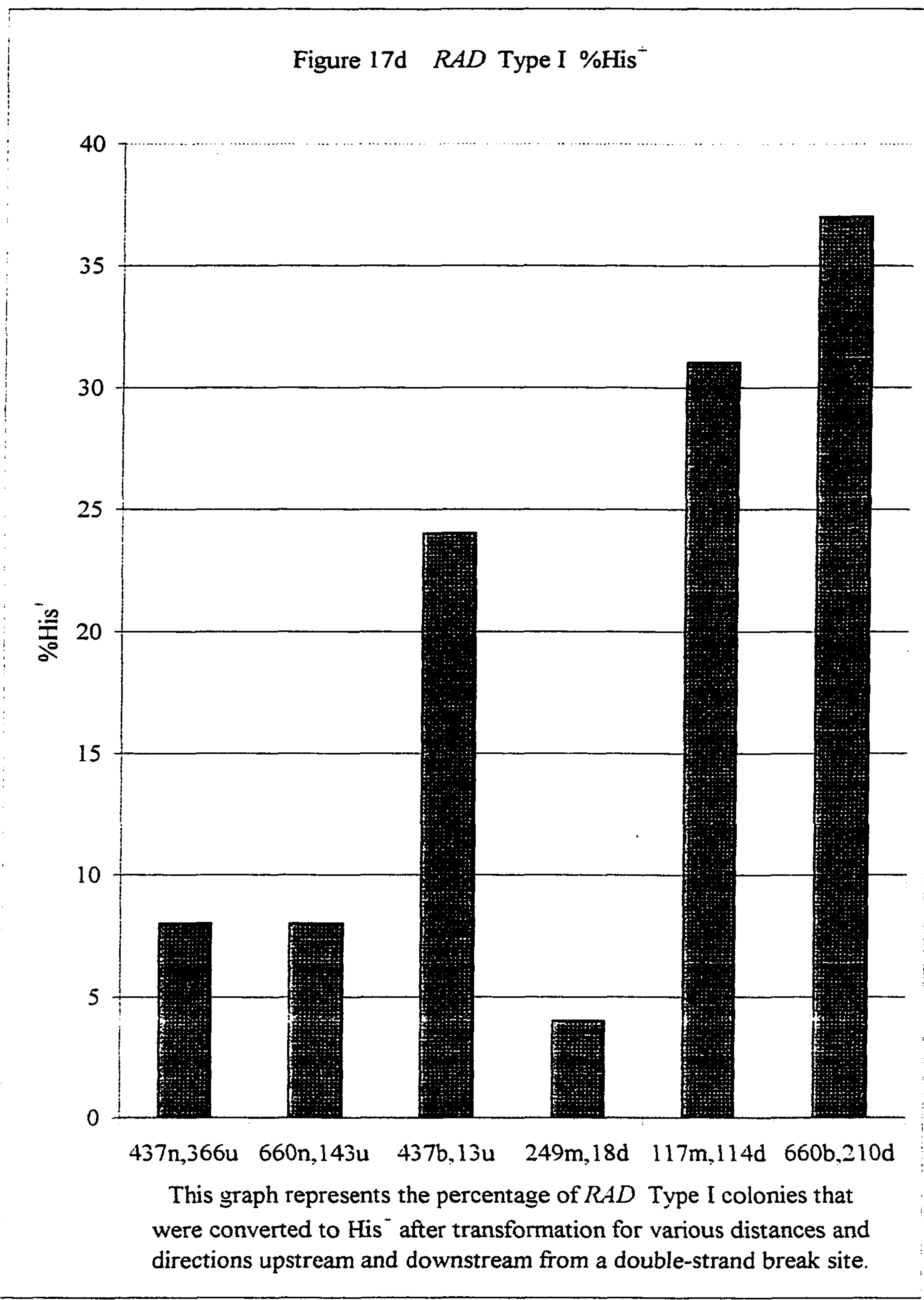

This graph represents the percentage of *RAD* Type I colonies that were converted to His⁻ after transformation for various distances and directions upstream and downstream from a double-strand break site.

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produced by *XbaI* digestion from the amplified *his3* alleles generated by digestion with restriction enzymes. These fragment sizes were used to identify the presence of plasmid or chromosomal *his3* alleles. They examine the direction of conversion between the chromosome and plasmid molecules. Figures 19 & 20 represent an analysis of colonies, which have an integrated plasmid and are converted to His$^-$.

Trp^-_s, His^-_s colonies were lysed and their genomic DNA amplified by PCR. The samples were then digested with *XbaI* and the fragments were separated by gel electrophoresis. Conversion may occur in both directions. Figure 18 demonstrates the possible size bands for plasmid to chromosome conversion, and for chromosome to plasmid conversion for each sample that can be seen on a gel after digestion.

The linearized plasmid may donate information to the uncut chromosome (plasmid cut) or the uncut chromosome may be the donor (chromosome cut). In some samples, conversion has gone in both directions and only *HIS3* alleles are generated (neither cut). Multiple integrations are demonstrated by the column labeled 'both cut' indicating that there are bands characteristic of both plasmid and chromosome patterns, in addition to a third copy of the intact *HIS3* allele.

Integration without conversion can also occur. This is demonstrated by samples 75 *MscI* and 622 *BsiWI* (figure 12). When the markers on the chromosome and plasmid occur on opposite sides of the double-strand break, a crossover event between them will link the marked ends of each gene together on one *his3* allele. The other *HIS3* allele will not carry either marker. The result will be a *HIS3* gene generated without conversion. This was observed with PCR analysis. When an allele carries one marker, two bands are seen

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on a gel after digestion. When an allele carries two markers, three bands are seen. This occurs because the larger of the latter two fragments is cut to yield two smaller fragments due to the addition of the additional restriction site marker from the other allele. It is only possible to see this result in a Type I arrangement. Both samples 75 *MscI* and 622 *BsiWI* are Type I arrangements. This occurred in four colonies of Type I arrangement, and did not occur in any samples of Type II arrangement.

Unstable Trp$^+$, His$^-$ colonies were also analyzed by PCR (table 9). They provide evidence that there is a mechanism of double-strand break repair that is independent of recombinational repair. Sequences were retained in non-conversion repair events. Such *his* genes retained the *XbaI* marker and displayed the corresponding band sizes on gel electrophoresis after digestion.

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Figure 18

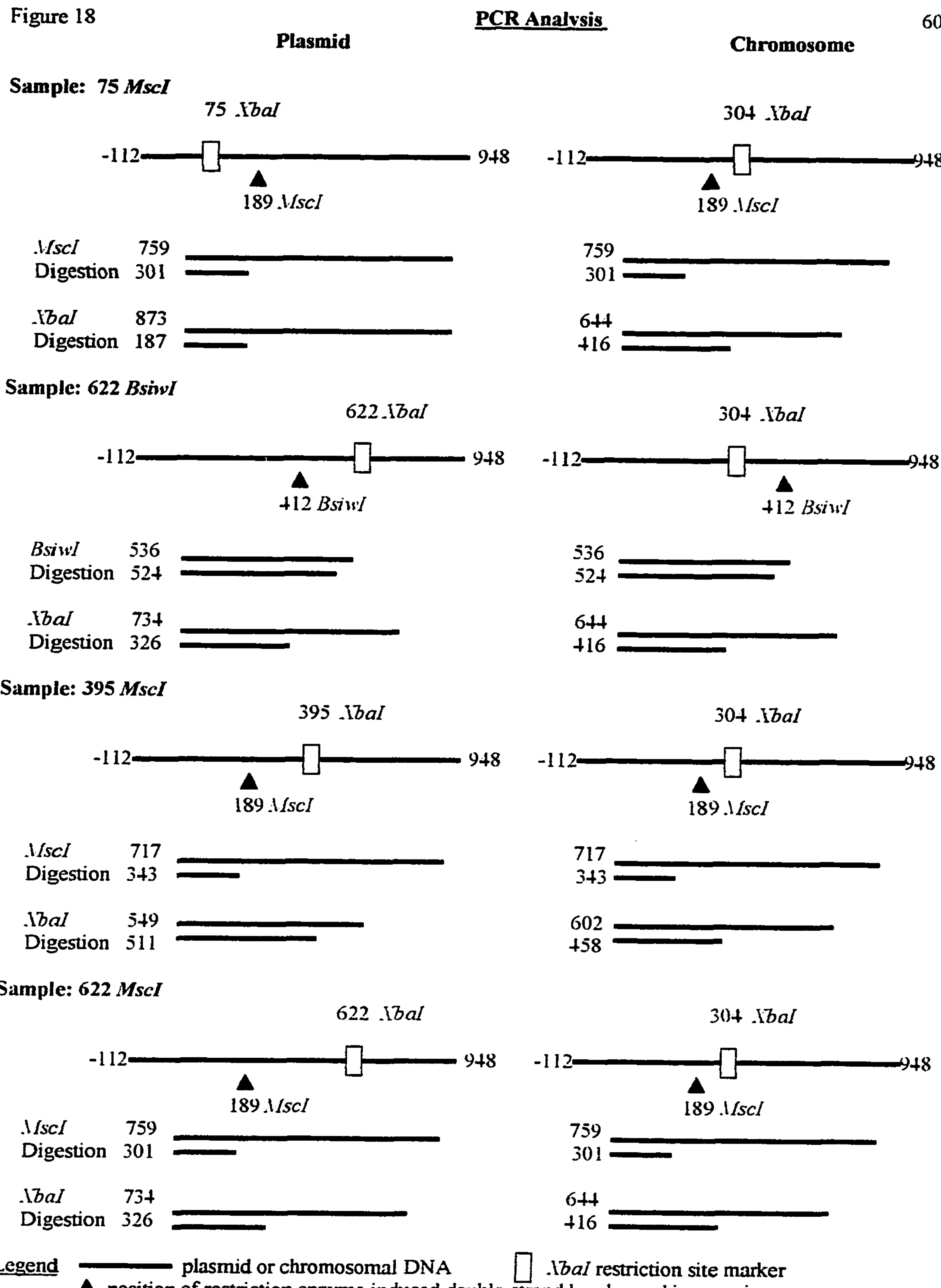

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Figure 19

Plasmid and chromosome alleles acting as donor in gene conversion for *RAD* Type I samples analyses by PCR.

This graph represents colonies that have an integrated plasmid and are converted to His$^-$. Therefore, each sample has at least two copies of the *HIS3* gene in functional or nonfunctional forms. Each PCR analysis showed an intact copy as well as a copy showing the *XbaI* digestion pattern of the plasmid (plasmid cut), the chromosome (chromosome cut), both plasmid and chromosome (both cut), or two functional copies (neither cut). The column labeled 'both cut' represents multiple integrations. Those samples must contain at least three copies of the *HIS3* gene. Samples 75 *MscI* and 622 *BsiWI* can produce a *HIS3* allele by integration without conversion (figure 12). This is represented by the column labeled 'both cut'.

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Figure 19 Plasmid and chromosome alleles acting as donor in gene conversion for *RAD* Type I samples analysed by PCR

Trp^{+}_{s}, His^{+}_{s}

40
35
30
25
20
15
10
5
0

	plasmid cut	chromosome cut	both cut	neither cut	his3 cut twice
■ Series1	35	10	8	5	4

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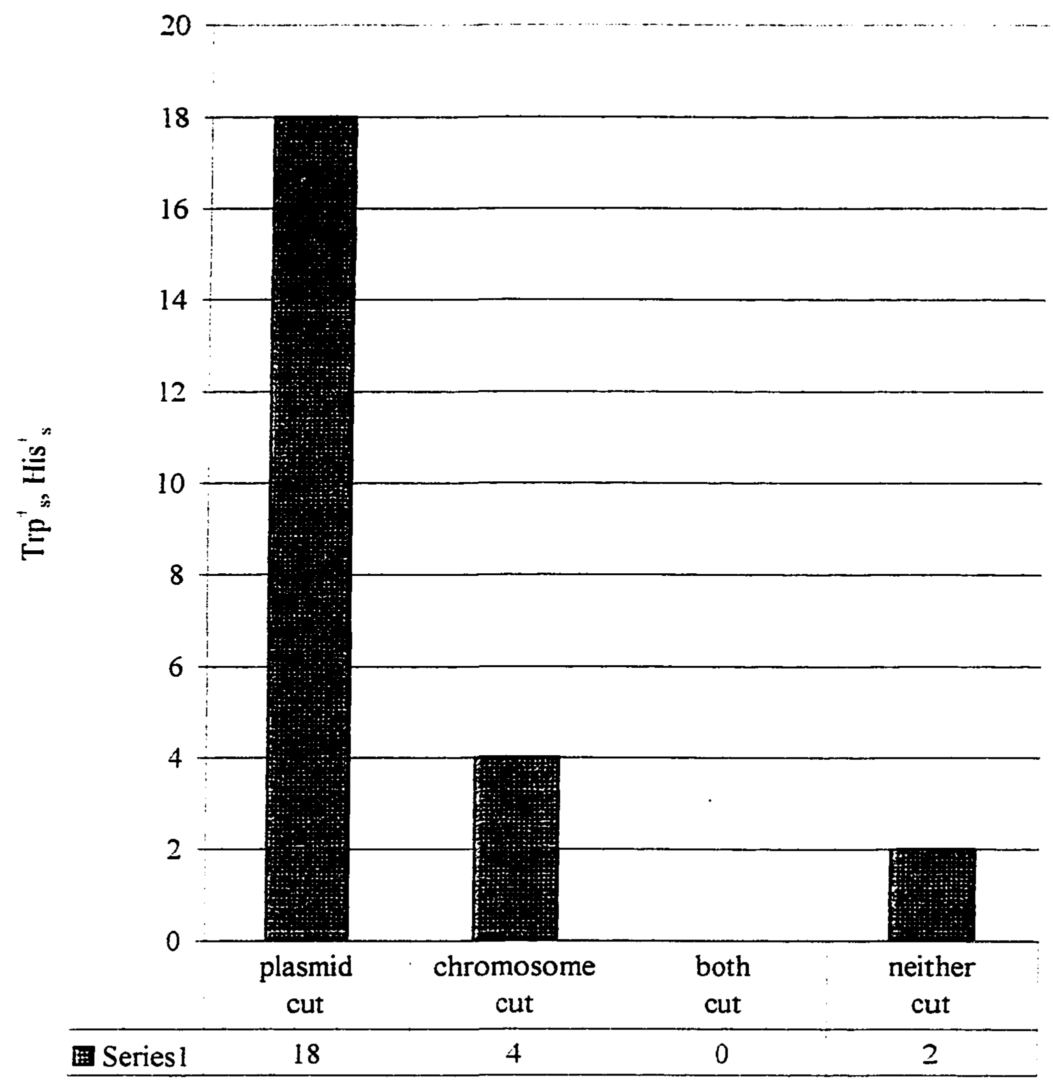
Figure 20 Plasmid and chromosome alleles acting as donor in gene conversion for *RAD* Type II samples analysed by PCR



This graph represents samples that have at least two copies of the *HIS3* gene. Each PCR analysis showed an intact copy as well as a copy showing the *XbaI* digestion pattern of the plasmid, chromosome, both plasmid and chromosome, or two functional copies.

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Table 9 Unstable His^- colonies analysed by PCR

Strain	Plasmid Cut	Chromosome Cut	Both Cut	Neither Cut
RAD	0	0	13	0
rad51	0	0	14	0
rad54	0	0	15	0
rad55	0	0	16	0
rad57	0	0	11	0

This table represents PCR analysis of unstable His^- colonies.

The column labeled 'both cut' lists the number of colonies from each strain in which two *his3* alleles were digested with the restriction enzyme *XbaI*.

Appendix 3 lists additional PCR analyses of His^- unstable colonies that include colonies that do not have two digested *his3* alleles.

Such analyses did not use a refined experimental technique that could detect chromosomal DNA in older colonies.

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Discussion

I have investigated the repair of double-strand DNA breaks in repair-proficient and the recombinational repair-deficient yeast strains *rad51*, *rad54*, *rad55*, and *rad57*. Gene conversion and reciprocal exchange associated with double-strand break repair were also investigated. The repair-deficient strains fell into two categories: 1) *rad55* and *rad57* were partially defective in repair and recombination, and 2) *rad51* and *rad54* were completely defective in recombination, but only partially defective in repair.

Gene conversion associated with double-strand break repair was examined in more detail by following conversion of markers placed at various distances from the site of the double-strand break. There were two classes of recombinants. They differed in the placement of mutations on the plasmid and chromosome with respect to the break. Type I arrangements, which carried markers placed on opposite sides of the break, showed high levels of conversion to His^-. Type II arrangements in which the break and the plasmid marker flanked the chromosome marker on opposite sides, showed low levels of conversion to His^-. These findings confirm the predictions of the double-strand break repair model of recombination.

The dependence of gene conversion on distance from the break was analyzed, but I found no significant effect over the range of 13 to 457 base pairs. Unexpectedly, there was a significant bias in the direction of gene conversion, with a greater incidence downstream than upstream within the *HIS3* gene.

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A) Repair and recombination in repair-proficient yeast

Previous investigators have found double-strand breaks to increase recombination frequency in yeast (Orr-weaver et. al. 1981, Silberman & Kupiec 1994), and mammalian cells (Elliot et. al. 1998, Taghian & Nicoloff 1997). In this system, double-strand breaks within the *HIS3* gene induce gene conversion to His$^-$ as shown by the comparison of linearized to uncut DNA in *RAD* cells (figure 8).

Gene conversion in Type I and Type II samples

The comparison of gene conversion to His$^-$ in Type I and Type II samples (figure 13, table 5) supports the double-strand break repair model. There is significantly more gene conversion in a Type I arrangement than in a Type II arrangement at the 0.001 level of probability.

As shown in figures 10 and 11, there are more ways for a Type I than a Type II arrangement to yield a His$^-$ phenotype. After resolvase separates the heteroduplex in a Type I arrangement, one DNA molecule has a marker sequence on only one strand. This can yield a His$^-$ phenotype if the marker is converted to the wild type, or a His$^-$ phenotype if the wild type is converted to the marker. After resolvase activity in Type II arrangements, the analogous molecule has a marker sequence on both strands. This yields four potential products of which only one can be a His$^-$ phenotype, and has different markers on opposite strands of the same molecule (figure 11). Mismatch repair to *HIS3* requires repair in opposite directions on the two strands by discontinuous conversion tracts. This is relatively infrequent. Continuous tracts are more common (Borts & Haber 1989, Sweetser et. al. 1994, Yi-Shin et. al. 1996). They result when the sequence of only one strand is copied to the other (figure 22). Consequently, there is a lower probability of

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His^- phenotypes being generated in Type II arrangements. This argument presumes that mismatch repair occurs randomly and independently at each site. Mismatch repair usually uses broken DNA as a recipient and unbroken DNA as the donor (Nelson et. al. 1996, Sweetser et. al. 1994, Weng et. al. 1996). This yields an even lower occurrence of His^- in Type II arrangements in practice.

Figure 21 depicts an alternative to mismatch repair that can result in a His^- phenotype. Double-strand break repair can result in a DNA molecule containing a marker on only one strand before mismatch repair (figure 10). Subsequent DNA replication will result in a DNA molecule containing the marker on both strands, and a DNA molecule without the marker on either strand. Cells containing this second DNA molecule would be His^-. On nonselective media, this would result in a sectored colony. Cells in one half of the colony would be His^-, while cells in the other half would be His^+.

Damaged and undamaged DNA molecules as donors in gene conversion

During the resolution of Holliday junctions, a portion of a strand from each DNA molecule is exchanged with the homologous portion from the other molecule (figures 1, 10, & 11). Mismatch repair occurs when the DNA exchanged to a molecule does not have an identical, complementary sequence to the other strand. If the bases on the received DNA are changed to complement the other strand, then information has not been transferred between the two DNA molecules. However, if the sequence on the other strand is changed to complement the sequence on the received DNA, then information has been exchanged between the molecules.

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Figure 21

Alternative to mismatch repair that leads to His^-

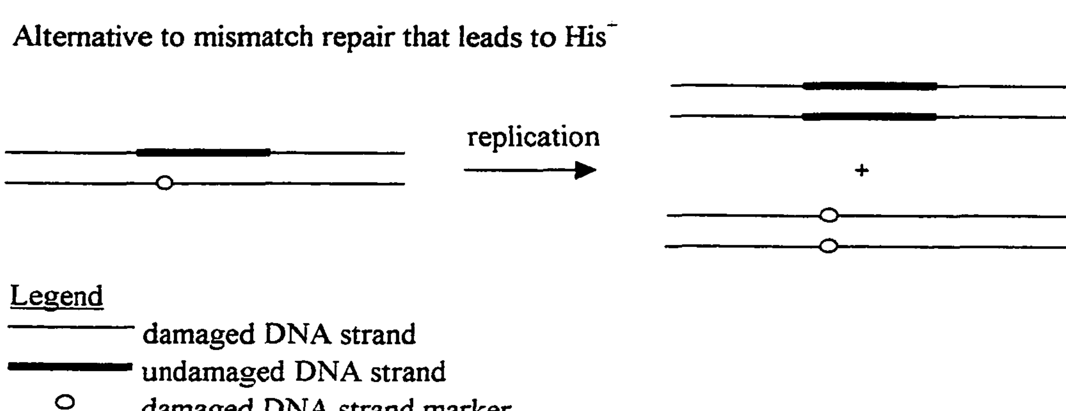

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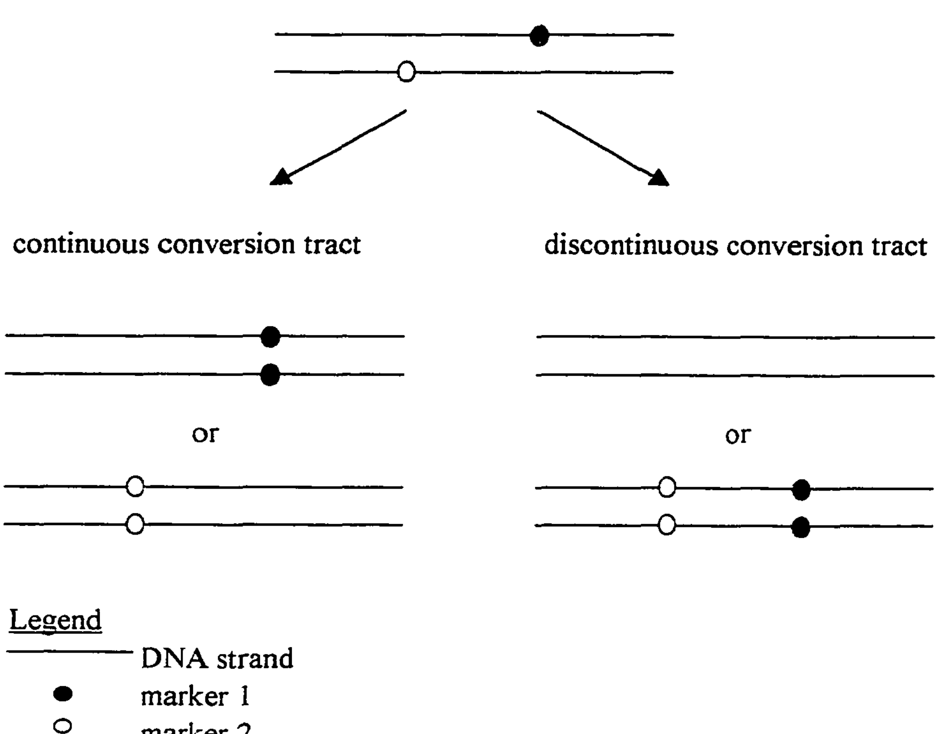
Figure 22
Effects of continuous and discontinuous conversion tracts on DNA repair



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When DNA double-strand break repair occurs, portions of each of the two molecules are exchanged. If bases on the DNA of the damaged molecule are changed by mismatch repair to complement the sequence of the DNA received from the undamaged molecule, then information is said to be passed from the undamaged to the damaged molecule. If bases on the DNA of the undamaged molecule are changed by mismatch repair to complement the sequence of the DNA received from the damaged molecule, then information is said to be passed from the damaged to the undamaged molecule. Either transfer of information can result in a His$^-$ phenotype in our experiments. Our experiments demonstrate that information can be transferred simultaneously from both uncut to linearized DNA and from linearized to uncut DNA.

Although both strands can equally donate sequence information in both Type I and Type II arrangements, a greater proportion of the Type I arrangements are observed as His$^+$ because there is a greater probability that such an exchange will have this result as previously discussed.

An analysis of Trp$^-$ stable, His$^-$ stable colonies by PCR (figures 19 & 20) shows that conversion occurs in both directions from the linearized plasmid to the uncut chromosome and from the uncut chromosome to the linearized plasmid. It also demonstrates that conversion can go in both directions in the same sample. This is demonstrated by the samples labeled 'neither cut'. Since the samples are stable His$^-$ and neither restriction site was cut, both sites had to have been repaired according to the complementary sequence on the other histidine gene. Although relatively more samples with the plasmid cut than with the chromosome cut were found in Type II arrangements, the difference was not significant.

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Using a *HIS3* system, Roitgrund et. al. (1993) also found that donation of DNA can occur from linearized plasmid to unbroken chromosome. In a *HIS3* system Silberman & Kupiec (1994) found that plasmid was unable to donate to chromosome. We have found donation from linearized plasmid to unbroken chromosome by PCR analysis.

According to the synthesis dependent strand annealing model (SSDA) (Paques et. al. 1998), DNA that is newly synthesized does not remain based paired to the template, but are displaced. This allows them to anneal. The total number of repeats in the recipient site may not have the same number of copies as the donor because alignment of complementary strands that are newly synthesized can occur in different registers. Our PCR analysis and the Trp^- unstable, His^- stable colonies in our genetic analysis (column labeled T^-_U, H^-_S in appendix 2) represent a transfer of information from the plasmid to the chromosome. This provides evidence against the synthesis-dependent strand annealing (SDSA) model because the model predicts transfer only from the undamaged to the damaged molecule.

Conversion tract lengths

In this study we were able to observe conversion tract lengths as short as 13 base pairs and as long as 457 base pairs from a damage site (figure 17c).

In analyses of conversion, we found no significant effect of conversion frequency on marker distance from the double-strand break site, whether comparing integrated and extra-chromosomal plasmids, Type I and Type II arrangements, or upstream and downstream directions. This suggests that the distances that we have used in our experiments may not be great enough to detect the effects of conversion tract length.

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Previous studies have observed average conversion tract lengths ranging from less than 500 bases (Sweetser et. al. 1994, Orr-Weaver et. al. 1988, Ahn et. al. 1986) to more than one kilobase (Grimm et. al. 1994, Borts & Haber et. al. 1989, Roitgrund et. al. 1993, Judd & Petes 1988). In studies where homology between DNA molecules was limited, conversion tracts were relatively shorter. When homology was greater, longer conversion tracts were seen.

Using a *HIS3* system, Ahn et. al., (1986) found that most conversions during spontaneous recombination are not co-conversions although both occurred. Frequency of co-conversion decreased with distance such that occurrence was infrequent past 1 kb. Average conversion length was approximately 500bp, and incidence of reciprocal recombination was greater when conversion tract lengths were greater than average.

Direction of gene conversion tracts (upstream and downstream)

We have found there to be more conversion downstream than upstream at the 0.01 level of significance in the *RAD* strain for Type II arrangements (figure 17, table 8). In the *rad55* and *rad57* strains, we have found there to be more conversion downstream than upstream at the 0.001 level of significance. See figure 16 for a comparison of *his3* alleles and digestion sites. All samples in this analysis required conversion from plasmid to chromosome at 104 or 115 base pairs from the double-strand break. Examination of samples containing different distances between chromosome and plasmid restriction markers yield similar results.

Phenotypic conversion to His$^-$ in Type I arrangements are not a reliable indicator for assessing repair differences upstream or downstream from a double-strand break. Type I arrangements could be converted to His$^-$ by a conversion tract in either direction when

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markers are placed on opposite sides of a double-strand break (figure 9). In a Type II arrangement, conversion to His$^+$ can only occur by a discontinuous conversion tract in one direction.

Mismatch repair bias was shown to be influenced by flanking sequences to favor G + C pairs (Brown & Jiricny 1989). In a *URA3* system, Yi-shin et. al. (1996) proposed that biased tract directionality may occur due to selection pressure against Ura$^-$ products that occur when frameshift mutations in donor alleles are transferred to recipient alleles. This is probably not a factor in our experiments because our cells were plated on a medium that lacked tryptophan to select for transformants, but supplied histidine. The production of histidine was later used to screen for conversion (figure 6). Tract directionality was also biased against Ura$^-$ in a study by Weng et. al. (1996). Interstrand crosslinks produced by psoralen have been shown to induce more conversion upstream than downstream in a *his3* system (Greenberg & Saffran, personal communicaton). This leads us to believe that our results are not an artifact of our system.

Ferguson and Holloman (1996) found directionally biased repair in the fungus *Ustilago maydis.* They interpreted the results to mean that DNA ends flanking a gap are subject to different types of processing, and proposed a model featuring a single migrating D-loop. This is based on observations that a heterologous insert placed within a gap in the coding sequence of two different marker genes inhibited repair if the DNA was cleaved at the promoter-proximal junction joining the insert and the coding sequence but not the promoter-distal junction.

Nelmes et. al. (1998) have shown that DNA double-strand break repair requires relocalization of repair proteins to the sites of DNA damage. Since repair is not localized

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to a specific region in the nucleus, repair enzymes may resemble transcription factors, and not replication enzymes. This is because the latter are localized at a specific site in the nuclear matrix. If repair enzymes resemble transcription factors, which synthesize in a five prime to three prime direction, repair machinery can operate in a directionality bias. This may be a strand bias since DNA is antiparallel. Strand bias is established for nucleotide excision repair. 8-oxoguainine endonuclease from human cells, *Schizosaccharomyces pombe* DNA endonuclease, and *Neurospora crassa* DNA endonuclease have been shown to incise five prime to damaged sites (reviewed in Sancar 1996).

If repair enzymes resemble replication enzymes, then conversion upstream may require the ligation of discontinuous fragments resembling Okazaki fragments. This can result in the greater amount of conversion seen downstream.

Our results offer further support in directionality of conversion tracts in wild type, *rad55*, and *rad57* strains. The double-strand break repair model may need to be modified to explain these observations. The molecular machinery of transcription which acts in a strand bias may be involved in double-strand break repair.

Crossing over (plasmid integration)

We do not expect the positioning of the markers to affect integration according to the model (reviewed in Szostak et. al. 1983, Shinohara & Ogawa 1995, Gilbertson & Stahl 1996, Stahl 1996). A comparison of Trp^- stable Type I versus Type II colonies (figure 14, table 6) supports the double strand break repair model. The chi-square value for a comparison between Type I and Type II arrangements for the *RAD* strain at one degree of freedom is not significant at the 0.05 level.

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Association of crossing over with gene conversion

We were able to detect crossing over accompanied by gene conversion by screening for His^{+} stable, Trp^{-} stable colonies (figure 6). Plating on non-selective media was used to dilute out the unintegrated plasmid. Out of 2,887 linearized colonies scored, 189 (6.5%) exhibited crossing over accompanied by gene conversion. Using PCR analysis (figures 19 & 20), we have confirmed the occurrence of gene conversion accompanied by integration. Previous investigators have detected crossing over accompanied by gene conversion (Aguilera & Klein 1989, Ahn et. al., 1986, Bethke & Golin 1994, Borts & Haber 1989, Kobayashi & Takahashi 1988, Kolodkin et. al. 1986, Muris 1997, Orr-Weaver & Szostak 1983, Rattray 1994).

In our experiments the incidence of conversion for integrated plasmids is greater than the incidence of conversion for extrachromosomal plasmids at the 0.001 level of probability for samples with Type I and Type II arrangements. This can be seen when comparing the samples %His^{-} unstable, Trp^{-} unstable to the samples %His^{-} stable, Trp^{-} stable. Samples 75M and 622B can yield His^{-} stable colonies upon integration without gene conversion. When these two samples are eliminated from the chi-square analysis, the incidence of conversion for integrated plasmids remains greater than the incidence of conversion for extrachromosomal plasmids at the 0.01 level of probability. Double-strand breaks can be repaired by crossover, conversion, end joining without crossover or conversion, and crossover accompanied by conversion. Since these results indicate that there is more conversion when integration occurs, the formation of a heteroduplex may increase the probability of crossing over, or crossing over may increase the probability of gene conversion.

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The double-strand break repair model predicts that crossing over should occur with a frequency of approximately fifty percent. Other types of recombination have shown lower frequencies. These include mating type switching, meiotic recombination, and intrachromosomal recombination. Mechanisms for resolution of the recombination intermediate including branch migration, topoisomerase activity, or replication are proposed to account for this (reviewed in Stahl 1996).

Using a mitotic *HIS3* system, Orr-Weaver & Szostak (1983) found that gene conversion accompanied by double-strand gap repair can occur with or without crossing-over. Also using a *HIS3* system, Ahn et. al. (1986) found that the incidence of reciprocal recombination was greater when conversion tract lengths were greater than the average of 500 bp. Using an intrachromosomal recombination system, Aguilera & Klein (1989) found that 50% of yeast mitotic gene conversions also had crossover. Kobayashi & Takahashi (1988) found conversion to be frequently accompanied by crossover in *Escherichia coli* during repair induced by a double-strand gap. Kolodkin et. al. (1986) found double-strand break induced meiotic recombination at the MATa locus in yeast to be associated with gene conversion. Bethke & Golin (1994) found spontaneous mitotic gene conversion to be accompanied by crossing over in yeast. Using restriction site heterologies, Borts & Haber (1987) found 59% of yeast meiotic crossovers to be associated with conversion. In an *ade2* intra-chromosomal recombination assay, 50 percent of recombinants occurred by gene conversion without crossing over, 35 percent by crossing over and 15 percent by crossover associated with conversion (Rattray & Symington, 1994). In *Schizosaccharomyces pombe*, using a *leu1* system for targeted integration, Muris (1997) found that most transformants had integrated plasmid while

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gene conversion occurred in less than 10 percent of transformants. In a mitotic *his1* system, gene conversion was shown to be negligible under *rad54* suppressed double-strand break repair (36 degrees Celsius) (Frankenberg-Schwager et. al., 1994).

An analysis of Trp$^+$ stable, His$^-$ stable colonies by PCR (figures 19 & 20) shows that integration without conversion can occur. This is demonstrated by samples 75 *MscI* and 622 *BsiWI* (figure 12). When the markers on the chromosome and plasmid occur on opposite sides of the double-strand break, a crossover event between them will link the unmarked ends of each gene together on one *his3* allele. The other allele will not carry either marker, and will be *HIS3*. This was observed by PCR analysis. When an allele carries one marker, two bands are seen on a gel after digestion. When an allele carries two markers, three bands are seen. It is only possible to see this result in a Type I arrangement such as 75 *MscI* or 622 *BsiWI*.

A PCR analysis of His$^+$, Trp$^-$ stable colonies from samples that had a Type I arrangement revealed that both chromosome and plasmid markers were retained in a single *his3* allele while a *HIS3* allele contained neither marker. This provides evidence that integration can occur independently of a conversion event or perhaps that the conversion tract was too short to be detected.

B) Repair and recombination in repair-deficient strains

Liefshitz et. al. (1995) proposed that recombinational repair has three functional subgroups: 1) *RAD52*, 2) *RAD51 & RAD54*, and 3) *RAD55 & RAD57*. Our data is consistent with this. We have found that *rad51* and *rad54* were completely defective in recombination while *rad55* and *rad57* were partly deficient.

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Aguilera (1995) states that there are three *RAD52* dependent pathways for repair; a *RAD51, RAD52, RAD57* dependent pathway for recombination, conversion, and reciprocal exchange; a *RAD1, RAD52* dependent deletions pathway; and a *RAD52* dependent recombination, conversion, reciprocal exchange pathway that is independent of *RAD51* and *RAD57*. Since we have found that *rad57* is partially deficient in recombination and conversion, it is consistent with the proposal by Aguilera regarding the role of this gene. Our data can support that *RAD57* is taking part in one pathway while it is not necessary for another.

Alternate pathways of double-strand break repair

The comparison of linearized versus uncut plasmids transformed into the five recombinational repair-deficient strains (figure 7, table 2) provides evidence that there is a mechanism of double-strand break repair that is independent of the *RAD52* epistasis group. Even though there is a significant decrease, each of these mutants shows repair of more than half that of the *RAD* strain. This shows that even though these products can take part in this type of repair, there is another mechanism in addition to homologous recombination that can act on double-strand breaks.

Orr-Weaver & Szostak (1983) found that linear plasmid DNA can be circularized even though it was digested with a restriction enzyme in a sequence without homology to a chromosomal sequence. They suggested that there is a recombination independent pathway for the repair of double strand breaks which functions through the ligation of cohesive ends. Glasunov et. al. (1995) measured repair of gaps in plasmid DNA and found that four hundred base pair gaps can be ligated by non-recombinational repair. This was shown to be cold sensitive (23 degree Celsius) in a *rad54* strain. Homology between

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linearized plasmid and chromosomal DNA was essential for repair of a double-strand gap without deletions.

In our PCR analysis of Trp^- unstable, His^- colonies, it was revealed that sequences were retained in non-conversion repair events. Such *his* genes retained the *XbaI* marker and displayed the corresponding band sizes on gel electrophoresis after digestion. Since the colonies were Trp^+ unstable, we know that the plasmids were repaired, but not integrated. If the plasmid was not repaired, the colony could not be Trp^-, and if the plasmid was integrated, it would remain Trp^- after growth on nonselective media. These results support a ligation mechanism for repair, because the plasmids could be re-cut by the original restriction enzyme while integration did not take place.

One possible alternative pathway involves the Ku protein which is also involved in V(D)J recombination in the mammalian immune system (Chu 1996). Ku is part of a DNA dependent protein kinase that binds to chromosomal ends (Weaver 1996). It rejoins the ends of DNA during immunoglobulin gene rearrangements to produce a variety of antibodies (Chu 1996). It may also be involved in the rejoining of broken chromosomal ends for DNA repair. It protects DNA from radiation, and has been identified in both yeast and mammals (Feldmann & Winnacker 1993, Lieber et. al. 1997, Rathmell & Chu 1994, Smider et.al. 1994, Taccioli et. al. 1994, Troelstra & Jaspers 1994). The *HDF1* gene in yeast has been identified as the homologue of the 70 kD subunit of the Ku protein. Siede et. al. (1996) have shown that while diploid yeast cells that are mutant in *HDF1* are not sensitive to ionizing radiation and methly methanesulfonate, in *rad52* mutants, inactivation of *HDF1* results in additional sensitization. They propose that *HDF1* plays a role in a non-recombinational repair process, and suggest a mechanism of

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double-strand break repair that involves direct DNA end-joining. Lieber (1999) states that non-homologous end joining is essential for viability in multi-cellular eukaryotes. Since Ku is thought to be the primary means of double-strand break repair in mammals and homologous recombination is the favored pathway in yeast, Siede et. al. (1996) suggest that it may have gained significance during the evolution of higher eukaryotes and was recruited for V(D)J recombination.

Both Ku and Rad52 bind to double-strand breaks. Ku directs the ends into a non-homologous end joining pathway while Rad52 directs them into homologous recombination (Van Dyck et. al. 1999).

DNA-dependent protein kinase (DNA-PK) and Ku function in DNA end-joining in V(D)J recombination and non-homologous double-strand break repair (Yaneva et. al. 1997). Ku is necessary in V(D)J recombination in both coding and signal joint formation (Kulesza & Lieber 1998). DNA-PK is activated for phosphorylation of protein targets when it binds to the terminal ends of DNA. Ku can stimulate DNA-PK when it independently binds to DNA alongside it. DNA-PK may interact with a calcium binding protein that may regulate kinase-phosphotase DNA end joining (Wu & Lieber 1997). Ku70 and KU 80 may function in V(D)J recombination and DNA repair that are independent of DNA-PK as well (Gao et. al. 1998, Kulesza & Lieber 1998). The genes RAD50 MRE11, XRS2, SIR2, SIR3, and SIR4 are essential for Ku-dependent double-strand break repair (Boulton & Jackson 1998).

Ku has a direct role in establishing a normal DNA end structure in yeast (Gravel et. al. 1998). It also maintains the integrity of the telomere structure by protecting the ends from

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nucleases and recombinases (Polotnianka et. a. 1998), and is necessary for silencing of genes near telomeres in yeast (Boulton & Jackson 1998).

Ku appears to participate in DNA replication by regulating the number of rounds of replication in the cell cycle and the structure of the initiation complex (Polotnianka et. a. 1998).

Plasmids linearized with *MscI* to produce blunt ends resulted in less repair than those linearized with *BsiwI* or *NsiI* which produce four base pair, five prime and three prime overhangs respectively (figure 7b). Schmidt et. al. (1994) reported greater topoisomerase mediated DNA ligation with base pairing ability between two molecules.

Double-strand break induced recombination

The proportion of gene conversion to the number of transformations is significantly lower in all Rad$^-$ strains studied than in recombination-proficient yeast (figure 18). The chi-square values comparing *RAD* to *rad51*, *rad54*, *rad55*, and *rad57*, are all significant at the 0.001 level of probability. This provides evidence that the *rad51*, *rad54*, *rad55*, and *rad57* gene products are involved in double-strand break induced conversion. However, *rad55* and *rad57* yeast are partially defective in recombination, while *rad51* and *rad54* strains showed profound deficiencies.

A comparison of the percentage of His$^+$ colonies to a percentage of Trp$^-$ stable colonies (figure 15, table 7) showed that conversion and integration decreased similarly in Rad$^-$ strains. The *rad* genes in this study are involved in both conversion and integration. This suggests that the recombinosome is needed for both processes.

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***RAD51* and *RAD54* are necessary for recombination**

RAD51, and *RAD54* have been shown to be essential in recombinational repair (Abe et. al. 1994, Aguilera 1995, Contopoulou et. al. 1987, Glazer et. al. 1989,1990, Glasunov et. al. 1989, Jha 1993, Moore 1978, Muris 1997, Nevo-Caspi & Kupiec 1994, Perera et. al. 1988, Rattray 1994, Schiestl et. al. 1990, Hays et. al. 1995, Vishnevetskaia et. al. 1983).

RAD51 has sequence homology to RecA (Lovett 1994), and is a functional homologue of RecA (reviewed in Friedberg et al 1995). Aided by RPA, *RAD51* can perform homologous pairing and low levels of strand exchange activity (Sung 1994). It forms helical filaments with single stranded DNA (Ogawa et al 1993) similar to those of RecA. Rad51 interacts with the *RAD55* gene product (Hays et. al. 1995) which aids the recombinosome by facilitating strand invasion into donor sequences (Sugawara et al 1995). The *RAD51* gene product also interacts with the *RAD52* gene product (Donovan et. al. 1994, Sharon et. al. 1995) which modulates its catalytic activities (Shen et al 1996), Rad52 may also anneal homologous single stranded DNA to a duplex (reviewed in Shinohara 1995).

RAD54 has homology to helicases although it has not been demonstrated to have helicase activity (reviewed in Friedberg et al 1995). *RAD54* is postulated to be part of the recombinosome (Jiang et al 1996). Rad51 and Rad54 proteins have been shown to interact (Clever et. al. 1997, Jiang 1996, Petukhova et. al. 1998), and form a direct association (Jiang, 1996, Golub 1997). Petukhova et. al. (1998) have shown that the Rad54 protein has double-strand DNA-dependent ATPase activity, and addition of Rad54

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to Rad51 strongly stimulates pairing between homologous single-stranded and double-stranded molecules of DNA.

The *E. Coli* RecA protein performs all these functions by itself (reviewed in Friedberg, 1995).

<u>*RAD51*</u> Double-strand breaks did not induce either conversion or integration in *RAD51* deficient yeast in our experiments. Previous investigators have found *rad51* to be deficient in repair of double-strand breaks (Abe et. al. 1994, Aguilera 1995, Contopoulou et. al. 1987, Glasunov et. al. 1989, Jha 1993, Moore 1978, Nevo-Caspi & Kupiec 1994, Schiestl et. al. 1990, Vishnevetskaia et. al. 1983). Other investigators have shown *RAD51* to stimulate the repair of double-strand breaks, or overcome repair deficiencies (Johnson & Symington 1995, Scully 1977, Sugawara et. al. 1995, Sung 1997, Vispe et. al., 1998, Yuan 1998). Ionizing radiation was shown to stimulate *RAD51* expression (Basile et. al., 1992, Schild et. al., 1995, Yuan 1998). *RAD51* has been shown to be involved in DNA double-strand break repair in other systems including *rhp51* in *Schizosaccharomyces pombe* (Muris 1997), human *RAD51* transgene in chicken B lymphocytes (Sonoda, 1998), and hamster CHO cells (Vispe et. al., 1998).

The role of the *RAD51* gene product appears not to include illegitimate recombination which takes place between nonhomologous DNA sequences (Tsukamoto et. al., 1996), and nonhomologous end joining (Moore & Haber, 1996). The *RAD51* gene product is also not needed in RNA-mediated recombination (Derr 1998), or direct repeat recombination (Mcdonald & Rothstein, 1994), but appears to play an inhibitory role. Like *Rad51*, the *RAD54* gene product does not appear to be involved in nonhomologous end joining (Moore & Habe, 1996), and illegitimate recombination (Tsukamoto et. al., 1996).

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In contrast to *RAD51*, *Rad54* appears to not only be unnecessary in direct repeat recombination, but may play an inhibitory role (Mcdonald & Rothstein, 1994).

Nonhomologous end joining did not require the *RAD51* and *RAD54* gene products in our experiments. Figure 7 portrays the repair of double-strand breaks in repair deficient strains. Although repair deficient strains show a lower rate of transformations for linearized versus uncut DNA than a repair proficient strain, significant transformation does take place. PCR analysis of His$^-$, Trp$^-$ unstable colonies (appendix 3) have shown conservation of the restriction site marker. This indicates a repair mechanism of homologous endjoining.

Schiestl et. al. (1994) report that restriction enzyme mediated events are independent of *RAD51*. Restriction enzyme mediated events involve activity of the restriction enzyme within the yeast cell, and cutting of chromosomal sequences as well as plasmids.

RAD54 Double-strand breaks did not induce either conversion or integration in *RAD54* deficient yeast.

Previous investigators have found *rad54* to be deficient in repair of double-strand breaks (Contopoulou et. al., 1987, Glazer et. al. 1989,1990, Glasunov et. al. 1989, Iadgarov & Kovel'tsova 1979, Jha et. al., 1993, Muris 1997, Perera et. al., 1988, Schiestl et. al. 1990). *rad54* strains were also found to be sensitive to radiation and DNA damaging agents (Iadgarov & Kovel'tsova 1979, Moore 1978, Zakharov et. al. 1983). Ho & Mortimer (1975) found a temperature sensitive *rad54* strain to have a similar x-ray survival response to a *RAD* strain at permissive temperature but was sensitive at restrictive temperatures. Petukhova (1998) found that the Rad54 protein has double-strand DNA-dependent ATPase activity, and addition of Rad54 to Rad51 strongly

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stimulates pairing between homologous single-stranded and double-stranded molecules of DNA.

Simon & Moore (1990) found that in *LEU*, *URA*, and *TRP* systems, there was no significant effect on recombination with undamaged single- or double-stranded circular DNA in *rad54* mutants.

In our study double-strand breaks did not induce conversion or integration in *rad51* and *rad54* strains. Previous investigators have not demonstrated this decrease in both repair events simultaneously, or in both mutants using the same system. Repair did occur in our experiments using the *rad51* and *rad54* strains, but not homologous recombination repair. Since the *RAD51* and *RAD54* gene products are not required for nonhomologous end joining repair, then the repair that occurred in our experiments was probably nonhomologous end joining repair. This can be tested by performing such experiments using strains that are deficient in *RAD51* or *RAD54*, and *HDF1* which performs in non-recombinational repair (Siede et. al. 1996). Since repair was significantly lower in the repair-deficient strains in our experiments than in the *RAD* strain (figure 7), we have evidence that nonhomologous end joining repair is not as efficient as homologous recombination repair in yeast.

Deletion of *RAD55* or *RAD57* decreases recombination

The *RAD55* gene product interacts with the *RAD51* gene product (Hays et. al. 1995, Johnson & Symington 1995) and aids the recombinosome by facilitating strand invasion into donor sequences, which would otherwise be inaccessible (Sugawara et al 1995). The *RAD55* gene product also interacts with the *RAD57* gene product and the two may help to stabilize the recombinosome at low temperatures (Hays et. al. 1995, Lovett 1987). Yeast

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Rad55 and Rad57 were shown to form a stable heterodimer and stimulate strand exchange when included with Rad51 and RPA (Sung 1997). Since they form a heterodimer, a mutation in either gene can be expected to have the same effect. Our results are consistent with this assertion.

rad55 and *rad57* yeast were found by several investigators to be sensitive to radiation and DNA damaging agents (Moore 1978, Schiestl et. al. 1990, Vishnevetskaia et. al. 1983). They have been found to be cold sensitive to double-strand breaks and ionizing radiation (Glaser & Glasunov 1997, Ho & Mortimer 1975, Johnson & Symington 1995, Lovett & Mortimer 1987, Perera et. al. 1988), and inverted-repeat recombination (Rattray & Symington 1995), suggesting that Rad55 and Rad57 stabilize the recombinosome at low temperatures. Mutants of *RAD57* have been found to be deficient in gene conversion (Aguilera 1995).

rad55 and *rad57* cells have been shown to have comparable rates to the wild type for illegitimate recombination (Tsukamoto et. al., 1996), and RNA-mediated gene conversion (Derr 1998).

Double-strand breaks induced both gene conversion and crossing over in *rad55* and *rad57* strains, although at significantly lower levels than in Rad$^+$ cells. There is no significant difference in double-strand break induced gene conversion between *rad55* and *rad57* strains. This supports the proposed structure of the recombinosome, and is consistent with previous reports in the literature.

The conversion to integration ratios were lower for the *rad55* and *rad57* strains than for the *RAD* strain (table 7). This was significant at the 0.001 and 0.01 levels of probability for Type I and Type II arrangements respectively in the *rad57* strain. It was

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not significant at the 0.05 level of probability for the *rad55* strain. This may be because the experiments using the *rad57* strain yielded a greater number of colonies, and therefore can calculate a greater significance. Further investigation with larger sample sizes may be necessary to obtain more accurate results. If no differences are found in conversion to integration ratios between Rad$^+$ and Rad$^-$ strains, it could mean that the products of *rad55* and *rad57* are involved in formation of the intermediate. If differences are found, then it could mean that the gene products are involved in the formation of different intermediates than the wild type, or they are involved in resolution of the intermediate.

Double-strand break repair has a fast phase that is completed in thirty to forty minutes and a slow phase that is completed within forty eight hours. Glasunov et. al. (1989) found that *rad55* inhibits the fast phase and *rad57* does not. Glaser et. al. (1989, 1990) found that *rad55* was not involved in the repair of double-strand gaps in plasmid DNA while *rad57* was deficient in this repair.

Mutations in *RAD55* and *RAD57* stimulated direct repeat recombination (Mcdonald & Rothstein 1994), and RNA mediated recombination (Derr 1998, Mcdonald & Rothstein 1994).

Borts et. al. (1986) found *rad57* mutants to make nearly as much recombined restriction fragments as the wild-type. The *RAD57* gene product was not found to be involved in cDNA-mediated recombination (Nevo-Caspi & Kupiec, 1994), but may act as an inhibitor in this pathway.

Schiestl et. al. (1994) found restriction enzyme mediated events to be independent of *RAD57*. Simon & Moore (1990) found no significant effect on recombination with single

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stranded or double stranded circular DNA in a *rad57* mutant in *LEU*, *URA*, and *TRP* systems. These studies analyze different aspects of recombination than the ones presented here.

In our study we found that both crossover and integration induced by double-strand breaks were significantly decreased in *rad55* and *rad57* strains when compared to a *RAD* strain. Previous investigators have not demonstrated this decrease in both repair events simultaneously, or in these two mutants using the same system. Our study demonstrates that conversion was decreased to a similar extent in both *rad55* and *rad57*. This provides evidence that the roles of Rad55 and Rad57 are linked.

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Conclusions

Double-strand breaks in plasmid DNA are repaired in both wild type and recombination-deficient strains of yeast. The repair efficiency is higher in recombination-proficient then in recombination-deficient yeast, but is similar in *rad51*, *rad54*, *rad55*, and *rad57* strains. The residual double-strand break repair seen in these strains indicates the presence of another double-strand break repair pathway independent of homologous recombination.

Double-strand breaks stimulate gene conversion and reciprocal exchange, in accord with the predictions of the double-strand break repair model. The recombination-deficient strains fall into two groups: *RAD51* and *RAD54* are required for both gene conversion and plasmid integration, while loss of *RAD55* or *RAD57* reduces, but does not eliminate recombination. Gene conversion was found to decrease more than plasmid integration in *rad55* and *rad57* yeast.

Gene conversion at distances of 13 to 457 bases from the double-strand break site was measured. No significant decrease with distance was observed, suggesting that the average conversion tract length in this system is greater than 400 bases. The observed frequency of conversion to His$^-$ depended on the arrangement of genetic markers with respect to the double-strand break: this frequency was lower in arrangements requiring discontinuous conversion tracts, indicating that these are rare. Both uncut (chromosomal) and linearized (plasmid) DNA can be donors in gene conversion events.

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Transformations: Number of His^+ and Trp^+ colonies grown on selection media.

Experiment		356 *RAD*			357 *RAD*		
		His	Trp	His/1000 Trp	His	Trp	His/1000 Trp
75	o	11	12,760	0.043	0	184	<0.1
	b	62	9,180	0.335	61	145	8.4
	m	207	4,150	2.49	131	78	33.6
	n	74	8,540	0.435	65	181	7.2
207	o	17	11,360	0.075	6	234	0.51
	b	31	3,670	0.42	22	107	4.1
	m	2	2,960	0.034	0	77	<0.26
	n	8	4,780	0.1	16	114	2.8
304	o	20	36,680	0.0273	14	1,913	0.146
	b	8	24,080	0.017	6	496	0.24
	m	16	26,480	0.03	2	422	0.095
	n	11	33,640	0.0165	13	1,074	0.24
395	o	10	12,230	0.041	2	279	0.14
	b	413	10,960	1.885	296	118	50
	m	102	6,370	0.8	94	83	22.7
	n	203	16,960	0.6	137	229	12
622	o	312	16,400	0.95	89	359	5
	b	399	8,960	2.2	297	121	49.1
	m	192	11,080	0.865	108	48	45
	n	231	14,080	0.82	175	743	4.7

Legend

o - circular DNA, b - linearized by digestion with *BsiWI*

m - linearized with *MscI*, n - linearized with *NsiI*

The numbers 75, 207, 304, 395, and 622 in the first column refer to the marker location on a *his3* allele. Sample 304 is used as a control for conversion to His^-.

The column labeled 'His' refers to the number of colonies that grew on plates with media that lacked histidine.

The column labeled 'Trp' refers to the number of colonies that grew on plates with media that lacked tryptophan.

The column labeled 'His/1000Trp' is a comparison of the number of colonies that grew on plates with media lacking histidine per thousand colonies that gew on plates lacking tryptophan.

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		361 *RAD*			370 *RAD*		
		His	Trp	His/1000Trp	His	Trp	His/1000Trp
75	o	94	1,844	0.86	24	861	0.56
	b	270	451	12	643	1138	11.3
	m	602	188	64	1,256	432	58.1
	n	187	252	14.8	289	1,044	5.5
207	o	35	759	0.9	12	289	0.83
	b	256	671	7.6	557	1,076	10.4
	m	8	275	0.6	0	0	0
	n	86	868	2	111	806	2.8
304	o	12	1,762	0.14	16	1,250	0.26
	b	44	1,504	0.59	37	1,302	0.56
	m	48	1,208	0.8	42	770	1.1
	n	11	3,640	0.33	13	1,074	0.24
395	o	20	1,091	0.37	7	1,214	0.12
	b	1,352	706	38.3	2,636	1,756	30
	m	349	240	29.1	429	534	16.1
	n	400	549	14.6	213	548	7.8
622	o	822	1,027	16	415	466	17.8
	b	978	371	52.7	1,492	487	61.3
	m	227	248	18.3	448	725	12.4
	n	482	748	12.9	158	3,040	1

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		360 *rad51*			352 *rad51*		
		His	Trp	His/1000Trp	His	Trp	His/1000Trp
75	o	2	71	0.56	1	60	0.33
	b	-	18	-	0	32	<0.63
	m	3	6	10	0	7	<2.9
	n	-	7	-	0	39	<0.51
207	0	1	29	2.2	1	82	2.4
	b	0	1	<20	0	20	<10
	m	0	9	<2.2	0	26	<0.77
	n	-	8	-	1	50	0.4
304	o	0	33	<0.61	1	248	0.081
	b	0	2	<10	1	190	0.11
	m	1	7	2.9	4	90	0.89
	n	-	37	0.54	6	163	0.74
395	o	0	23	<0.87	0	205	<0.1
	b	0	1	<20	1	27	0.74
	m	0	0	0	1	3	6.7
	n	0	14	<0.14	0	85	<0.24
622	o	79	27	59	43	61	14
	b	2	5	8	1	20	1
	m	-	3	<13	0	7	<2.9
	n	22	16	28	18	71	51

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		363 *rad51*		
		His	Trp	His/1000 Trp
75	o	0	4,275	<0.0047
	b	0	3,033	<0.0066
	m	1	8	2.5
	n	0	3,827	<0.0052
207	o	2	4,026	0.0099
	b	0	3,813	<0.0052
	m	0	3,657	<0.0055
	n	0	4,101	<0.0049
304	o	0	4,692	<0.0043
	b	2	3,530	0.011
	m	2	4,498	0.0089
	n	2	4,952	0.0081
395	o	0	5,575	<0.0036
	b	0	4,385	<0.0046
	m	0	4,366	<0.0046
	n	0	4,632	<0.0043
622	o	96	5,084	0.38
	b	2	12	3.3
	m	0	2	10
	n	38	5,571	0.136

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		365 *rad54*			371 *rad54*		
		His	Trp	His/1000 Trp	His	Trp	His/1000 Trp
75	o	1	1	20	24	502	0.96
	b	1	2	10	38	892	0.85
	m	0	0	-	15	195	1.54
	n	0	0	-	5	329	0.3
207	o	0	3	<6.7	26	264	1.97
	b	0	2	<10	36	367	1.96
	m	0	0	-	0	0	-
	n	0	0	-	18	224	1.61
304	o	0	5	<4	13	880	0.3
	b	0	1	<20	27	541	1
	m	0	0	-	44	400	2.2
	n	0	7	2.9	14	790	0.35
395	o	0	4	<5	2	746	0.051
	b	0	2	<10	21	548	0.77
	m	0	1	<20	2	100	0.4
	n	0	1	<20	4	192	0.42
622	o	22	2	220	328	144	45.6
	b	3	0	-	15	122	2.46
	m	1	0	-	22	77	5.71
	n	13	5	52	380	409	18.6

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		366 *rad55*			367 *rad55*		
		His	Trp	His/1000 Trp	His	Trp	His/1000Trp
75	o	1	1	20	24	502	0.96
	b	0	9	<2	0	8	<2.5
	m	1	1	20	9	7	26
	n	2	10	4	0	5	<4
207	o	0	18	<1.1	1	22	0.9
	b	0	4	<5	2	10	4
	m	0	12	<1.7	1	16	1.25
	n	0	10	<2	0	4	<5
304	o	2	115	0.35	1	42	0.48
	b	0	48	<0.42	1	11	1.8
	m	2	21	1.9	2	15	2.6
	n	2	48	3.5	4	81	0.99
395	o	0	4	<5	0	11	<1.8
	b	9	13	14	24	10	48
	m	2	0	-	1	5	4
	n	1	6	3.3	3	-	-
622	o	41	15	55	105	8	260
	b	4	5	16	18	1	360
	m	2	0	-	7	0	-
	n	20	22	18	-	-	-

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		368 *rad57*			369 *rad57*		
		His	Trp	His/1000 Trp	His	Trp	His/1000 Trp
75	o	0	253	<0.158	0	186	<0.215
	b	0	162	<0.25	0	50	<0.8
	m	3	40	1.5	0	18	<2.2
	n	0	64	<0.6	0	73	<0.55
207	o	0	316	<0.1	0	122	<0.33
	b	2	95	0.84	2	40	2
	m	0	82	<0.5	0	83	<0.48
	n	0	154	<0.3	0	88	<0.45
304	o	2	713	<0.1	2	249	<0.32
	b	0	382	<0.1	0	176	<0.23
	m	1	101	0.4	0	49	<0.82
	n	3	1015	0.118	0	252	<0.16
395	o	0	313	<0.1	0	133	<.3
	b	5	76	3	3	71	1.69
	m	0	29	<1	0	7	<5.7
	n	2	129	0.6	0	82	<0.49
622	o	173	309	22.3	15	207	2.9
	b	9	55	6.5	2	25	3.2
	m	1	23	2	0	4	10
	n	22	208	4.2	17	100	6.8

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Appendix 2

Number of His$^+$ and Trp$^+$ colonies out of 100 grown transformations on selecton medi

	356 RAD								361 RAD						
	total	$T^+_u H^-_u$	$T^-_s H^-_u$	$T^+_u H^+_s$	$T^+_s H^+_s$	$T^-_u H^-$	$T^+_s H^-$		total	$T^+_u H^-_u$	$T^-_s H^+_u$	$T^+_u H^-_s$	$T^-_s H^+_s$	$T^+_u H^-$	$T^-_s H^-$
75o	100	0	0	0	0	100	0	75o	100	1	0	0	0	99	0
75b	100	0	0	0	7	93	0	75b	100	0	1	4	4	70	21
75m	100	1	4	1	39	54	1	75m	99	10	6	7	10	58	8
75n	100	0	1	11	21	67	0	75n	100	1	0	0	1	90	8
207o								207o	100	12	0	0	0	88	0
207b								207b	95	1	1	3	4	61	25
207m								207m	99	4	0	0	0	95	0
207n								207n	100	1	1	0	0	93	5
304o	100	0	0	0	0	100	0	304o	100	2	0	0	0	96	2
304b	100	0	0	0	1	99	0	304b	100	2	0	0	0	47	51
304m	100	0	0	0	0	100	0	304m	100	0	0	0	0	55	45
304n	100	0	0	0	0	100	0	304n	100	0	0	0	0	55	45
395o	100	0	0	0	0	100	0	395o	100	6	1	0	0	93	0
395b	100	1	0	1	24	73	1	395b	100	7	1	4	8	62	18
395m	100	0	1	1	20	78	0	395m	100	1	2	0	9	55	33
395n	100	0	0	0	8	92	0	395n	99	1	2	2	5	67	22
622o	100	0	0	0	2	98	0	622o	100	1	0	2	1	95	1
622b	100	5	9	0	31	54	0	622b	100	2	1	0	27	58	12
622m	100	2	0	2	19	77	0	622m	100	0	0	0	6	79	15
622n	100	0	0	0	18	82	0	622n	99	0	0	0	6	88	5

The column labeled $T^-_U H^-_S$ represents a transfer of information from the plasmid to the chromosome. This provides evidence against the synthesis-dependent strand annealing (SDSA) model.

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370 RAD

	total	T^{+}_{u} H^{-}_{u}	T^{+}_{s} H^{+}_{u}	T^{+}_{u} H^{+}_{s}	T^{+}_{s} H^{-}_{s}	T^{+}_{u} H^{-}	T^{+}_{s} H^{-}
75o	100	0	0	0	0	100	0
75b	100	0	0	0	2	83	15
75m	100	2	0	3	24	49	22
75n	100	2	0	1	8	63	26
207o	100	1	0	0	0	100	0
207b	100	0	0	3	0	63	34
207m							
207n	100	1	0	0	1	82	16
304o	99	0	0	0	0	99	0
304b	100	0	0	0	0	48	52
304m	100	0	0	0	1	55	44
304n	100	0	0	0	0	63	37
395o	100	0	0	1	0	100	0
395b	99	5	2	5	15	66	7
395m	100	1	0	7	7	67	18
395n	100	2	0	0	4	54	40
622o	100	1	0	0	0	96	3
622b	100	2	1	6	34	47	10
622m	99	1	2	2	9	45	41
622n	98	3	1	2	4	67	23

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362 rad51

	total	T^+_u H^+_u	T^+_s H^+_u	T^+_u H^+_s	T^+_s H^+_s	T^+_u H^-	T^+_s H^-
75o	100	0	0	0	0	100	0
75b	100	0	0	0	0	100	0
75m	62	0	0	0	0	62	0
75n	100	0	0	0	0	100	0
207o	100	0	0	0	0	100	0
207b	89	0	0	0	0	87	2
207m	98	0	0	0	0	98	0
207n	100	0	0	0	1	100	0
304o	99	0	0	0	0	99	0
304b	98	0	0	0	0	98	0
304m	99	0	0	0	0	99	0
304n	98	0	0	0	0	98	0
395o	82	0	0	0	0	82	0
395b	100	0	0	0	0	97	3
395m	19	0	0	0	0	17	2
395n	100	0	0	0	0	100	0
622o	200	0	0	0	0	200	0
622b	95	0	0	0	0	95	0
622m	35	0	0	0	0	30	5
622n	99	0	0	0	0	99	0

363 rad51

	total	T^+_u H^+_u	T^+_s H^+_u	T^+_u H^+_s	T^+_s H^+_s	T^+_u H^-	T^+_s H^-
75o	97	0	0	0	0	97	0
75b	47	0	0	0	0	47	0
75m	25	0	0	0	0	25	0
75n	88	0	0	0	0	88	0
207o	58	0	0	0	0	58	0
207b	39	0	0	0	0	39	0
207m	44	0	0	0	0	44	0
207n	39	0	0	0	0	39	0
304o	100	0	0	0	0	100	0
304b	100	0	0	0	0	100	0
304m	29	0	0	0	0	29	0
304n	100	0	0	0	0	100	0
395o	96	0	0	0	0	96	0
395b	25	0	0	0	0	25	0
395m	3	0	0	0	0	3	0
395n	31	0	0	0	0	31	0
622o	96	0	0	0	0	96	0
622b	27	0	0	0	1	26	0
622m	9	0	0	0	0	9	0
622n	86	0	0	0	0	86	0

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364 rad54

	total	T^{+}_{u} H^{-}_{u}	T^{+}_{s} H^{+}_{u}	T^{+}_{u} H^{-}_{s}	T^{+}_{s} H^{+}_{s}	T^{+}_{u} H^{-}	T^{+}_{s} H^{-}
75o	83	0	0	0	0	83	0
75b	95	0	0	0	0	95	0
75m	31	0	0	0	0	31	0
75n	47	0	0	0	0	47	0
207o	69	0	0	0	0	69	0
207b	94	0	0	0	0	94	0
207m	73	0	1	0	0	72	0
207n	99	0	0	0	0	99	0
304o	99	0	0	0	0	99	0
304b	81	0	0	0	0	81	0
304m	74	0	0	0	0	73	1
304n	98	0	0	0	0	98	0
395o	98	0	0	0	0	97	1
395b	47	0	0	0	0	47	0
395m	6	0	0	0	0	6	0
395n	47	0	0	0	0	47	0
622o	76	0	0	0	0	76	0
622b	45	0	0	0	0	45	0
622m	23	0	1	0	0	22	0
622n	90	0	0	0	0	90	0

371 rad54

	total	T^{+}_{u} H^{+}_{u}	T^{+}_{s} H^{-}_{u}	T^{+}_{u} H^{+}_{s}	T^{+}_{s} H^{-}_{s}	T^{+}_{u} H^{-}	T^{+}_{s} H^{-}
75o	100	0	0	0	0	100	0
75b	100	0	0	0	0	100	0
75m	100	0	0	0	0	100	0
75n	100	0	0	0	0	100	0
207o	100	0	0	0	0	100	0
207b	100	0	0	0	0	100	0
207m							
207n	100	0	0	0	0	99	1
304o	100	0	0	0	0	100	0
304b	99	0	0	0	0	99	0
304m	98	0	0	0	0	98	0
304n	100	0	0	0	0	100	0
395o	100	0	0	0	0	100	0
395b	98	0	0	0	0	97	1
395m	97	0	0	0	0	95	2
395n	100	0	0	0	0	100	0
622o	100	0	0	0	0	100	0
622b	100	0	0	0	0	100	0
622m	100	0	0	0	0	99	1
622n	100	0	0	0	0	100	0

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366 rad55	total	T^{+}_{u} H^{+}_{u}	T^{+}_{s} H^{-}_{u}	T^{+}_{u} H^{+}_{s}	T^{+}_{s} H^{+}_{s}	T^{+}_{u} H^{-}	T^{+}_{s} H^{-}
75o	100	0	0	0	0	100	0
75b	46	0	0	0	0	45	1
75m	28	0	0	0	1	27	0
75n	28	0	0	0	0	27	1
207o	93	0	0	0	0	93	0
207b	39	0	0	0	0	36	3
207m	93	0	0	0	0	93	0
207n	97	0	0	0	0	95	2
304o	96	0	0	0	0	94	2
304b	93	0	0	0	0	84	9
304m	59	0	0	0	0	51	8
304n	93	0	0	0	0	85	8
395o	97	0	0	0	0	97	0
395b	45	0	0	0	2	38	5
395m	12	0	0	0	0	9	3
395n	43	0	0	0	0	42	1
622o	98	0	0	0	0	98	0
622b	41	0	0	1	2	35	3
622m	37	0	0	0	1	33	3
622n	100	0	0	0	1	98	1

367 rad55	total	T^{+}_{u} H^{+}_{u}	T^{+}_{s} H^{+}_{u}	T^{+}_{u} H^{-}_{s}	T^{+}_{s} H^{+}_{s}	T^{+}_{u} H^{-}	T^{+}_{s} H^{-}
75o	99	0	0	0	0	99	0
75b	50	0	0	0	0	47	3
75m	45	0	0	0	1	40	4
75n	35	0	0	0	0	33	2
207o	91	0	0	0	0	91	0
207b	51	0	0	0	1	46	4
207m	91	0	0	0	0	91	0
207n	44	0	0	0	0	43	1
304o	95	0	0	0	0	94	1
304b	92	0	0	0	0	82	10
304m	95	0	0	0	0	81	14
304n	92	0	0	0	0	84	8
395o	78	0	0	0	0	78	0
395b	72	0	0	1	3	63	5
395m	37	0	0	1	3	28	5
395n	60	0	0	0	1	57	2
622o	86	0	0	0	0	86	0
622b	28	1	1	0	4	21	1
622m	0	0	0	0	0	0	0
622n	0	0	0	0	0	0	0

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368 rad57

	total	T^+_u H^+_u	T^-_s H^-_u	T^+_u H^-_s	T^-_s H^+_s	T^+_u H^-	T^+_s H^-
75o	91	0	0	0	0	90	1
75b	77	0	0	0	1	70	6
75m	37	0	0	2	1	29	5
75n	48	0	0	0	2	42	4
207o	83	0	0	0	0	82	1
207b	76	0	0	0	0	69	7
207m	64	0	0	0	0	64	0
207n	100	0	0	0	0	100	0
304o	98	0	0	0	0	98	0
304b	96	0	0	1	0	86	9
304m	72	0	0	0	0	63	9
304n	98	0	0	1	0	87	10
395o	96	0	0	0	0	95	1
395b	86	0	0	2	0	83	1
395m	29	0	0	0	0	22	7
395n	97	0	0	0	0	94	3
622o	98	0	0	0	0	98	0
622b	45	0	0	0	1	42	2
622m	21	0	0	0	0	18	3
622n	99	0	0	1	0	98	0

369 rad57

	total	T^+_u H^+_u	T^+_s H^-_u	T^-_u H^-_s	T^-_s H^-_s	T^-_u H^-	T^+_s H^-
75o	98	0	0	0	0	98	0
75b	100	0	0	0	0	94	6
75m	97	0	0	1	14	77	5
75n	99	0	0	1	0	90	8
207o	92	0	0	0	0	92	0
207b	33	0	0	0	1	32	0
207m	93	0	0	0	0	93	0
207n	94	0	0	0	0	94	0
304o	98	0	0	0	0	98	0
304b	98	0	0	0	0	92	6
304m	100	0	0	0	0	63	37
304n	97	0	0	0	0	93	4
395o	88	0	0	0	0	88	0
395b	61	1	0	0	2	56	2
395m	90	0	0	0	6	41	43
395n	99	0	0	0	2	88	9
622o	100	0	0	0	0	100	0
622b	98	0	0	0	3	92	3
622m	6	0	0	0	0	3	3
622n	91	0	0	0	1	88	2

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Appendix 3

Polymerase Chain Reaction

Type I DSB-repair

Digestion Experiment #	Phenotype	Strain	Plasmid Cut	Chromosome Cut	Both Cut	Neither Cut
75 MscI	Trp^-_s, His^-_s	RAD	5	4	6	3
370			28%	22%	33%	17%
75 MscI	Trp^-_s, His^-_s	rad57	5	4	2	1
369			42%	33%	17%	8%
---------	------------	---------	---------	-----------	--------	-----
75 MscI	Trp^-_u,His^-	RAD	4	3	12	0
370			21%	16%	63%	0%
75 MscI	Trp^-_u,His^-	RAD	0	0	13	0
370			0%	0%	100%	0%
75 MscI	Trp^-_u,His^-	RAD	4	10	2	0
370			25%	63%	13%	0%
75 MscI	Trp^-_u,His^-	rad51	20	0	0	0
362			100%	0%	0%	0%
75 MscI	Trp^-_u,His^-	rad51	0	0	14	0
362			0%	0%	100%	0%
75 MscI	Trp^-_u,His^-	rad54	18	8	4	0
371			60%	27%	13%	0%
75 MscI	Trp^-_u,His^-	rad54	0	0	15	0
371			0%	0%	100%	0%
75 MscI	Trp^-_u,His^-	rad55	0	0	16	0
366			0%	0%	100%	0%

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75 MscI	Trp^{+}_{u},His^{-}	rad57	0	0	11	0
368/369			0%	0%	100%	0%
622 BsiwI	Trp^{-}_{s},His^{-}_{s}	RAD	30	6	2	2
370			75%	15%	5%	5%
622 BsiwI	Trp^{-}_{s},His^{-}_{s}	rad55	2	2	0	2
366/367			33%	33%	0%	33%
622 BsiwI	Trp^{-}_{s},His^{-}_{s}	rad57	3	1	0	0
368/369			75%	25%	0%	0%

Type II DSB-repair

Digestion Experiment #	Phenotype	Strain	Plasmid Cut	Chromosome Cut	Both Cut	Neither Cut
395 MscI	Trp^{-},His^{+}	RAD	18	4	0	2
361/371			75%	17%	0%	8%
395 MscI	Trp^{+}_{s},His^{+}_{s}	rad54	1	0	0	1
371			50%	0%	0%	50%
395 MscI	Trp^{-}_{s},His^{-}_{s}	rad55	1	0	0	2
366/367			33%	0%	0%	67%
395 MscI	Trp^{-}_{s},His^{-}_{s}	rad57	1	0	0	0
			100%	0%	0%	0%
622 MscI	Trp^{-},His^{-}	RAD	9	0	1	3
361/370			69%	0%	8%	23%
622 MscI	Trp^{-},His^{-}	rad51	12	0	0	0
362			0%	0%	0%	0%
622 MscI	Trp^{-}_{s},His^{-}_{s}	rad55	9	0	0	2
366/367			82%	0%	0%	18%

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Appendix 4

Chi-Square Computations

Computations were made using Pierson's Standard Statistics (Levin & Fox, 1994)

Criticl values: Level	0.05	0.02	0.01	0.001
1 degree of freedom	3.814	5.412	6.635	10.827
2 degrees of freedom	5.991	7.824	9.21	13.815

Repair of double-strand breaks (figure 7, table 2)

Transformations, linearized versus uncut

comparison	chi-square	degrees of freedom	probability level	difference
RAD versus *rad51*	17,976	1	0.001	positive
RAD versus *rad54*	2,106	1	0.001	positive
RAD versus *rad55*	114	1	0.001	positive
RAD versus *rad57*	1,088	1	0.001	positive

The effects of double-strand breaks on gene conversion (figure 8, table 3)

Transformations: His^+ / 1000 Trp^+ damaged per His^+ / 1000 Trp^+ undamaged

comparison	chi-square	degrees of freedom	probability level	difference
RAD versus *rad51*	5,072	1	0.001	positive
RAD versus *rad54*	640	1	0.001	positive
RAD versus *rad55*	560	1	0.001	positive
RAD versus *rad57*	24	1	0.001	positive

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Gene conversion, Type I versus Type II (figure 13, table 5)

$\%His^+$ Type I versus $\%His^+$ Type II

comparison	chi-square	degrees of freedom	probability level	difference
RAD Type I versus *RAD* Type II	80.57	1	0.001	positive
RAD Type I & Type II versus *rad55* Type I & Type II	128.2	2	0.001	positive
RAD Type I & Type II versus *rad57* Type I & Type II	199.46	2	0.001	positive
Type I *rad55* versus Type I *rad57*	0.0904	1	0.05	negative
Type II *rad55* versus Type II *rad57*	0.057959	1	0.05	negative
rad55 Type I versus Type II	3.5	1	0.05	negative
rad57 Type I versus Type II	5.4	1	0.02	positive

Plasmid integratin Type I versus Tpe II (figure 14, table 6)

$\%Trp^-_s$ Type I versus % Trp^-_s Type II

comparison	chi-square	degrees of freedom	probability level	difference
RAD Type I versus *RAD* Type II	2	1	0.05	negative
RAD Type I & Type II versus *rad55* Type I & Type II	383.024	2	0.001	positive
RAD Type I & Type II versus *rad57* Type I & Type II	179.25	2	0.001	positive

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Gene converson and plasmid integration, Type I versus Type II

Comparison of gene conversion and integraton in repair-deficient strains.

%His$^-$ versus %Trp^+_s (figure 15, tables 7 & 7b)

comparison	chi-square	degrees of freedom	probability level	difference
Type I His$^+$/Trp^-_s versus Type II His$^-$/Trp^-_s	38.961	1	0.001	positive
Type I *RAD* %His$^-$/%Trp^-_s versus *rad55* & *rad57* %His$^+$/%Trp^-_s	2.08	2	0.05	negative
Type II *RAD* %His$^-$/%Trp^+_s versus *rad55* & *rad57* %His$^-$/%Trp^-_s	8.889	2	0.01	negative
Type II *RAD* %His$^+$/%Trp^-_s versus *rad55* & *rad57* %His$^-$/%Trp^-_s (sample 395m eliminated)	3.156	2	0.05	negative
RAD Type I %His^-_u, Trp^-_s versus %His^-_s, Trp^-_s	59.615	1	0.001	positive
Above with samples 75m, 622b eliminated	8.783	1	0.01	positive
RAD Type II %His^+_u, Trp^+_s versus %His^+_s, Trp^-_s	24.511	1	0.001	positive
RAD Type I %His^-_u, Trp^-_u versus %His^-_s, Trp^-_s (with samples 75m, 622b eliminated)	6.25	1	0.02	positive
RAD Type II %His^+_u, Trp^-_u versus %His^-_s, Trp^-_s	29.4	1	0.001	positive
His$^-$/Trp^-_s *RAD* Type I versus His$^-$/Trp^-_s *rad55* Type I	1.179	1	0.5	negative
His$^-$/Trp^-_s *RAD* Type I versus His$^-$/Trp^-_s *rad57* Type I	0.89	1	0.5	negative
His$^-$/Trp^-_s *RAD* Type II versus His$^-$/Trp^-_s *rad55* Type II	539.03	1	0.001	positive
His$^-$/Trp^-_s *RAD* Type II versus His$^-$/Trp^-_s *rad57* Type II	8.071	1	0.01	positive

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Comparison of gene conversion upstream versus downstream of a double-strand break (figure17, table8)

%His$^-$, Type II, upstream versus downstream

comparison	chi-square	degrees of freedom	probability level	difference
RAD upstream versus downstream	7.516	1	0.01	positive
RAD versus *rad55*	13.879	2	0.001	positive
RAD versus *rad57*	21.283	2	0.001	positive

Plasmid and chromosome alleles acting as donor in gene conversion (figures 19 & 20)

Type I	13.888	1	0.001	positive
Type II	8.909	1	0.01	positive

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