DEVELOPMENT OF NEW ASSAYS TO IDENTIFY *SACCHAROMYCES CEREVISIAE* GENES REQUIRED FOR EFFICIENT REPAIR OF A SINGLE SITE-SPECIFIC DNA DOUBLE-STRAND BREAK

THESIS

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CHAPTER I

INTRODUCTION

Chromosomes are highly condensed DNA molecules that exist within the nucleus of all eukaryotic cells. These chromosomes are commonly thought of as "blueprints" for the heritable, genetic information that DNA carries, and they are ultimately responsible for the replication and proliferation of cells. At all times these biomolecules are tremendously vulnerable to various types of damage that can be caused by endogenous or exogenous factors. General endogenous factors feature processes such as oxidation, where highly reactive oxygen species attack the bases, sugars and negatively charged phosphate groups of DNA to abstract an electron, as well as cleavage by nucleases. Physical or chemical agents, such as ionizing radiation, bleomycin, or methyl methanesulfonate (MMS), represent exogenous factors that affect DNA. Consequences of these harmful factors are extensive, but the most severe types of damage can generate a lethal double-stranded break (DSB). If these chromosomal breaks persist, the cell is subject to destructive effects, including persistent activation of cell cycle checkpoints, accumulation of mutations, decrease in chromosomal DNA stability and death of the cell (1). Cancer may even result if these double-stranded breaks (DSBs) are improperly fixed, leading to mutations in oncogenes and tumor suppressor genes.

In particular, since repair of DSBs is imperative for the maintenance of cells, through evolutionary events, cells have developed two separate repair mechanisms that are able to restore such breaks and ultimately save the cell. These pathways are known as homologous recombination and non-homologous end-joining (NHEJ) (1-5). Homologous recombination and NHEJ have been shown to be vastly conserved pathways in all eukaryotes, from yeast to humans. Genes of the budding yeast *Saccharomyces cerevisiae* are greatly homologous to human genes, yet yeast is a lower eukaryotic organism in which genes can be easily manipulated (6-7). Thus, studies conducted in budding yeast systems have historically been used to identify new genes, as well as to analyze the function of genes that were formerly identified (8). In addition, *Saccharomyces cerevisiae* can alternate between growth in either haploid or diploid states, which facilitates creation and analysis of genetic mutants. Also, the fact that many genes are considered to be non-essential has allowed an extensive number of genes to be inactivated and the consequences to be studied in yeast. Moreover, under optimal growth conditions, yeast undergoes cell division approximately every 90 min, and therefore replicates faster than higher eukaryotes (9). All of these beneficial qualities have classified yeast as a superlative model for studying both homologous recombination and NHEJ repair pathways (5-8, 10-11).

The two pathways are distinct in their means of repair, as well as which protein complexes are employed, with the exception of one particular protein complex (12-13). Exclusive NHEJ protein complexes include Yku70/Yku80, Sir2/Sir3/Sir4, and Dnl4/Lif1/Nej1 (1, 4). Proteins exclusive to homologous recombination repair include Rad51, Rad52, Rad54, Rad55, Rad57, Rad59, the Rfa complex, as well as other

uncharacterized proteins (Figure 1). The protein complex that is required for both repair mechanisms, composed of Mre11, Rad50 and Xrs2, is commonly referred to as the Mrx complex (14).

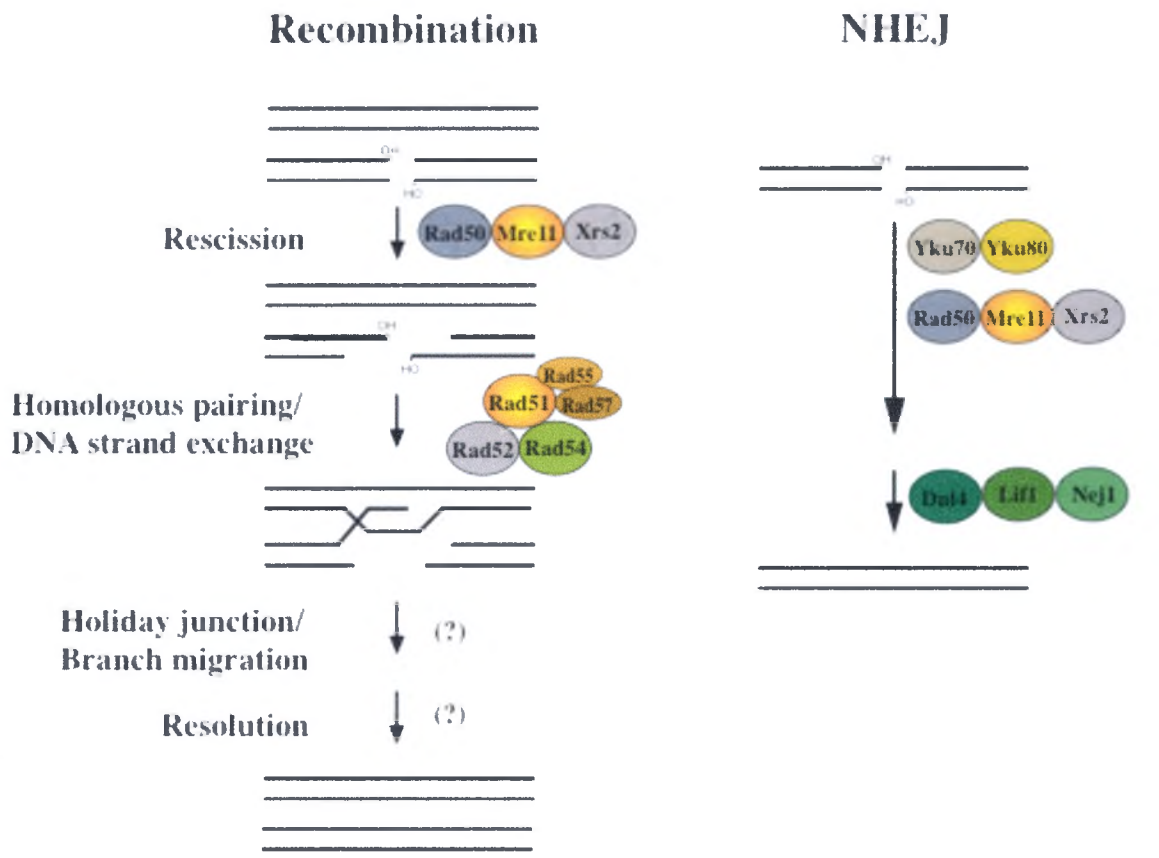

Figure 1. Illustration of the proposed mechanisms and proteins involved in both known DSB repair pathways.

While both homologous recombination and NHEJ pathways are typically utilized in all eukaryotic organisms, usually one of the mechanisms is predominant among a particular species. Specifically, both homologous recombination and NHEJ are found to restore DSBs in yeast cells, but homologous recombination is the primary system of

repair (4). Moreover, NHEJ would only be used extensively in yeast when homologous recombination is inactivated (15). In addition, the templated yet indirect repair mechanism of homologous recombination is considered to be highly accurate, unlike the untemplated, error-prone NHEJ pathway (15-18).

Homologous recombination is the most complex and least understood of the two mechanisms. Previous work has shown that this repair method begins with the binding of the Mrx complex where each individual protein plays its own separate role. First, the coiled-coil protein Rad50 not only binds ATP, but also displays a dependence on ATP for the binding of DNA (19-20). The fibrous structure of Rad50 allows the Mrx complex to take on an arrangement shaped like a headphone when bound to broken ends of DNA (Figure 2).

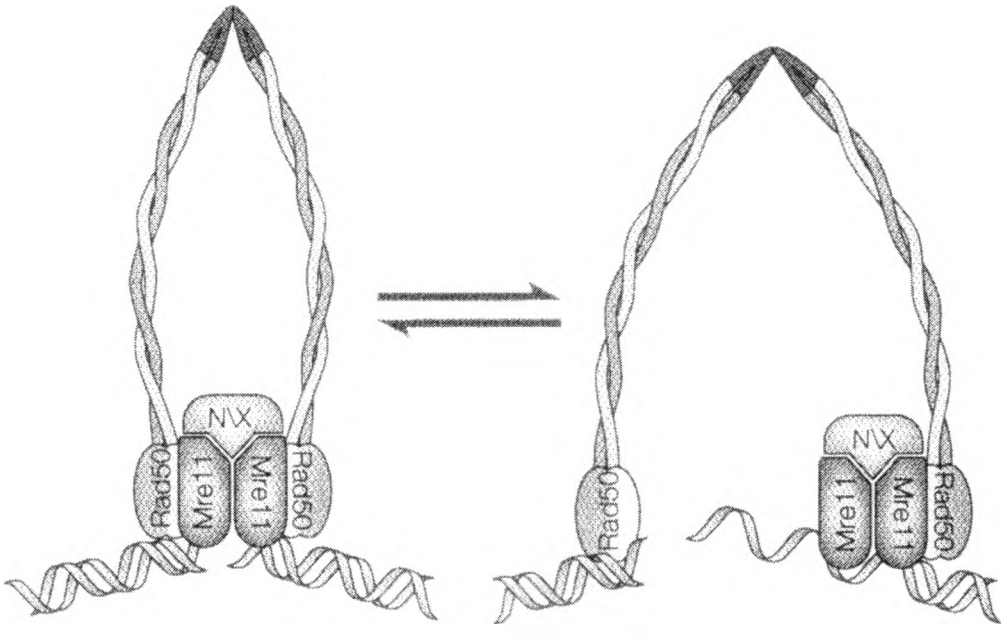

Figure 2. Representation of the Mrx complex. Upon binding to DSBs of DNA, this complex adopts a hypothesized "headphone" structure.

This configuration then promotes the Mrx complex to draw the broken ends into close enough proximity for repair (19). After the binding of the complex to the broken DNA ends, Mre11 uses its nuclease activity to generate single-stranded DNA 3' overhangs (4, 19). Little is known about the function of Xrs2 because it has not been well conserved, and it is for this reason that Rad50 and Mre11 are often thought of as the core proteins of the Mrx complex (19).

After Mrx nuclease activity produces 3' overhangs, these single-stranded "tails" are used as substrates for Rad51, Rad52, and Rad54, which catalyze strand invasion of the "tails" to similar sequences on either an intact sister chromatid or homologous chromosome. Subsequently, large X-shaped structures known as Holliday junctions are formed. These Holliday junctions move down the DNA melting base pairs between the chromosome that is being invaded and forming base pairs between the 3' tails and the complementary sequence on the homologous chromosome. The proteins and mechanisms responsible for synthesizing new DNA, cleaving the Holliday junctions, and ultimately restoring the DNA to its original double-stranded state remain unidentified (4).

Specific biochemical functions have also been identified for some of the latter proteins involved in homologous recombination. For example, Rad51 is a recombinase protein that is dependent on the binding of DNA for its catalytic activity during the "presynaptic" phase of recombination. Upon the binding of Rad51 to a single-stranded DNA (ssDNA) 3'tail, Rad51 uses its ATPase activity to initiate pairing of the ssDNA to a homologous chromosome and to begin strand exchange. Another homologous recombination protein whose function has become established is Rad52. Exclusive to

eukaryotes, Rad52 binds with high affinity to ssDNA and forms a complex with Rad51. During this "synaptic" phase, Rad52 acts as a mediator between Rad51 and Rpa (a ssDNA binding factor) for the purpose of conducting effective strand exchange. One other protein that plays a significant role in homologous recombination is Rad54. This multifaceted protein, like Rad51, is a DNA-dependent ATPase. Furthermore, Rad54 interacts with Rad51 to facilitate efficient pairing of homologous DNA and may also assist in nucleosome movement to promote access to the DNA (14).

More recent studies have identified cell-cycle checkpoints, sister chromatid cohesion and nucleosome remodeling as important processes in DNA repair, particularly with homologous recombination (21-26). After a DSB ensues and Mrx is recruited to process the broken ends, Rpa coats the exposed ssDNA, which leads to the activation of a kinase signal cascade event. First, the presence of Rpa is recognized by checkpoint protein Ddc2, which then interacts with the Rpa. Subsequently, Ddc2 interacts and forms a complex with Mec1, a crucial checkpoint kinase. The Mec1-Ddc2 complex is then able to recognize and associate with the site of DNA damage (22). Next, Mec1 and a more minor checkpoint kinase, Tel1, are involved in the phosphorylation, and thus, activation of Rad53 and Chk1. Full checkpoint activation depends on the presence of three other checkpoint complexes, Ddc1-Mec3-Rad17, Rad24-RFC and Rad9-Ssa, which are also required in activating Rad53 (21-22). Finally, the activation of both Rad53 and Chk1 signals the cell to arrest until the damage is repaired (22).

Once the cell has arrested growth, cohesins localize at the site of DNA damage for postreplicative repair (23-26). These large ring-like structures are gathered by the Scc2/Scc4 complex onto chromosomes, and Eco1 further acts on this complex by an

unknown mechanism to establish cohesion (25). Kim *et al.* (23) suggest that for homologous recombination, cohesion assists in enhancing the efficiency of repair in chromosomes. This requirement causes an increase in cohesion of sister chromosomes in response to DNA damage to the cell.

In contrast to homologous recombination repair, Shim *et al.* (27) has identified a multi-subunit protein necessary for DSBs in yeast to be properly restored via the NHEJ pathway. The Rsc complex is responsible for repositioning nucleosomes away from the chromosome end so that subsequent repair proteins may gain appropriate access to the break site to promote efficient repair (27).

Mating-type switching is a type of homologous recombination mechanism that is executed in budding yeast in order to regulate gene expression and switching between haploid and diploid states. Generally, for laboratory purposes, yeast subsists as either haploid a or haploid α cells, but yeast cells may also exist as more stable a/α diploids. Whether a haploid cell exists as a or α is determined by which mating type is being expressed at the mating-type (*MAT*) locus on chromosome III (Figure 3). Besides what is expressed at the *MAT* locus of a chromosome, two other silent cassettes, *HMR* and *HML,* are present at the ends of the chromosome. These additional loci contain unexpressed genetic information for a (*a1* and *a2* genes) or α (*α1* and *α2* genes), respectively. Induction of mating-type switching takes place when a DSB is introduced at the *MAT* locus. HO endonuclease is the particular enzyme that produces such specific cleavage (Figure 3) (9, 28-29).

Once a DSB is initiated by HO at the *MAT* locus, Mrx processes the ends to produce 3' single-stranded tails in the same manner as homologous recombination. For

example, HO cleavage at *MATa* (Figure 3) initiates a type of homology search, whereby genes at HMLα are recruited to the break site for repair. When repaired, the *MATa* allele will be replaced by that of the opposite type, becoming *MATα* (Figure 3) (28). This type of recombination event is called gene conversion because during restoration of the break the HM site transfers its genetic information to the *MAT* locus with no reciprocal exchange to the HM site (9, 28).

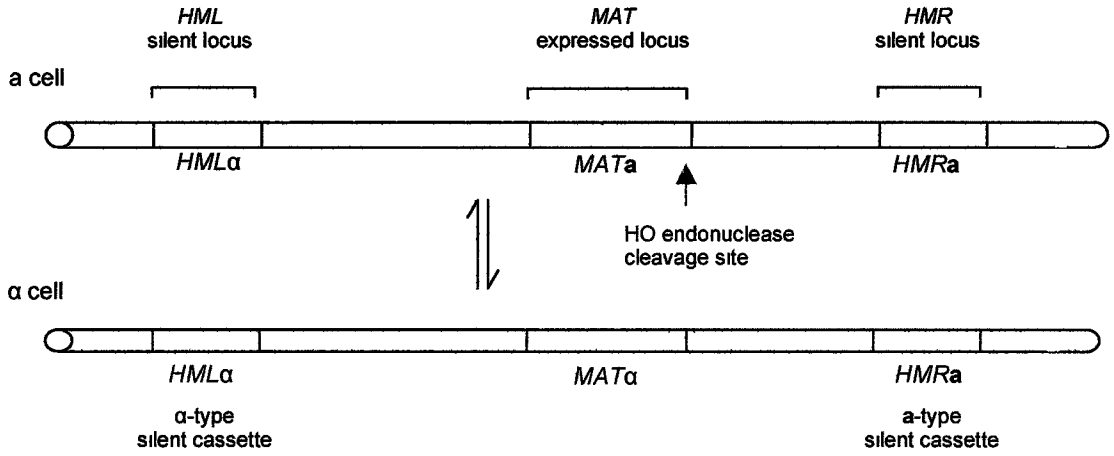

Figure 3. Schematic of *Saccharomyces cerevisiae* chromosome III. This figure illustrates the expressed *MAT* locus and silenced *HML* and *HMR* loci. (Adapted from Watson, J. D. *et al.* 2004, Molecular Biology of the Gene, 5th ed.)

Yeast cells that have fundamental recombination genes such as *RAD50*, *MRE11*, or *RAD51* inactivated display many of the same observable characteristics. These characteristics include tremendous sensitivity to the previously mentioned exogenous factors that cause DSBs (e.g. X rays or various chemicals), as well as defects in homologous recombination assays. Some of the same phenotypes of cancer-prone individuals have been seen in cells of humans that have genetic flaws in two of these genes, human *MRE11* and *XRS2* (4).

Previously, a set of 5,000 mutant yeast strains were screened in an attempt to identify new genes that encode proteins necessary for chromosomal DSB repair (5, 10). Individual mutant strains were screened, each of which contained a single, discrete inactivated yeast gene, to identify those needed for resistance to gamma radiation. All known non-essential yeast genes were represented among these separate mutants. Results indicated that at least 169 of the yeast genes were essential in order to withstand radiation. Prior to these findings, 17 of the 169 genes had already been linked to the homologous recombination pathway. Most of the genes had not previously been linked to DNA repair. Interestingly, mutant cells with these 17 genes inactivated were sensitive to gamma radiation, and they also shared characteristics causing them to be overly sensitive to the chemical agents MMS and bleomycin (5, 10, 30). Through the methylation of guanines and adenines, MMS creates bulky DNA adducts that lead to inhibitory effects on DNA synthesis and even cell death, if unrepaired. Several repair pathways, including homologous recombination are required for the restoration of DNA lesions induced by MMS (30). Bleomycin is an antibiotic used in combination with other drugs for some cancer treatments. Binding of this drug to oxygen and metals, such as iron causes a free radical complex to form, thereby inducing a variety of lesions to DNA, including DSBs (31).

Prior to the current study, the remaining 152 (169 minus 17) genes needed for gamma radiation resistance had not been tested for possible involvement in chromosomal DSB repair (1, 5). Interestingly, previous work (5, 10, 30) demonstrated that upon inactivation of 85 of these genes, sensitivity to MMS and bleomycin was also observed indicating that they have characteristics similar to the 17 known recombination genes

described above. More recently, one of the 85 mutants containing an inactivated *ASF1* gene was tested in the Lewis lab and was positively identified as a component in a DNA synthesis step of recombination (1). Therefore, the remaining 84 genes that have not been studied have a high probability of being involved in DSB repair, possibly by homologous recombination repair.

Since mating-type switching is primarily a DNA homologous recombination event, many studies have exploited the highly specific function of HO endonuclease in an attempt to gain insight into DSB repair via homologous recombination. HO is an enzyme that naturally exists in yeast that is normally expressed in cells, causing them to constantly switch mating type. In lab strains used for research, the HO gene is always inactivated so that cells do not switch and remain either *MATa* or *MATα*. HO endonuclease cuts at a 45 base pair recognition site, producing 4 nucleotide, 3' overhangs. By transforming yeast cells with a plasmid whose HO gene is inducible on galactose media (*GALp::HO*), mutant cells that are deficient in DNA repair may be characterized by their growth response on galactose when HO is expressed (29, 32, 47). Earlier work has shown that *rad51* or *rad52* mutant cells containing a pGalHO plasmid grow normally on glucose, but cannot grow on galactose media because repair of the HO-induced DSB at the *MAT* locus is impaired (1).

The two most common promoters implemented for regulating gene expression are *GAL1* and *GAL10*. *GAL1* is often the preferred promoter when the effects of high expression rates are of interest due to the dramatic three orders of magnitude increase when exchanging glucose for galactose (33-34). Induction of *GAL* promoters can be more carefully regulated by using alternate carbon sources, such as raffinose, in

conjunction with differing concentrations of galactose. Raffinose offers an advantage over glucose since it does not interfere with *GAL* promoter induction, whereas, on glucose, basal levels of expression can be detected (33). When yeast cells transformed with a plasmid containing a GAL::HO promoter fusion are transferred to galactose, DSBs are induced, resulting in elevated levels of recombination (29).

New, rapid quantitative assays have been developed in Dr. Lewis' lab (1, 35) that monitor recombinational repair between a DNA plasmid containing a DSB and an unbroken chromosome with homologous sequence inside cells. In these studies a plasmid containing a strategically induced DSB within a particular gene was used in order to stimulate repair by homologous recombination. The entire plasmid was then transformed into cells, followed by integration *via* a homologous repair mechanism, resulting in the function of the broken gene being restored (Figure 4) (1, 35, 36). In the example shown in Figure 4, the plasmid was cut within *HIS3*, and, after integration into yeast chromosome XV, a *his3-Δ1* mutant strain was converted to $HIS3^{+}$. Initial results with this system demonstrated that recombination was reduced 4- and 50-fold, respectively, in *rad50* and *rad51* mutants (35).

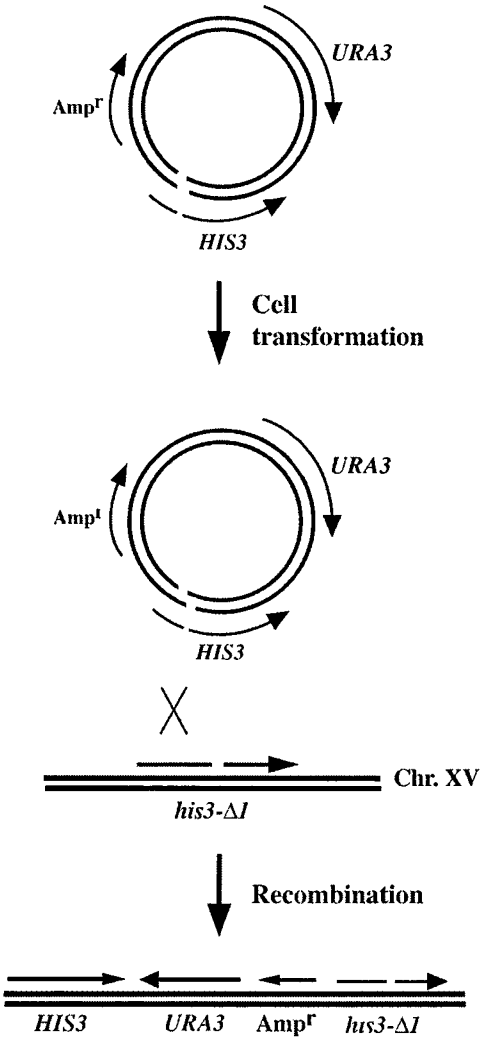

Figure 4. Representation of the recombination assay used to monitor repair efficiency between a plasmid containing a DSB and an intracellular homologous chromosome.

The primary goals of the current study were constructing an integrating plasmid containing three selective markers, optimizing a homologous recombination assay for DSB repair, and using the optimized assay to identify new genes involved in DNA repair by homologous recombination. Initial experiments in this study involved optimizing a

recombination assay based on the type of break that is induced and the overall size of the plasmid. New plasmids were created that were either integrating plasmids or gap-containing plasmids that were used for quantitative studies to investigate which plasmid and/or induced cut site yields the most efficient assay for monitoring DNA repair. The assay that produced the best distinction between wild-type and repair-deficient cells was used to further investigate repair defects in new yeast mutants. The HO endonuclease expression characterization method was utilized in order to prescreen the 84 candidates potentially involved in the homologous recombination repair pathway based on their previously identified sensitivity to gamma rays, MMS, and bleomycin.

Specifically, 10 new yeast mutants out of a total of 84 known to be sensitive to gamma, MMS and bleomycin were identified as sensitive to expression of HO endonuclease inside cells. Testing of these 10 HO-sensitive strains revealed that two exhibited aberrant recombination. Results of this study have thus identified two new genes affecting the homologous recombination pathway.

CHAPTER II

MATERIALS AND METHODS

I. MATERIALS

General Reagents

Tris base and sodium chloride were purchased from VWR International (West Chester, PA). Lithium acetate hydrate, magnesium chloride, calcium chloride, Hoechst 33258, glutamic acid (monopotassium salt) were obtained from Sigma Chemical Company (St. Louis, MO). Ammonium sulfate (granular), glycerol, polyethylene glycol (PEG-4000), boric acid, dimethylsulfoxide (DMSO) and sodium acetate were purchased from Mallinckrodt AR (Paris, Kentucky). Ethidium bromide was purchased from Shelton Scientific, Incorporated (Shelton, CT). Ethylenediamine tetraacetic acid (EDTA) and agarose were obtained from EMD Chemicals, Incorporated (Gibbstown, NJ). Triton X-100 and both monobasic and dibasic forms of potassium phosphate were purchased from Mallinckrodt Baker, Incorporated (Phillipsburg, NJ).

Bacteriological and yeast media

Ampicillin, D-(+)-glucose, D-galactose, raffinose, and all amino acids were acquired from Sigma Chemical Company (St. Louis, MO). Bacto agar, bacto yeast nitrogen base, bacto tryptone, bacto peptone, and bacto yeast extract were purchased

from Becton Dickinson Microbiological Systems (Sparks, MD). SCS110 competent *E. coli* cells were obtained from Stratagene (La Jolla, CA).

Restriction enzyme digestion, creation of blunt ends and ligation of DNA fragments

All restriction enzymes, DNA Polymerase I (Klenow), T4 DNA Ligase and 10X T4 Ligase Buffer were obtained from New England Biolabs (Beverly, MA). A mixture of deoxynucleotide triphosphates (dNTPs) was purchased from Takara Bio, Incorporated (Otsu, Japan).

Cell culture solutions and media

Yeast cells were cultivated on YPDA (rich) media (1% bacto yeast extract, 2% bacto peptone, 2% glucose, 2% bacto agar, 0.5% adenine) for general, non-selective growth. All percentages are w/v except nutritional plates describing galactose concentrations, which are v/v. For evaluation of mitochondrial function, yeast cells were grown on YPG (1% bacto yeast extract, 2% bacto peptone, 2% bacto agar, 3% glycerol). Synthetic media with drop-out mix (17% yeast nitrogen base without amino acids or ammonium sulfate, 0.5% ammonium sulfate, 2% glucose, 2% bacto agar, plus all essential amino acids minus amino acids used for selection) was used to grow yeast cells for plasmid selection. Raffinose (1%), raffinose plus galactose (1% and 3%, respectively) and galactose (2%) plates were made using synthetic media.

Yeast strains

The parent haploid yeast strain used for all experiments was BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) (37). Pilot experiments involving HO endonuclease sensitivity also used YLKL650 (as BY4742, but *mre11Δ*::G418^r). The 5,000 strain yeast deletion library was obtained from Open Biosystems (Huntsville, AL).

II. METHODS

Plasmid DNA purification

Plasmid DNA was extracted from *E. coli* cells using a boiling lysis method (38).

Yeast transformation

Either the quick DMSO-based transformation method of Soni *et al.* (39) or the high efficiency method of Gietz *et al.* (40) adopted for yeast transformations.

E. coli cell transformations

The following method was used to transform plasmid DNA into either DH5α or SCS110 *E. coli* cells. To a Falcon 2059 tube, 100µl of cold KCM buffer (100 mM KCl, 30 mM $CaCl_2$, 50 mM $MgCl_2$) 7-8 µl of plasmid DNA and 100 µl of thawed competent DH5α cells were added. The mixture was placed on ice for 10 min, followed by 10 min of immersion at 25-30°C. To the tube, 0.9 ml of SOC broth was added. The broth mixture was then shaken for ~45 min at 37°C. Aliquots of differing volumes were spread to LB plates containing ampicillin (Amp).

For transforming DNA into *E. coli* Top 10 cells, 100 µl of cells was mixed with 1-5 µl of plasmid in a 1.5 ml tube. The mixture was placed on ice for 20 min, followed by immersion at 42°C for 1 min, and then placed back on ice for 2 min. To the mixture, 1 ml of LB or TB broth was added. The broth mixture was then shaken for ~1hr at 37°C. Aliquots were spread to LB + Amp plates.

Construction of pLKL88Y

Restriction digestion of pLKL68Y and pRS303. Both pLKL68Y (*CEN/ARS HIS3 URA3 LEU2*) and pRS303 (integrating vector, *HIS3*) (41) were digested with *Fsp*I to create a new plasmid retaining all three genes from pLKL68Y, but lacking the CEN/ARS region. For the digestion of pLKL68Y the following were added to a 1.5 ml microfuge tube: 15 µl pLKL68Y, 61µl ddH_2O, 20 µl KGB, 4µl *Fsp*I (20 units). For the digestion of pRS303 the following were added to a 1.5 ml microfuge tube: 10 µl pRS303, 66 µl ddH_2O, 20 µl KGB, 4 µl *Fsp*I (20 units). The reaction mixture was incubated for 2-5 h at 37°C. Samples from each reaction were run on a 0.6% preparative agarose gel to purify the vector fragments of interest.

Gel purification of pLKL68Y and pRS303 fragments. A sample of each digest was loaded onto a 0.6% agarose gel and electrophoresed at 110 V for 1.5 h. The gel was stained with ethidium bromide followed by destaining with ddH_2O. A UV light source allowed viewing and removal (using Millipore tweezers) of the larger fragments for both pLKL68Y and pRS303. To extract the DNA from the gel, each removed gel plug was transferred to a 1.5 ml microfuge tube containing a 1 ml pipette tip packed with glass wool. In a microcentrifuge, the tubes were spun for 1 min at approximately 10,000-

11,000 x g. The glass wool-packed pipette tips were removed and discarded, and the tubes were recentrifuged at full speed for 1 min. The supernatant was then transferred to a new 1.5 microfuge tube and precipitated with 10 µl 3 M NaOAc and 250 µl 100% ethanol using standard methods.

Ligation of the pLKL68Y and pRS303 vector fragments. A Hoefer fluorometer (Hoefer Pharmacia Biotech Inc., California) was used to measure the concentration of each fragment using 2 µl of sample. The purified fragments were added together at a molar ratio of 1:1 (vector:insert) and were co-precipitated with reaction volumes as follows: 1.2 µl (81 ng/µl) pRS303, 3.8 µl (26 ng/µl) pLKL68Y, 15 µl ddH_2O, 2 µl 3 M NaOAc, 55 µl 100% EtOH. The mixture was spun for 10 min at full speed, washed with 70% EtOH and spun an additional 3 min. The wash was removed and the pellet was dried for 10 min using a Savant SC110 Speedvac (Savant, New York). The pellet was resuspended in the following: 12.5 µl ddH_2O, 1.5 µl 10X T4 ligase buffer, 1.0 µl T4 DNA ligase (400 units). Incubation of the mixture took place for 4-5 hr at room temperature (RT). The reaction mixture was transformed into DH5α cells; individual colonies produced on LB+Amp plates were used to complete DNA minipreps. Plasmids containing all these functional genes (*HIS3*, *URA3* and *LEU2*) and no CEN/ARS region were termed pLKL88Y.

Transformation of DH5α with pLKL88Y. The DNA ligation was transformed into *E. coli* DH5α cells as previously described. Aliquots of 50 µl, 100 µl, 200 µl and 300 µl were spread to LB + Amp plates.

Recombination assays using integrating plasmids containing variable selective markers

Preparation of plasmid DNA to be transformed. Plasmids pRS303, pLKL37Y and pLKL88Y were separately transformed into SCS110 *E. coli* cells. DNA minipreps were performed from resulting colonies on LB+Amp plates. Each plasmid was then digested with 5µl *Bcl*I (75 units) in 1X KGB in 100µl at 50°C for 3-5 h. The digested DNA was precipitated and quantitated (as previously described) and a sample of each miniprep was run on a 0.6% gel to verify complete digestion.

Transformation. The high efficiency transformation method was used to transform *HIS3* vector pRS313 (41) (200 ng per transformation), *Bcl*I-pRS303 (500 ng), *Bcl*I-pLKL37Y (500 ng) and *Bcl*I-pLKL88Y (500 ng) into yeast strains BY4742 (Brachmann 1998), *mre11* and *rad51*. Transformaton efficiency was calculated based on colony counts as the number of recombinants per µg of DNA normalized to the efficiency of transformation using the unbroken vector pRS313.

Construction of pLKL90Y and pLKL91Y

Restriction digestion and fill-in of pLKL69Y. Two separate double-digests were performed on pLKL69Y (*CEN/ARS HIS3 URA3 LEU2*) to remove the *URA3* gene from the plasmid, resulting in an increased number of unique sites in the *HIS3* gene region. Initially, pLKL69Y was double-digested with *Bam*HI and *Sma*I. A separate double-digest was conducted on pLKL69Y using *Nco*I and *Sma*I. For the digestion of pLKL69Y with *Bam*HI and *Sma*I the following were added to a 1.5 ml microfuge tube: 6 µl pLKL69Y, 69 µl ddH_2O, 20 µl 5X KGB buffer, 2 µl *Bam*HI (40 units) and 3 µl *Sma*I (60 units). For the digestion of pLKL69Y with *Nco*I and *Sma*I the following were added to a

1.5 ml microfuge tube: 6 µl pLKL69Y, 68 µl ddH_2O, 20 µl 5X KGB buffer, 3 µl *Nco*I (30 units) and 3 *Sma*I (60 units). The reaction mixtures were incubated for 2-5 hr at 30°C. To each reaction mixture the following were then added: 12.6 µl H_2O, 4 µl 5X KGB buffer, 1 µl of Klenow DNA polymerase (5 units), 2.4 µl 2.5 mM dNTPs. Each reaction mixture was incubated at room temperature for 20 min, followed by 20 minute incubation at 65°C. Samples from each reaction were run on a 0.6% preparative agarose gel to purify the vector fragments of interest.

Gel purification of pLKL69Y fragments. The gel containing pLKL69Y digests was stained with ethidium bromide followed by destaining with ddH_2O. A UV light source was used to allow viewing and removal (using Millipore tweezers) of the larger fragments (which contained *HIS3* and *LEU2*, but not *URA3*) for both pLKL69Y samples. Each removed gel plug was transferred to a 1.5 ml microfuge tube containing a pipette tip packed with glass wool to purify the DNA as described above. Each mixture was precipitated with 10 µl 3 M NaOAc and 250 µl 100% EtOH. Both mixtures were centrifuged at full speed for 10 min, washed with 70% EtOH and respun for 3 min. The wash was removed from each tube and the pellets were dried for 10 min in the speedvac.

Self-ligation of pLKL69Y fragments The dried pellets were each resuspended in the following: 12.5 µl ddH_2O, 1.5 µl 10X T4 ligase buffer, 1.0 µl T4 DNA ligase (400 units). Incubation of the mixture took place for 2-4 hr at room temperature. The reaction mixture was transformed into Top 10 cells as described above; individual colonies produced on LB+Amp media were used to complete DNA minipreps. The plasmids were digested with *Bam*HI/*Sma*I and *Nco*I/*Sma*I to confirm loss of *URA3*, and the resulting plasmids were termed pLKL90Y and pLKL91Y, respectively.

Gap repair recombination assay

Preparation of plasmid DNA to be transformed. pLKL90Y was digested with *Bse*RI using the following reaction volumes: 25 µl pLKL90Y, 51 µl ddH_2O, 20 µl 5X KGB buffer, 4 µl *Bse*RI (16 units). The reaction mixture was incubated at 37°C for 1.5 hr. To the reaction mixture, 3 µl of *Bsm*I (30 units) was added and the mixture was further incubated at 65°C for 1.5 hr. The DNA was precipitated and quantitated, and complete digestion was verified by gel electrophoresis.

Transformation. The high efficiency transformation method was used to co-transform *URA3* vector pRS316 (100 ng) and *Bse*RI/*Bsm*I-cut pLKL90Y (300 ng) into wild-type (WT), *mre11* and *rad51* strains. Plate growth media selected for Leu^+ and/or His^+ cells. Transformation efficiencies were calculated as described above.

To further investigate whether Leu^+ colonies were repaired by homologous recombination or by NHEJ, transformant colonies were subsequently patched to both plates without leucine and plates without histidine.

Recombination assays using the integrating plasmid created by BclI-digestion of pLKL37Y

The high efficiency transformation method was used to co-transform *LEU2* vector pRS315 (100 ng) and *Bcl*I-cut pLKL37Y (800 ng) WT, *mre11* and *rad51* cells. Transformation efficiencies were calculated as above.

Recombination assays using the integrating plasmid created by BseRI-digestion of pLKL37Y

Preparation of plasmid DNA to be transformed. pLKL37Y was digested with *Bse*RI under the following conditions: 80 µl pLKL37Y DNA, 230 µl ddH_2O, 80µl 5X KGB buffer, 10 µl *Bse*RI (40 units). DNA minipreps were prepared from the resulting colonies. The digested DNA was precipitated, verified by gel electrophoresis and quantitated by fluorometry as described above.

Transformation. The high efficiency transformation method was used to co-transform *LEU2* vector pRS315 (100 ng) and *Bse*RI-cut pLKL37Y (500 ng) into WT, *mre11*, *rad50*, *rad51*, *rad52*, *rad54*, *rad57* and *rad59*. Transformation efficiencies were calculated as before.

Development of an HO endonuclease survival assay for screening of strains from the yeast deletion strain library collection

Transformation of mutants with pGALHO. Selected mutants from the yeast gene deletion library (Open Biosystems; Huntsville, AL) were transformed with pGALHO (*URA3*) (47) using the rapid DMSO-based method. Three colonies from each transformation were patched to glucose plates lacking uracil.

Screening method used to identify repair-deficient strains. All pGALHO mutants were patched to selective media containing 1% raffinose (Raff) to alleviate glucose repression of the GAL10 promoter. These patches were then replica-plated, using velvets, to a 1% Raff plate. This plate was then immediately used as a master plate to perform another replica-plating onto a series of selective plates lacking uracil (Ura) in the

following order: 1% Raff, 1% Raff + 3% galactose (Gal) and 2% Gal. Mutants that were able to grow on all three media were considered to have WT sensitivity (+). Mutants which grew on Raff and on Raff + Gal, but not on gal alone were categorized as moderately sensitive (-). Mutants that could only grow on Raff were considered to be highly sensitive (- - -). All moderately and highly sensitive mutants were subsequently transformed with the vector pRS316. All mutants that were moderately sensitive HO were patched to Raff – Ura plates. In each case, mutant cells containing pRS316 and pGALHO were patched side-by-side. These patches were double imprinted using the same order of selective media as the initial screen above.

Dilution pronging survival assays. All mutants determined to be sensitive to HO endonuclease activity expression from replica-plating were more quantitatively investigated by survival pronging assays. Both mutants containing pGalHO and mutants containing pRS316 were initially cultivated on 1% Raff – Ura plates by allowing growth at 30°C for 3 days or at RT for 4 days. Then the cells were harvested and diluted 1/40 in H_2O, followed by brief sonication using a Vibra-cell sonicator supplied by Sonics and Materials Inc. (Newtown, CT). Next, the cells were loaded into a 0.1 mm deep Reichert Bright-Line hemacytometer (Buffalo, NY), viewed and counted using a Lomo HT-30.01 microscope (St. Petersburg, Russia). A concentration of 2 - $3x10^7$ cells was added to H_2O in a sterile 96-well microtiter dish for a total volume of 220 µl per well. Five-fold serial dilutions were then made (40 µl into 160 µl H_2O) for a total of 6 rows across the length of the dish. Finally, the cells were pronged onto control plates containing 1% Raff – Ura and selective plates containing 1% Raff + 3% Gal as well as 2% Gal. After 3-4 days of growth at 30° C, the plates were evaluated for sensitivity to HO-induced DSBs.

CHAPTER III

RESULTS AND DISCUSSION

The focus of this research project was to characterize new genes that are required for repair of DNA DSBs *via* homologous recombination. The project involved two parts. First, an assay needed to be developed that was capable of identifying mutant yeast strains with reduced ability to repair a single HO endonuclease-induced DSB on chromosome III. The presumption was that many of these mutants would be defective in homologous recombination. In the second part, a new plasmid-targeting assay for recombination efficiency was created to screen the HO-sensitive mutants for general defects in homologous recombination. Previous research showed that an integrating plasmid with two selective markers increased the fold effects observed in DSB repair compared to a plasmid containing one selective marker, which generated a better way of distinguishing gene involvement in homologous recombination repair (L.K. Lewis, unpublished results). Therefore, exploring the effect of an integrating plasmid with three selective markers was of interest. Furthermore, optimizing the recombination assay for screening potential recombination-deficient mutants was of highest concern.

Several systems were designed and tested during development of the plasmid recombination assay. These systems included multiple plasmid-targeting systems (where plasmid DNA transformed into cells integrates into a host chromosome) as well as gap

repair assays. These latter assays involve transformation of cells with plasmid DNA containing a deletion of part of a gene. After entry into the cell, the missing sequence, or gap, is filled by homologous recombination with a chromosome in normal cells but not in recombination-defective mutant cells.

Rationale for investigating recombination efficiency with integrating plasmids containing one, two or three selective markers

The first approach to creation of a plasmid recombination assay capable of clearly distinguishing recombination efficiencies between wild-type and recombination-deficient mutants followed up on earlier work in the Lewis lab. This work revealed that conducting a recombination assay using an integrating plasmid with two selectable markers decreased recombination in known recombination mutants more than an integrating plasmid with only one selectable marker. Therefore, recombination assays comparing the effects of using an integrating plasmid with one, two or three selectable marker(s) was performed.

Before the assays could be employed, construction of a three-gene integrating plasmid was completed. A laboratory plasmid, pLKL68Y (*CEN/ARS HIS3 URA3 LEU2*), already had three genes but also contained a centromere (*CEN*) and an origin of replication (ARS) that needed to be removed. By double-digesting both pLKL68Y and pRS303 (an integrating vector that only has the *HIS3* gene) with the restriction enzyme *Fsp*I, gel-purifying and ligating the fragments of interest, the desired plasmid was constructed (see Figure 5).

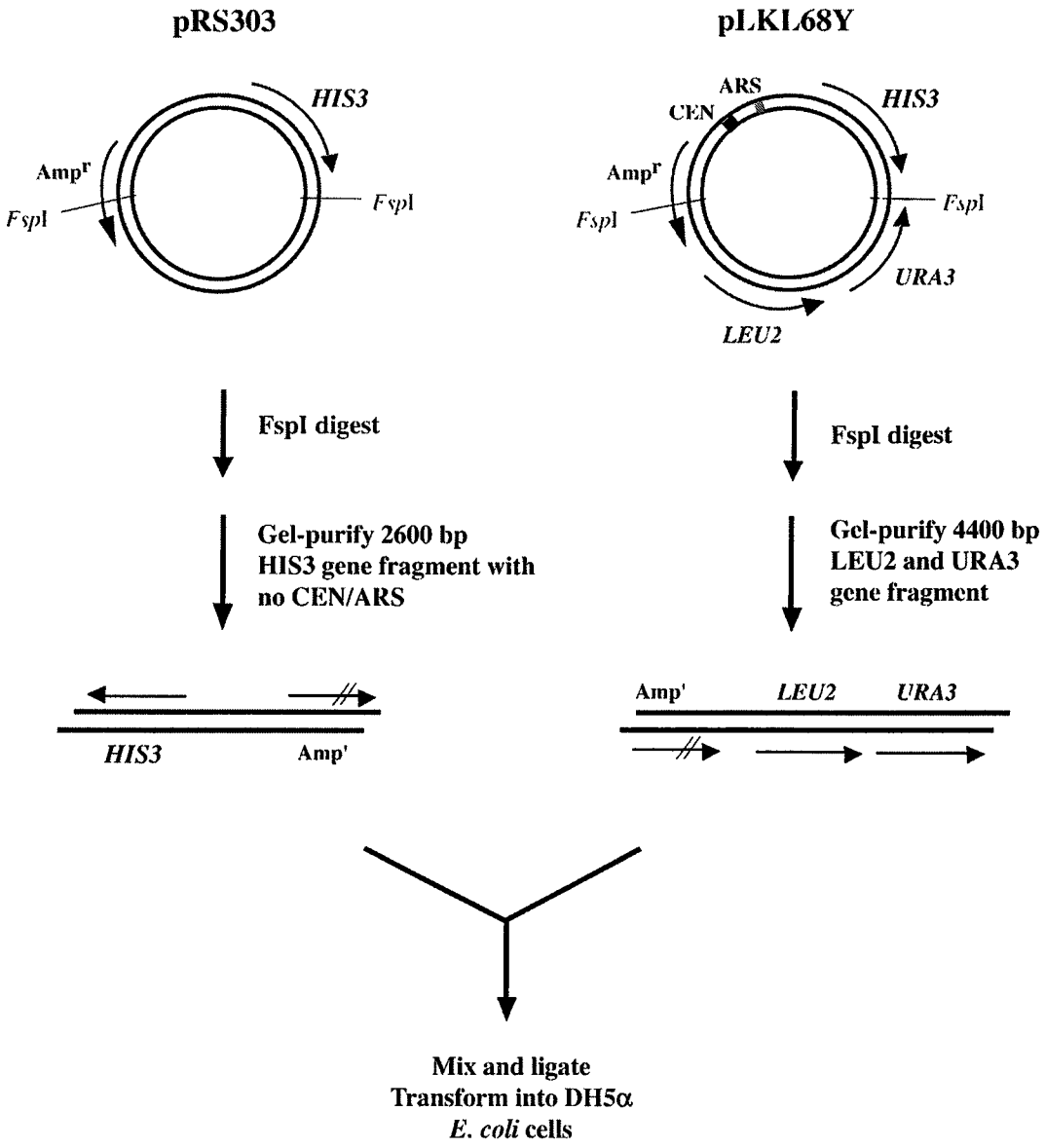

Figure 5. Schematic illustrating the process of constructing pLKL88Y. This plasmid was made from the preexisting plasmids pRS303 and pLKL68Y.

A 4400 base pair (bp) fragment of pLKL68Y containing *LEU2* and *URA3* was excised from a 0.6% agarose gel and a 2600 bp fragment of pRS303 containing *HIS3* was also gel purified. The fragments were ligated, transformed into *E. coli* DH5α cells and plasmids

containing the correct configuration were identified by gel electrophoresis analysis of miniprep DNAs. The resulting plasmid was designated pLKL88Y (Figure 6). Transformation of pLKL88Y into wild-type (WT) yeast cells was done to ensure that the plasmid did not retain a *CEN* or an *ARS*, which, if present, would allow the plasmid to exist as an episome in the nucleus. This information was confirmed when no colonies were produced on selective plates lacking histidine, uracil or leucine. Subsequent experiments demonstrated that the *HIS3*, *URA3* and *LEU2* genes remained functional (see below).

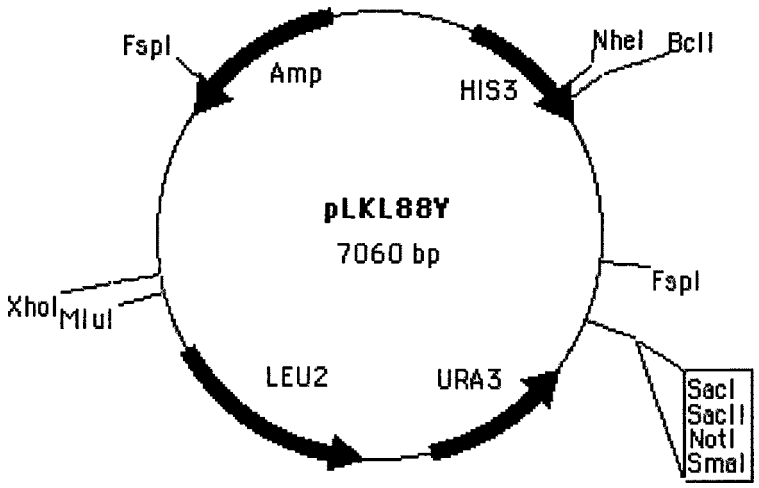

Figure 6. Schematic showing integrating plasmid constructed containing three selectable markers and no *CEN* or *ARS*. Restriction sites of interest are displayed at their appropriate locations.

BclI-plasmid digestion of one-, two- and three-gene plasmids induces homologous recombination at the his3-Δ1 locus in yeast cells

When restriction enzyme *Bcl*I recognizes the DNA sequence T'GATCA, cleavage occurs producing complementary 4-nucleotide (nt) 5' overhangs. Plasmids pRS303 (*HIS3*), pLKL37Y (*HIS3 URA3*) and pLKL88Y (*HIS3 URA3 LEU2*) were purified from SCS110 (*dam*$^-$) *E. coli* cells because *Bcl*I digestion is inhibited by *dam* methylation. These plasmids were then individually digested with *Bcl*I to strategically cut each plasmid at a unique site within the *HIS3* gene. This cut site was selected in order to generate broken ends with homology on both sides to the *HIS3* gene. Portions of the end sequences of each plasmid are identical to sequences within the chromosomal *his3-Δ1* gene of BY4742 yeast cells. This digestion therefore creates a single DSB that contains 57 bp of plasmid to chromosomal homology on one side of the break and 312 bp of homology on the other side. Thus, the *Bcl*I-digested plasmids can be used as integrating fragments for recombination assays (Figure 7).

As shown in the figure, the broken ends of the plasmid can undergo strand exchange, or homologous recombination, with the mutant *his3* allele on the chromosome. This event results in integration of the entire plasmid into the *his3* locus, producing a duplication of the *his* genes where one allele is functional and the other is not. Because all of the genes become stably integrated into chromosome XV, the cells go from His^- Leu^- Ura^- (requiring histidine, leucine and uracil to be able to grow) to His^+ Leu^+ Ura^+, so that the cells are capable of forming colonies on plates lacking these three nutrients.

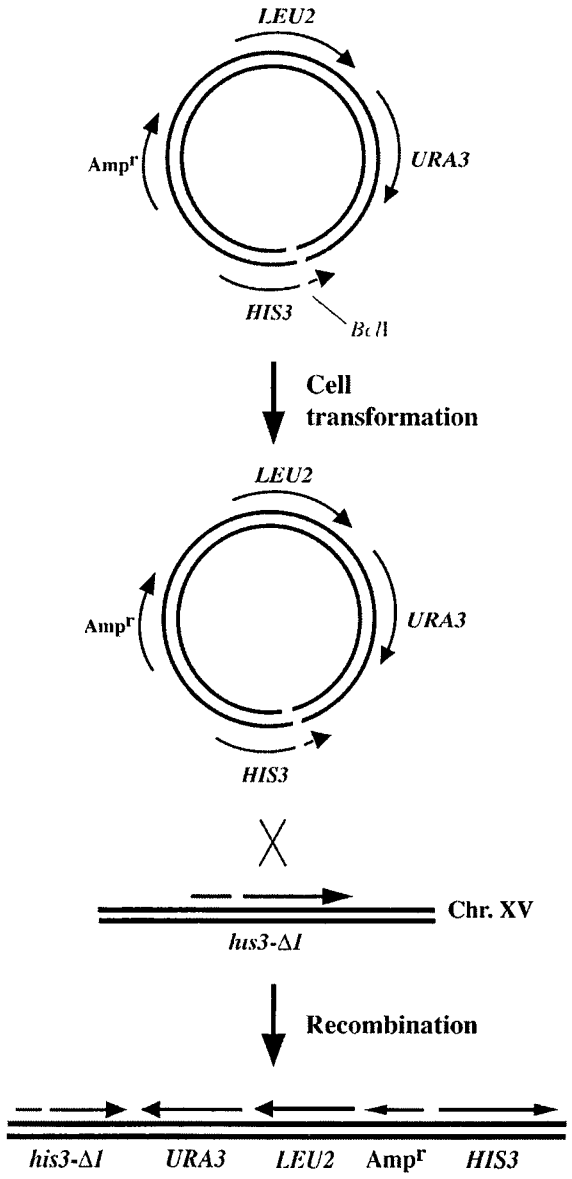

Figure 7. Schematic depicting induction of homologous recombination repair by transforming yeast cells with *Bcl*I-cut pLKL88Y. As the broken fragment integrates into the chromosome, *HIS3* function is restored. The "X" indicates the site of strand exchange.

Integrating plasmid with three selective markers does not increase recombination deficiency

Recombination assays were performed by transforming 500 ng of *Bcl*I-digested pRS303, pLKL37Y or pLKL88Y into WT cells and into known recombination-defective *mre11* and *rad51* mutant cells. In these experiments, cells were also transformed with 200 ng of the unbroken *CEN/ARS* plasmid pRS313. The number of transformant colonies per μg of DNA achieved with the broken plasmids (formed by recombination) was normalized to the number achieved with pRS313 (resulting simply from transformation of the stable *CEN/ARS* plasmids into the cells). This normalization was done to take into account possible differences in general transformation efficiencies among WT, *mre11* and *rad51* strains.

As seen in Figure 8, repair efficiency in *mre11* cells was modestly decreased by 5.0-, 4.2- and 3.4-fold with pRS303 (one gene) (A), pLKL37Y (two genes) (B) and pLKL88Y (three genes) (C), respectively. Fold reduction observed in *rad51* cells was 12.5 for both pRS303 and pLKL37Y, and repair efficiency was further decreased to 25-fold with pLKL88Y (Figure 8). Although these results indicated that levels of reduction for *mre11* mutants were not significantly different by using a plasmid containing one, two or three genes, more differences were seen with the three-gene plasmid pLKL88Y in *rad51* cells. Unfortunately, these reductions were not superior to prior studies where significant decreases in repair were seen with a two-gene integrating plasmid containing both *HIS3* and *URA3* (L.K. Lewis, unpublished results). Furthermore, the overall uncut plasmid transformation efficiency was high (~200,000 transformants per μg for uncut

vector pRS313), however, the experiments consistently produced low numbers of recombinant colonies, making statistical analysis difficult.

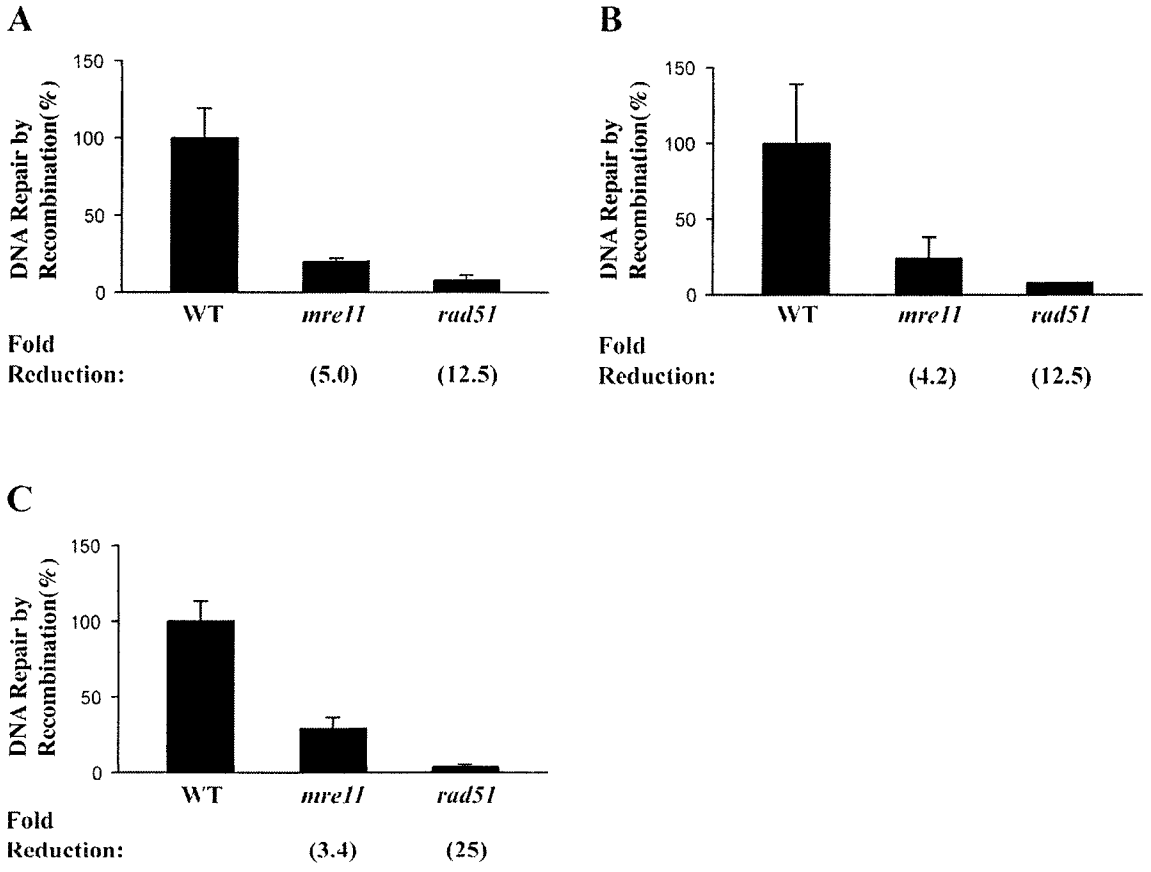

Figure 8. Inactivation of *MRE11* and *RAD51* genes caused a modest decrease in repair by recombination. Fold reductions for *Bcl*I-cut plasmids with one, two or three selective marker(s) are shown in A (pRS303), B (pLKL37Y) and C (pLKL88Y), respectively. Error bars indicate standard deviations.

Rationale for conducting a gap repair recombination assay

Previous work has indicated that a greater number of recombinant colonies per μg of transformed DNA are consistently produced with gap repair assays compared to those of plasmid-integrating recombination assays (42). Because the recombinant colony counts were unacceptably low in the previously attempted integration assays, a recombination assay *via* gap repair (gene conversion) was executed in an attempt to generate a higher number of recombinants. This test was done by introducing a gap, or large deletion into an independent, stable plasmid that contains both a *CEN* and an *ARS*, which allows the plasmid to exist in the cell as a non-integrating circle. The assay works because normal cells will repair, or fill in, the gap in the plasmid using homologous sequences in the *his3-Δ1* gene on chromosome XV by recombination.

Construction of plasmids pLKL90Y and pLKL91Y for use in gap repair assays

Prior to conducting the assays, two new plasmids were constructed in order to produce the right combination of unique restriction sites. The laboratory plasmid pLKL69Y (*CEN/ARS HIS3 URA3 LEU2*) was double-digested with *Bam*HI/*Sma*I OR *Nco*I/*Sma*I to remove all or a portion of the *URA3* gene, respectively. Following digestion and filling in with Klenow DNA Polymerase, the DNA fragments lacking *URA3* were purified from a 0.6% agarose gel (see Figure 9) and self-ligated. By generating these new plasmids a unique *Bsm*I site was created within the plasmid *HIS3* gene. The new *Bam*HI/*Sma*I-pLKL69Y and *Nco*I/*Sma*I- pLKL69Y plasmids were termed pLKL90Y and pLKL91Y, respectively (Figure 10).

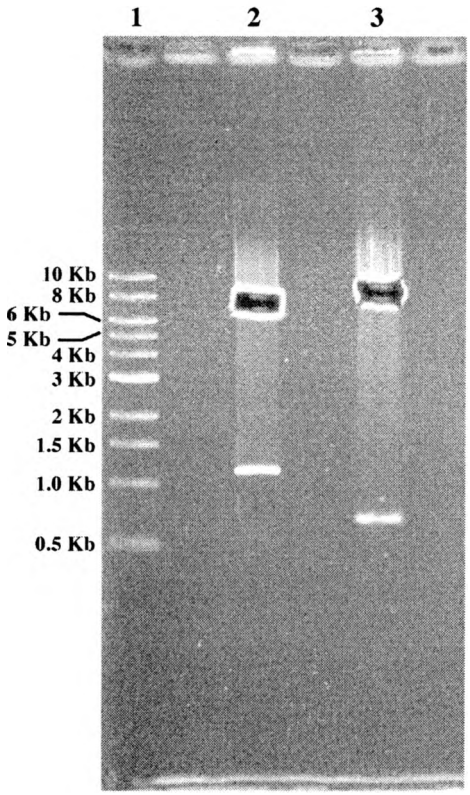

Figure 9. Gel purificiation of digested pLKL69Y DNA fragments. The digested fragments were electrophoresed on a 0.6% agarose gel after double-digestion with *Bam*HI/*Sma*I (lane 2) or *Nco*I/*Sma*I (lane3). Digestion resulted in successfully excising either all or part of *URA3* as seen in lanes 2 and 3, respectively. Comparison of the DNA fragments to the molecular weight standard, shown in lane1, indicated that all fragments migrated at their expected molecular weights.

Digestion of either pLKL90Y or pLKL91Y with *Bse*RI (GAGGAG[10/8]) and *Bsm*I (GAATGC[1/-1]) (see Figure 10) produces a deletion of 417 bp. The ends of the broken plasmid have the sequences shown below (and see Figure 11A).

*Bse*RI:

----------CTATTA$^{3'}$

----------GATA$^{5'}$

*Bsm*I:

$^{5'}$CATTC----------

$^{3'}$TCGTAAG----------

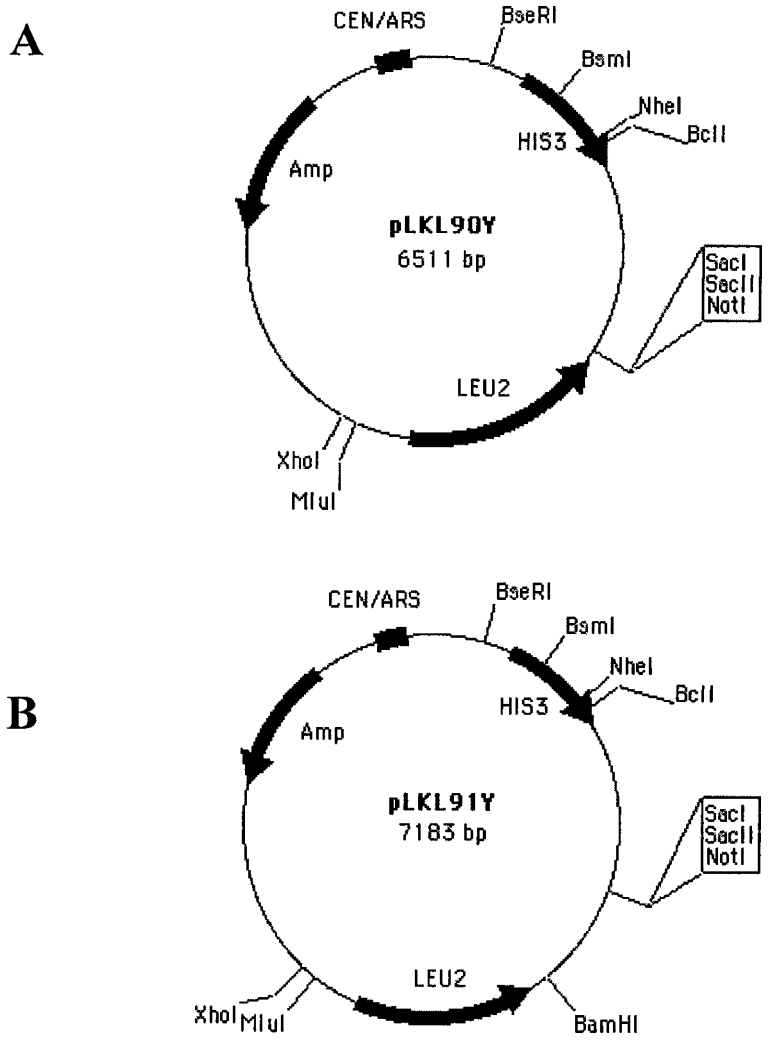

Figure 10. Illustration of plasmids constructed for gap repair assays. These plasmids were made to generate a unique *Bsm*I site with the *HIS3* gene. Plasmids resulting from digestion of pLKL69Ywith *Bam*HI/*Sma*I (A) and *Nco*I/*Sma*I (B), filling in the sticky ends and self-ligation.

The broken ends have 119 bp (upstream of the *Bse*RI cut site) and 92 bp (downstream of the *Bsm*I cut site) of homology with the *his3-Δ1* locus on chromosome XV and can be repaired by recombination. This exchange results in restoration of the *HIS3* gene on the plasmid and the plasmid-containing cells become Leu^+ His^+ (Figure 11B). Alternatively, the broken plasmids can be repaired without recombination by joining of the ends to each other *via* NHEJ. This would also produce a stable, plasmid-containing cell, but the cells would become Leu^+ His^- because the *HIS3* gene on the plasmid was not restored (see the upper pathway in Figure 11B).

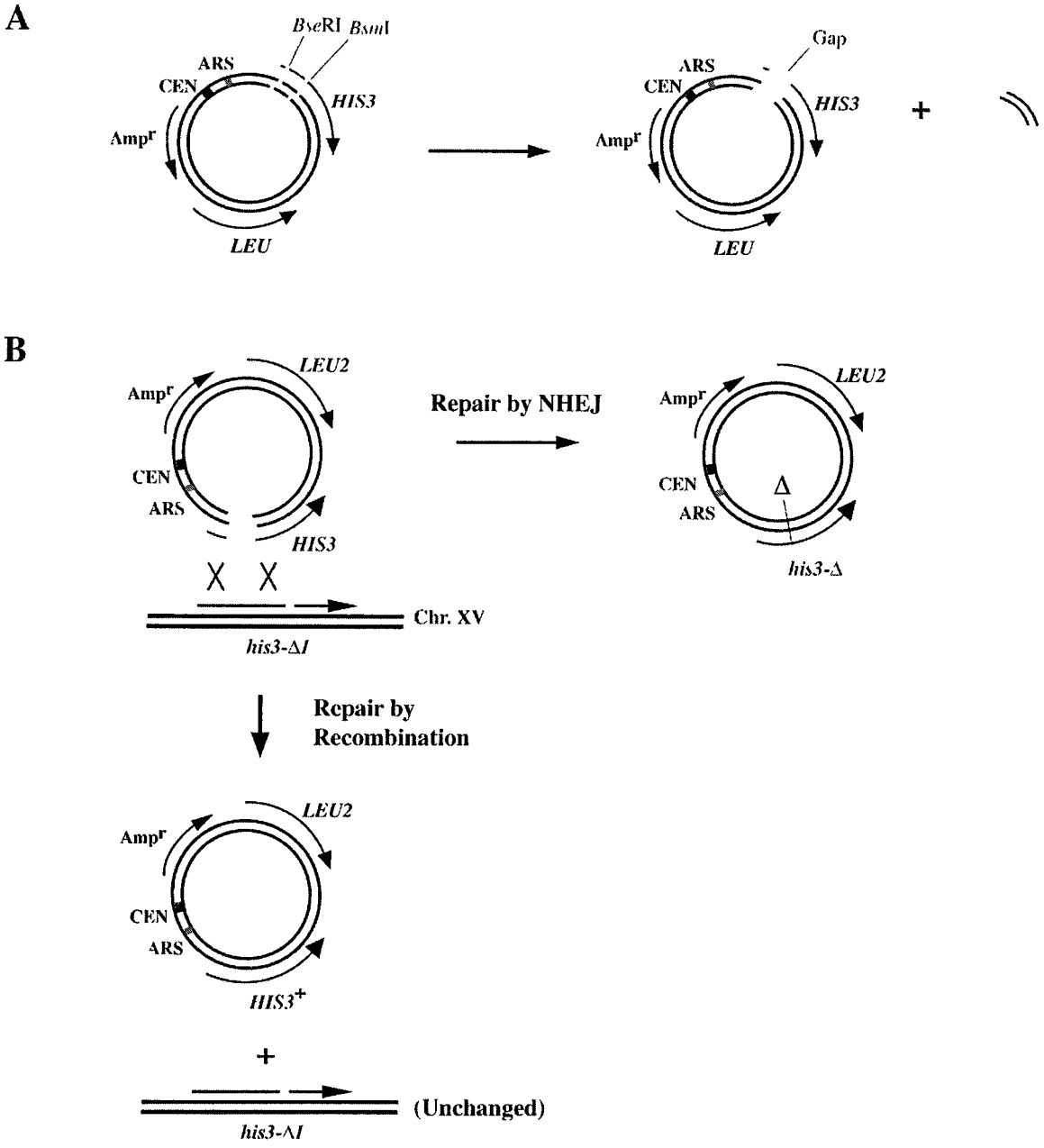

Figure 11. Schematic illustrating the two possible mechanisms for gap repair. Digestion of pLKL90Y with *Bse*RI and *Bsm*I excises a 400 bp fragment, creating a gap (A). Repair of the gap by recombination restores the function of *HIS3* without altering chromosomal information while NHEJ joins the broken ends, repairing the plasmid structure so that *HIS3* is inactivated (B). The two pathways can be monitored by selecting for $Leu^+ His^+$ vs. $Leu^+ His^-$ transformant colonies.

Gap repair assays can simultaneously monitor recombination and NHEJ

In preparation of the assays, pLKL90Y was double-digested with *Bse*RI and *Bsm*I to create a gap of 417 bp in the 5' end of the coding region of *HIS3*. Assays were performed with 300 ng of cut-pLKL90Y DNA per transformation as well as WT and *mre11* cells. Initially, cells were plated to media selective for only Leu^+, resulting in transformants generated as a mixture of recombination and NHEJ. Results indicated that total DNA repair (HR and NHEJ), monitored as total Leu^+ colonies, was reduced by 6.3-fold in *mre11* mutants relative to WT cells (Figure 12A).

In order to investigate what fraction of the transformants from the gap repair assay were restored by homologous recombination versus NHEJ, a total of 50 Leu^+ WT and *mre11* transformants were randomly chosen to be subsequently patched to selective plates lacking leucine and selective plates lacking histidine. Interestingly, 82% of *mre11* transformants were Leu^+ His^+ (repaired by recombination) while only 32% of WT transformants were repaired this way. Because the expression of *MRE11* is vital to NHEJ (the pathway is nearly abolished), but recombination is only partially defective, the majority of *mre11* transformants were repaired by recombination though at a lower level than WT. However, in WT cells, either pathway was available to restore the plasmid. NHEJ appeared to be the primary means (2/3 of repair events), possibly aided by the fact that there are 1-nt complementary ends (the last base of the 2-nt overhang created with *Bse*RI is an A and the last base of the 2-nt overhang created by *Bsm*I is a T, which might form an A:T bp). Although homologous recombination is usually the predominant repair pathway for DSBs in yeast, WT cells in this assay may have favored NHEJ because of this complementarity.

Gap repair assays monitoring only recombination events demonstrated no improvement in number of recombinants or fold reduction in DNA repair

Another gap repair assay was conducted under the same conditions in WT and *mre11* cells, selecting for transformants that were both Leu^+ His^+ as a result of only homologous recombination repair. In this way, any plasmids that may have been repaired *via* NHEJ were undetected because they always lead to a deletion in the *HIS3* gene. Thus, the assay was adapted to directly monitor homologous recombination repair. A 1.5-fold reduction in repair was observed in *mre11* cells, which was not significant enough to be clearly distinguished from that of WT (Figure 12B). Although the total number of recombinants slightly increased using this assay, the variability in the results was high. Therefore, the values obtained for gap repair could not be accepted with confidence.

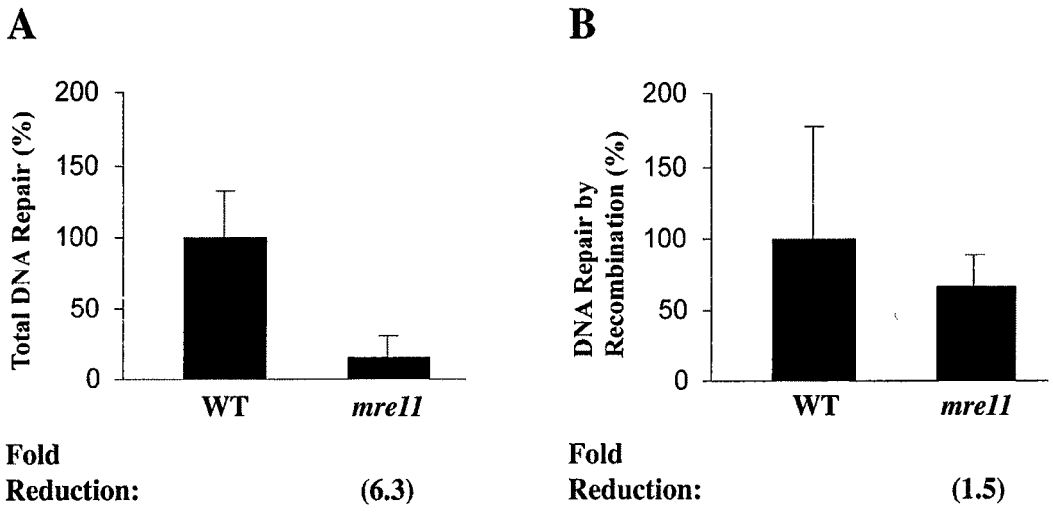

Figure 12. Effect of gap repair on recombination in *mre11* cells. (A) Leu^+ recombinants that were generated by either NHEJ or gene conversion recombination were significantly reduced in repair. (B) Leu^+ His^+ recombinants that were produced only by gene conversion revealed no significant reduction in repair.

Increased concentration of BclI-cut pLKL37Y DNA does not improve integration assay colony counts

Because integration assays using a plasmid with two selectable markers digested with *Bcl*I were routinely successful in previous studies (1, 35), this assay was revisited with the concentration of *Bcl*I-pLKL37Y increased from 500 ng per transformation to 800 ng per transformation. All other assay conditions were performed as for Figure 8. As seen in Figure 13, repair efficiency for *mre11* cells was decreased 8.3-fold. However, the number of recombinant colonies per plate was too low to be statistically valid, with less than 10 colonies per plate. Only a 4.2-fold reduction in repair was observed in *rad51*, which were inconsistent with a 100-fold reduction detected in a similar assay conducted in previous research (35). It was unclear why *Bcl*I-cut pLKL37Y repeatedly gave such low number of colonies per plate, but when cut with a different enzyme, this plasmid did yield a higher number of recombinants (described below).

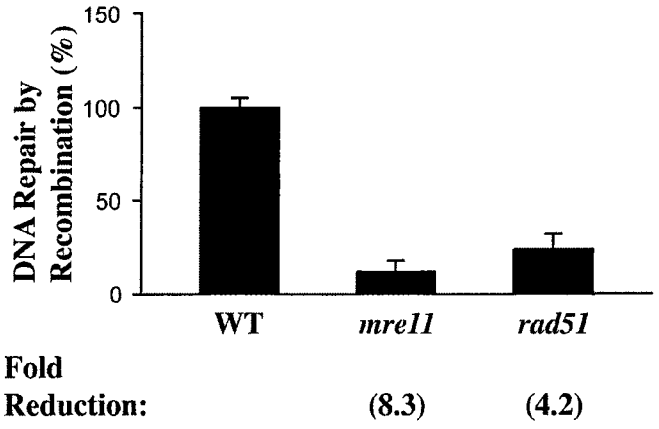

Figure 13. Inactivation of *MRE11* and *RAD51* genes resulted in reduced recombination efficiency using 800 ng of *Bcl*I-cut pLKL37Y DNA as an integrating plasmid.

Rationale for conducting BseRI-digested pLKL37Y integration assays

Since recombination assays using pLKL37Y digested with *Bcl*I failed to produce reproducibly acceptable results (colony counts were too low to yield good statistics, and *rad51* mutants did not display the large reduction in recombination seen in other published assays), a different restriction site within the *HIS3* gene was chosen for digestion. Because prior studies have shown that efficiency of recombinations increases when homology at DSB ends is increased (43), *Bse*RI was chosen for the digestion of pLKL37Y. By selecting *Bse*RI, homology between plasmid *HIS3* and chromosomal *his3-Δ1* increased from 57 bp and 312 bp on either side of the break with *Bcl*I to 119 bp on one side of the break and 497 bp on the other side. Although the *HIS3* coding sequence ranges from bp 503 to 1162 in pLKL37Y, homology exists between the plasmid *HIS3* gene and the chromosomal *his3-Δ1* within yeast cells beginning at bp 191 and ending at bp 1363 on the plasmid. Thus, *Bse*RI introduces a break in pLKL37Y that is upstream of its coding sequence, rather than within it, but the resulting ends retain extensive homology to the *his3-Δ1* locus on chromosome XV (Figure 14).

*Bse*RI was not initially chosen to conduct integration assays due to the 2-nt overhangs that it generates, raising concerns of negatively affecting the number of recombinants. Although all DSBs stimulate recombination, it is know that blunt-ended or nearly blunt-ended DSB due not stimulate recombination as efficiently as those with longer overhangs (4). Most previous assays had used ends with 4-base overhangs. However, pLKL37Y was digested with *Bse*RI and used for new integration assays to test whether an increase in homology at the ends could increase recombinants.

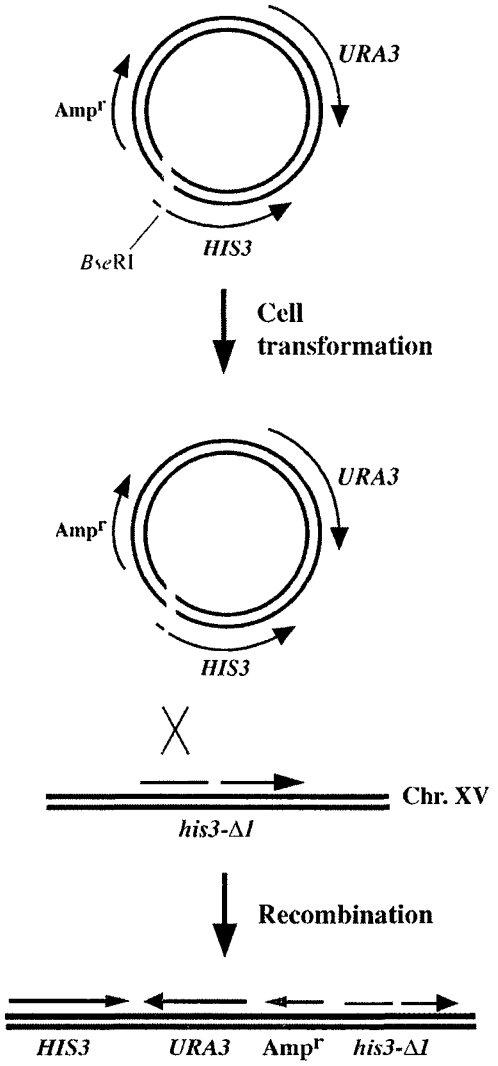

Figure 14. Schematic depicting induction of homologous recombination repair by transforming yeast cells with *Bse*RI-cut pLKL37Y. As the broken fragment integrates with the chromosome, *HIS3* function is restored and cells also become *URA3*.

The number of recombinants obtained in the integrating assay is increased with BseRI-digested pLKL37Y

A pilot test recombination assay was initially performed using 500 ng per transformation of *Bse*RI-cut pLKL37Y DNA in WT and *mre11* cells. Recombination efficiency increased in *mre11* mutants to more than 100 transformants per μg compared to previous *Bcl*I-pLKL37Y integration assays, where efficiency ranged from only 3-26 transformants per μg. In addition, a 5.3-fold reduction in DNA recombination repair efficiency was observed in *mre11* cells relative to WT cells (Figure 15). This result was consistent with the previous 4-fold reduction seen for *mre11* mutants in the Lewis lab (35). The results with *Bse*RI-pLKL37Y, on both colony counts and DNA repair by recombination, indicated this assay was sufficient for screening large numbers of unknown mutants for their potential involvement in the homologous recombination DNA repair pathway.

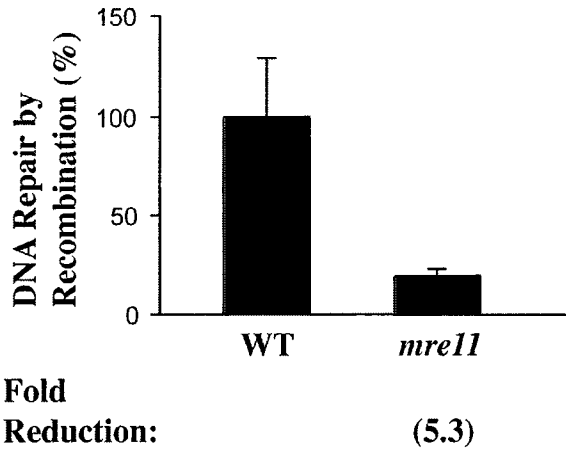

Figure 15. Demonstration that *mre11* mutants exhibit a reduction in DNA repair by using *Bse*RI-cut pLKL37Y as an integrating plasmid.

Use of a novel HO endonuclease survival assay to identify new DSB repair mutants

The two primary goals of this project were to screen a collection of new yeast mutants to identify those that could not repair endonuclease-induced DSBs efficiently and then to determine which members of this group are specifically defective in DSB repair by homologous recombination. To accomplish the first part, a new assay involving testing of sensitivity to *in vivo* expression of HO endonuclease was employed. When HO is expressed in yeast cells, it makes a single DSB at a 45-bp target sequence on chromosome III that is repaired poorly in known recombination mutants (discussed below).

Previous work in the Bennett lab (5, 10) screened 5,000 yeast mutants, each with a different gene inactivated, and identified 169 genes that are required for cellular resistance to gamma radiation. They subsequently showed that a total of 78 yeast mutant strains were sensitive to ionizing radiation, MMS and bleomycin (all three). These three treatments can each induce DSBs in DNA, though their mechanisms of action are all different. The 78 strains included mutants known to be involved in homologous recombination (e.g. *rad50*, *mre11*, *rad51*, *rad52*, *rad54*, *rad55*, *rad57* and *rad59*) plus many genes not previously linked to DSB repair. These 78 mutants were selected for screening with HO endonuclease. An additional 9 mutants that were sensitive to MMS, gamma, UV and hydroxyurea in an earlier study conducted by Chang *et al.* (30) were also screened (*dun1*, *mec3*, *mms2*, *mms4/ybr099c*, *npl6*, *rad5*, *rad17* and *rad24*). Furthermore, 5 other mutants previously implicated in homologous recombination from earlier DSB induction studies were also tested, which included *rad1*, *rad9*, *rad10*, *rdh54* and *exo1* strains. (19, 44-46) A total of 9 mutants previously implicated in DSB repair by

NHEJ were also screened (*dnl4*, *lif1*, *nej1*, *rad27*, *sir2*, *sir3*, *sir4*, *yku70* and *yku80*) (4). Lastly, because *mec3* and *rad17* cells were part of the original 78 unknown mutants to test, and because Ddc1 exists in a trimeric checkpoint protein complex with Mec3 and Rad17, *ddc1* mutants were also tested (22). The complete collection thus included 101 total mutants, 17 of them previously linked to recombination and 84 uncharacterized.

In order to identify new genes required for DNA repair by homologous recombination, a new HO sensitivity assay was developed and optimized for screening the 84 potential repair deficient mutants. Because HO endonuclease stimulates mating-type switching, and mating-type switching is primarily a homologous recombination event (32), this assay was used to narrow the number of possible repair mutants that needed to be screened using the *Bse*RI-pLKL37Y integrating assay.

In order to induce a single, site-specific DSB in the mutant cells, each strain was transformed with pGALHO, a galactose inducible plasmid for the expression of HO endonuclease. Expression of HO is primarily off in cells grown on glucose or raffinose (Raff) and can be induced by transferring cells to galactose (Gal) media using this system. Furthermore, it is possible to "sub-induce" HO to obtain intermediate levels of DSBs by growing cells in Raff + Gal. The ability to repair HO-induced DSBs could then be tested by monitoring the individual mutants' growth on media containing Gal (full induction) and on Raff + Gal (sub-induction) (29, 47).

Identification of mutants sensitive to HO endonuclease that are growth-inhibited on media containing galactose

HO endonuclease sensitivity was initially tested by replica-plating cells for the purpose of monitoring growth of pGALHO-transformed mutants on media with and without Gal. This technique consisted of first growing patches of the unknown mutants, known recombination-deficient strains and WT cells independently transformed with pGALHO and control vector pRS316 on 1% Raff plates lacking uracil (Ura). Raff was selected as the carbon source instead of glucose because subsequent induction in Gal is known to be faster when cells come from Raff plates. Cells on 1% Raff were replica-plated to 1% Raff + 3% Gal plates lacking Ura to test the effects of partial HO expression on the mutants. Finally, replicas were also made onto 2% Gal – Ura plates to investigate the mutants' phenotypes after full HO endonuclease induction. The patches were transferred to each new plate using "double-imprinting", i.e., by touching the cells of a master plate to a sterile velvet cloth and making a single replica to a Raff – Ura plate (illustrated in Figure 16). This was followed by immediately using this plate as a new master and replica-plating a second time to fresh plates of selective media using a second velvet. This approach was adapted and modified from a similar double-imprinting methodology developed earlier in the Lewis lab by graduate student Jennifer Summers.

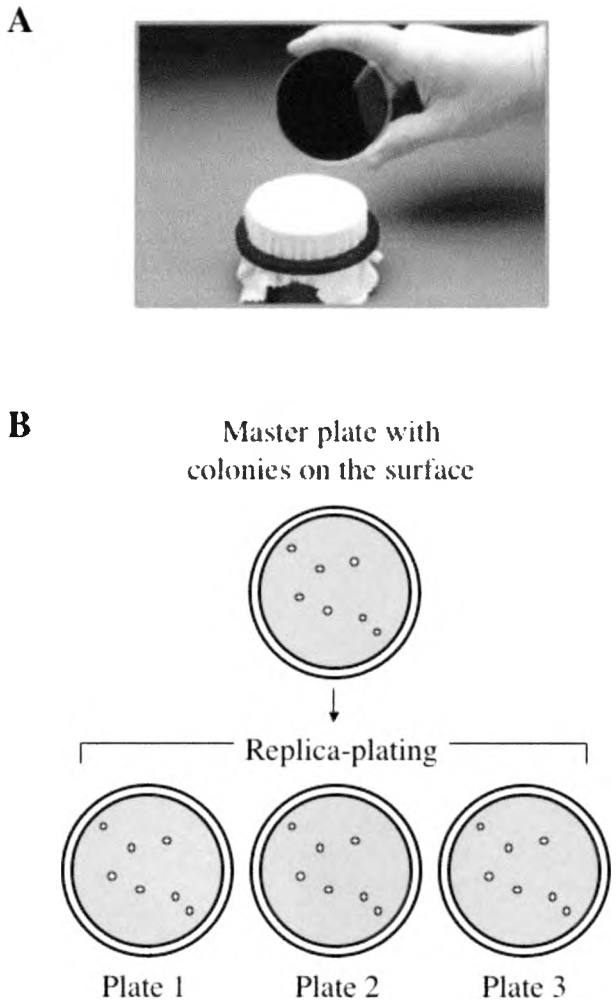

Figure 16. Illustration of the tools and technique implemented for replica-plating. After colonies are transferred to new plates using a sterile velvet (A), replicas contain a pattern of colonies identical to that of the master plate (B).

Based on the amount of cell growth on galactose media compared to growth of pRS316-containing mutants, the pGALHO-mutants were classified as either non-inhibited (+, grew well on all plates), modestly inhibited (-, grew well on Raff + Gal – Ura plates, no growth on Gal – Ura plates) or severely inhibited (- - -, only grew well on Raff – Ura, with poor or no growth on Raff + Gal and Gal plates). In these tests, growth of most of the 101 mutants tested was unaffected by HO endonuclease activity.

Figure 17 shows an example of one experiment. The top of each plate contains two patches (single streaks) of an HO-sensitive control *rad52* strain. The *rad52* patches on the right have the vector pRS316 and grow on all plates. The *rad52* cells on the left contain pGALHO and grow efficiently on Raff but not on Raff + Gal or Gal plates because HO-induced DSBs cannot be repaired in these mutants. Six previously uncharacterized mutants (*not4*, *dia4*, *ygl218w*, *spt10*, *rad18*, *srv2*), all containing pGALHO were tested in the experiment. *not4* cells did not grow on Raff + Gal or Gal and were categorized as (- - -). *ygl218w*, *dia4*, *srv2* and *spt10* mutants were inhibited only on Gal plates and were designated (-). Growth of *rad18* cells was not inhibited and these cells were scored (+). Analysis of all 101 mutants suggested that a total of 16 were HO sensitive, with 1 classified as (-) and 15 classified as (- - -). The mutant classified as (-) was *adk1*, and those classified as (- - -) included *dcc1*, *dun1*, *grr1*, *hfi1*, *htl1*, *kre22*, *mot2*, *mms2*, *mms4/ybr099c*, *nat3*, *pat1*, *rad6*, *rad24* and *slx8* mutants.

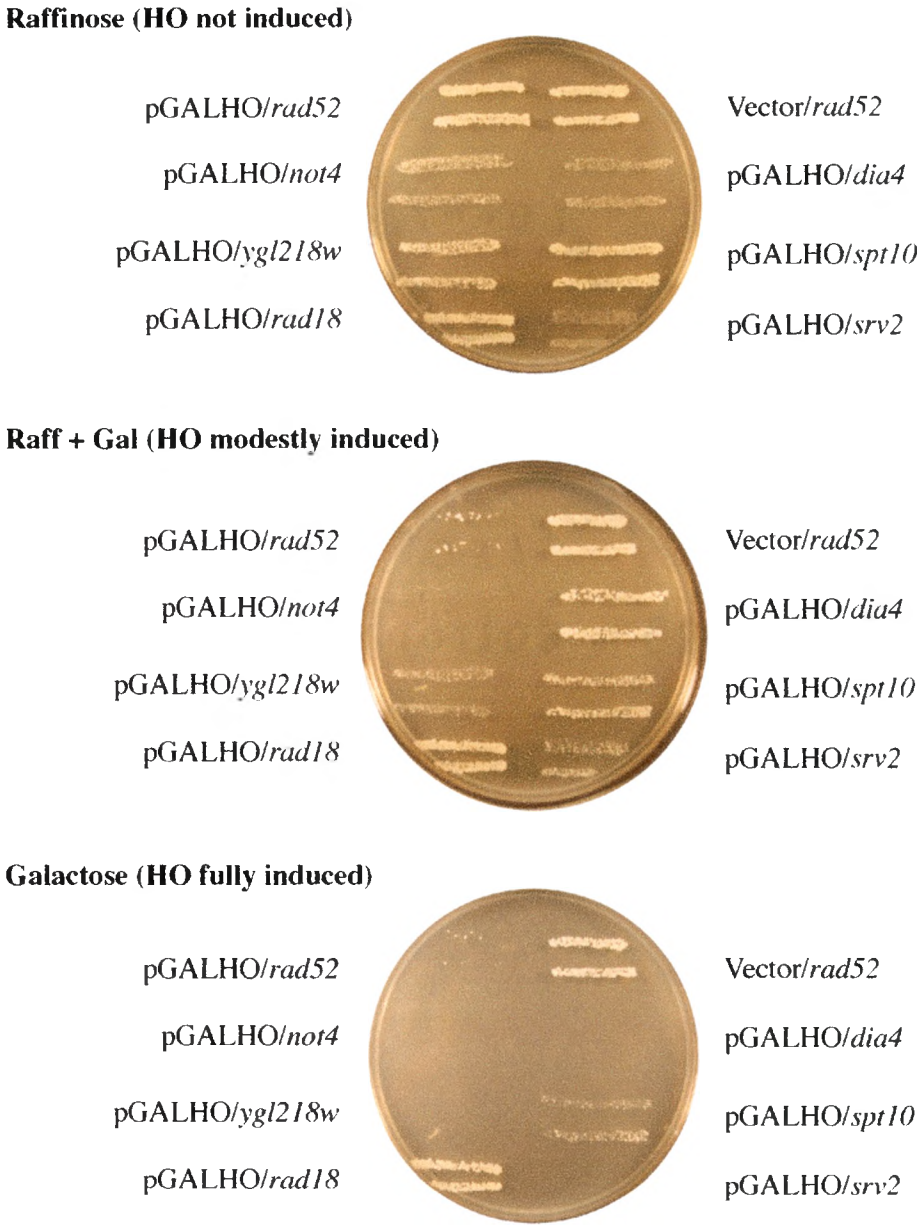

Figure 17. Double-imprints of unknown mutants displaying inhibitory effects of HO endonuclease expression. Plates used were Raff – Ura, Raff + Gal – Ura and Gal – Ura. See text for discussion.

Analysis of putative HO-sensitive mutants using dilution pronging survival assays

All 16 mutants implicated as sensitive in the HO endonuclease double-imprint assay were retested using a more quantitative dilution pronging survival assay to further investigate the extent of HO endonuclease sensitivity, and therefore, the potential involvement in DSB repair. By testing 5-fold serial dilutions, pronging survival assays cannot only detect sensitivity to HO expression, but can more accurately indicate the degree to which a mutant is affected. This assay was used to monitor the effects of HO endonuclease activity by examining whether cellular growth was inhibited and/or if cell death was induced.

Either 2×10^7 or 3×10^7 cells were inoculated into a 96-well microtiter plate, from which a series of 5-fold dilutions were made for each pGALHO plasmid-containing mutant and its respective pRS316 control. These diluted cells were pronged to 1% Raff – Ura, 1% Raff + Gal – Ura and 2% Gal – Ura plates. Cellular growth of HO-expressing mutants was compared to that of pRS316 vector-containing cells. The mutants with pGALHO were categorized as either non-inhibited (+, grew like their vector control), modestly inhibited (-, < 5-fold killing and/or slower growth relative to the vector control on plates with Gal) or severely inhibited (- - -, > 5-fold killing and inhibition of growth on plates with Gal).

Several known recombination mutants display HO endonuclease sensitivity

The HO-sensitivity of 8 well-established recombination pathway mutants (*rad50*, *rad51*, *rad52*, *rad54*, *rad55*, *rad57*, *rad59* and *mre11*) plus 3 others (*rem50*, *mus81*, *rdh54*) implicated in recombination was studied next. The survival of recombination-

defective *rad51*, *rad52* and *rad54* cells was reduced more than 625-fold compared to that of WT cells on 1% Raff + 3% Gal plates and on 2% Gal plates (Figure 18) (four columns of less growth at 5-fold dilution per column is 5 x 5 x 5 x 5 = 625). These results indicated that HO expression caused *rad51*, *rad52*, and *rad54* cells to exhibit an extremely sensitive phenotype (Table 1). This data suggests that proper function of these genes is crucial to mating-type switching by homologous recombination. These findings are consistent with previous reports which indicate these genes are highly important in recombinational DSB repair (4, 20).

Survival of *mre11*, *rad50* and *rad55* cells was decreased ≤ 5-fold (relative to WT cells containing pGALHO) on 1% Raff + 3% Gal plates and on 2% Gal plates (Figure 18). The results suggest that these mutants are only modestly defective in mating-type switching (Table 1). Previous studies have supported this data by proposing that inactivation of these genes is, in part, countered by the presence of "back-up" proteins (14, 35).

As seen in Figure 18, *rad57* and *rad59* cells grew similar to wild-type cells when HO was expressed, indicating that these mutants play little or no role in mating-type switching. This idea is further supported by the suggestions that Rad57 and Rad59 are less significant proteins that participate in recombination repair (12). Growth of the 3 additional gene implicated in recombination, *rem50*, *mus81* and *rdh54*, were also unaffected by HO. Results with the 8 established recombination gene mutants are summarized in Table 1.

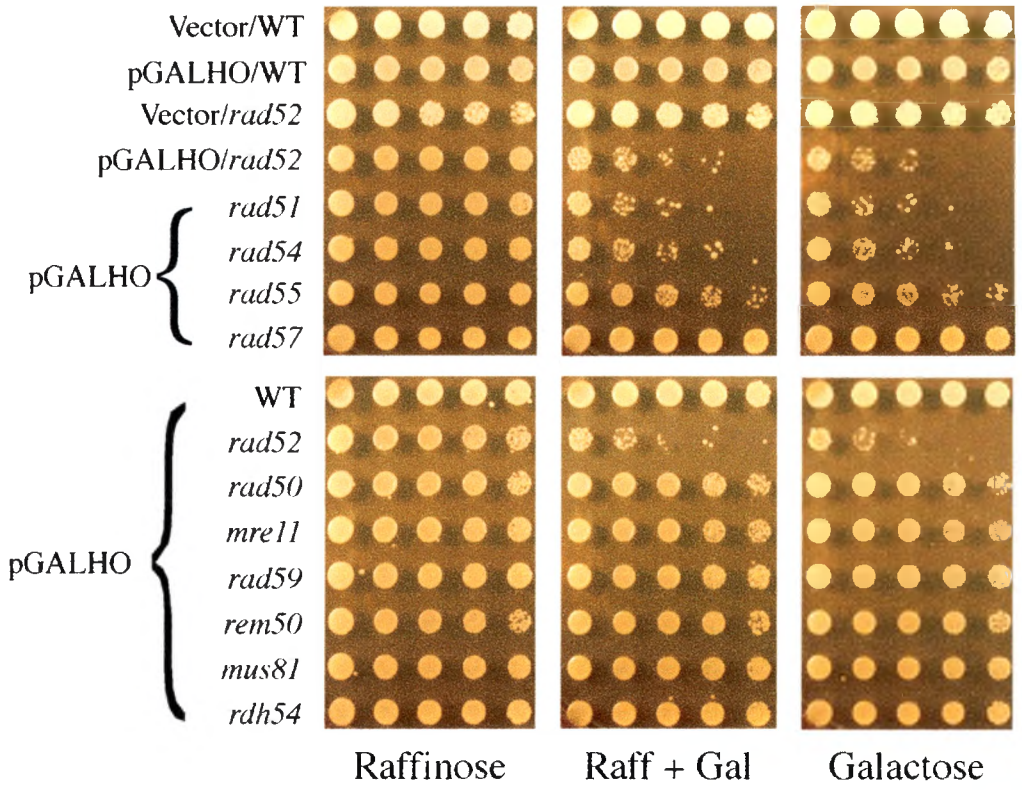

Figure 18. Dilution pronging survival assays of well-known recombination mutants and others previously implicated in DNA repair. Vector or pGALHO-containing cells grown on Raff-Ura plates were harvested and pronged to Raff – Ura, Raff + Gal – Ura and Gal – Ura plates. Expression of HO endonuclease induced killing or growth inhibition in known recombination mutants (*rad51*, *rad52*, *rad54*, *rad55*, *rad50* and *mre11*). The genes *RAD57*, *RAD59*, *REM50*, *MUS81* and *RDH54* have been implicated in recombination, but strains with these genes inactivated exhibited no obvious effects in response to HO activity.

Table 1. Summary of HO-sensitivity in *S. cerevisiae* mutants previously identified as deficient in DSB repair

Gene	Effect of HO expression[a]	Description
rad50	-	Homologous Recombination, telomere stability
mre11	-	Homologous Recombination, telomere stability
rad51	- - -	Homologous Recombination
rad52	- - -	Homologous Recombination
rad54	- - -	Homologous Recombination
rad55	-	Homologous Recombination
rad57	+	Homologous Recombination
rad59	+	Homologous Recombination

[a] Symbols: +, wildtype growth; -, modest growth inhibition; - - -, strong growth inhibition

The HO endonuclease assay identifies new mutants that are sensitive to a single, site-specific DSB

In order to test the 16 unknown mutants suggested by the double-imprinting replica-plating assays more quantitatively for HO endonuclease sensitivity, the dilution pronging survival assay was applied. A total of 10 mutants were determined to be either growth-inhibited or killed due to HO endonuclease activity. For example, *slx8* and *pat1* cells transformed with pGALHO both demonstrated approximately a $\geq$ 25-fold decrease in survival on 1% Raff + 3% Galplates and on 2% Gal plates (Figure 19A). Thus, the *SLX8* and *PAT1* genes appear to be critical for mating-type switching and may even be more crucial for the process than other known recombination genes, such as *MRE11*.

As seen in Figure 19B, *grr1* mutants exhibited strong killing, and *htl1* and *not4* cells showed modest killing on Gal plates. Results for *grr1* imply that the product of this

gene is essential for mating-type switching and may be as significant as *RAD52*. Survival of *mms2*, *dun1* and *rad24* mutants was suppressed 625-fold, 125-fold and ~ 5-fold, respectively, after induction of HO on Gal plates (Figure 19C). All 10 genes are listed along with brief descriptions in Table 2.

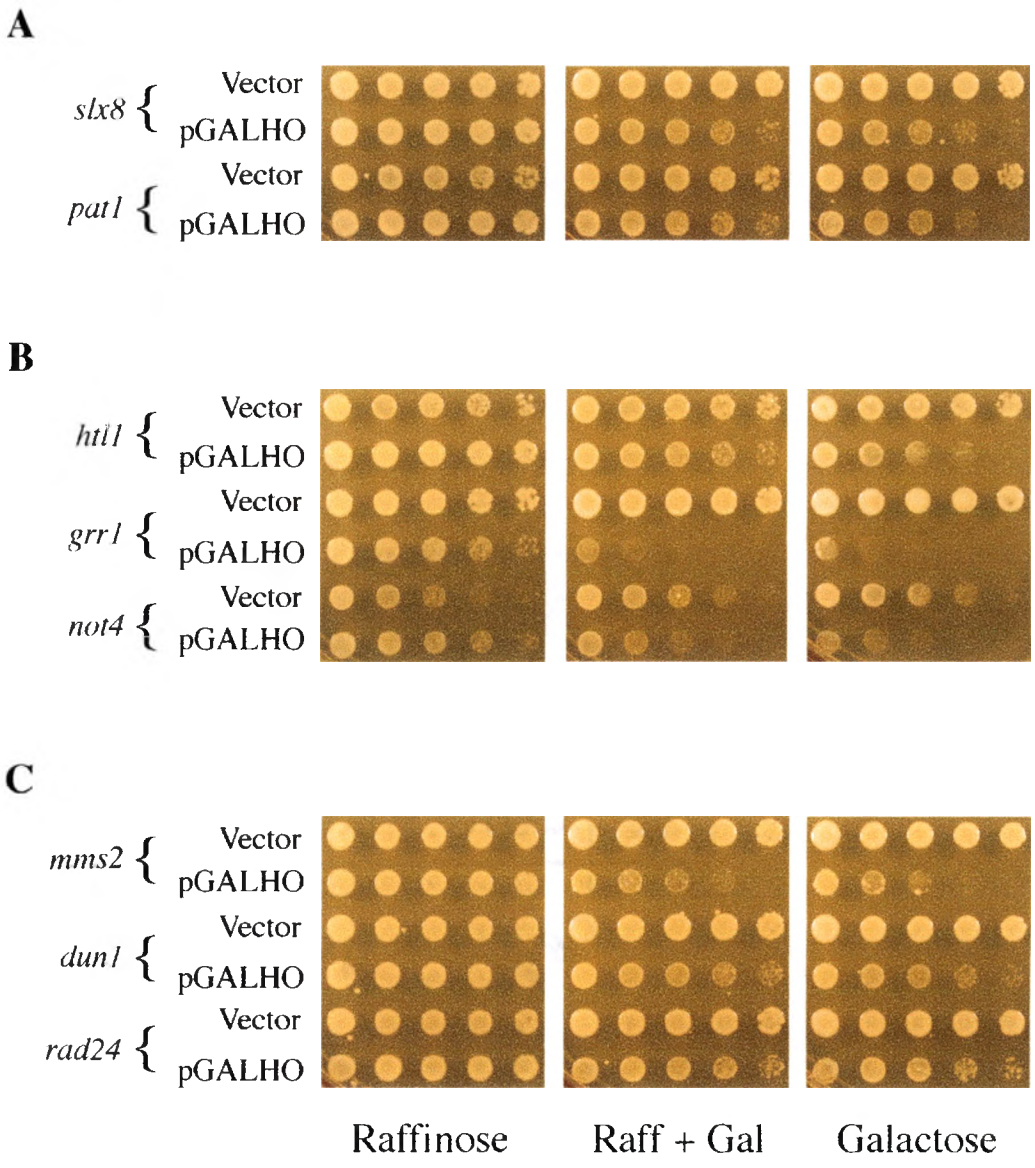

Figure 19. Dilution pronging survival assays of HO-sensitive mutants. Vector or pGALHO-containing cells grown on Raff-Ura plates were harvested and pronged to Raff, Raff + Gal and Gal plates as for Figure 17.

Table 2. *S. cerevisiae* mutants sensitive to HO endonuclease expression

Gene / ORF	Description
dcc1 / YCL016c [a]	Sister chromatid cohesion and telomere length maintenance
dun1 / YDL101c [a]	DNA damage checkpoint, postreplicative DNA repair
grr1 / YJR090c	Carbon catabolite repression, divalent cation transport, high affinity glucose transport, morphogenesis, sulfite detoxification
htl1 / YCR020w-B	Chromatin remodeling, transcriptional regulation/elongation, chromosome stability, establishment of cohesion, telomere maintenance
mms2 / YGL087c	Postreplication DNA repair, contributes to ubiquitin-protein ligase activity
mot2 / YER068w	Multiple transcription roles, degradation of mRNA
nat3 / YPR131c [a]	Acetylation of N-terminal residues of proteins
pat1 / YCR077c	mRNA decapping factor associated with Topoisomerase II
slx8 / YER116c	Substrate-specific ubiquitin ligase
rad24 / YER173w [a]	DNA damage checkpoint, loads Rad17-Mec3-Ddc1 onto DNA

[a] *dcc1*, *dun1*, *nat3* and *rad24* mutants exhibited only modest effects on growth/survival; all other mutants displayed 5-fold or more killing when HO was expressed. Descriptions are from the *Saccharomyces* Genome Database (www.yeastgenome.org).

All other mutants showed no effect from HO expression, indicating that these genes do not play critical roles in mating-type switching (listed in Table 3). Other mutants selected for analysis, listed in Table 4, were not tested due to a variety of technical reasons. Growth of six of these strains was compromised because of complications with sugar processing. The mitochondria were compromised in two of the untested mutants, affecting their growth ability in Gal and therefore proper analysis of effects caused by HO expression. Four of the other mutants not tested were not available

in the yeast deletion strain library collection used here, and *cdc40* cells could not be tested because they are *URA3* but should be *ura3*.

Table 3. *S. cerevisiae* mutants not sensitive to HO endonuclease activity

adk1	*ctf8*	*mec3*	*rad17*	*spt10*
akr1	*ddc1*	*mms4*	*rad18*	*srb5*
anc1/taf14	*dhh1*	*mms22*	*rad27*	*ume6*
asf1	*exo1*	*mus81*	*rad61*	*vid21*
bck1	*fab1*	*not5*	*rdh54*	*vid31*
bem1	*fun12*	*npl6*	*ref2*	*vph2*
bdf1	*gos1*	*nup84*	*rem50*	*yaf9*
bud32	*hfi1*	*nup120*	*rlr1*[b]	*ybr099c*
bur2[a]	*hof1*	*pol32*	*rpb9*	*ybr100w*
cax4	*hpr1*	*rad1*	*rpl31a*	*ylr235c*
cbc2/sae1	*hpr5*	*rad5*	*rsc1*	
chl1	*jem1*	*rad6*	*ruv161*	
cmn67	*kre22*	*rad9*	*sae2*	
ctf4/pob1	*mct1*	*rad10*	*sgs1*	

[a] *bur2* cells containing pGALHO consistently grew better on Gal than control *bur2* cells containing only vector

[b] Isolates of *rlr1* grew better on galactose than on raffinose.

Table 4. *S. cerevisae* mutants not tested

Sugar[a]	Mitochondria[b]	Not in library[c]	Ura^{+d}
apn1	*mdm10*	*cdc28*	*cdc40*
clc1	*mdm20*	*ddc2*	
dia4		*rvs167*	
nup133		*xrs2*	
srv2			
ygl218w			

[a] Compromised growth on raffinose, uninhibited or excessive growth on raffinose/galactose, no growth on galactose only

[b] Mitochondria are compromised affecting growth on particular carbon sources

[c] Strains not included in the yeast deletion strain library

[d] Strain produces URA; conflicts with pGALHO selective marker

Optimized recombination assay implicates mating-type switching genes in homologous recombination repair

A total of 10 genes were identified as potentially involved in mating-type switching due to their sensitivity to HO endonuclease expression. To establish whether any of these genes were involved in DSB repair by homologous recombination, the new *Bse*RI-pLKL37Y integrating recombination assay was employed to test each of the mutants. Results of several control assays using known recombination mutants are shown in Figure 20. All of the mutants tested were significantly reduced in recombinational repair compared to WT cells. The strong reductions in recombination proficiency in *rad51*, *rad52* and *rad54* mutants (125-167 fold) are consistent with previous studies (4, 14) and the HO endonuclease sensitivities shown in Table 1. The more modest reductions in the other recombination mutants are also in accord with past studies.

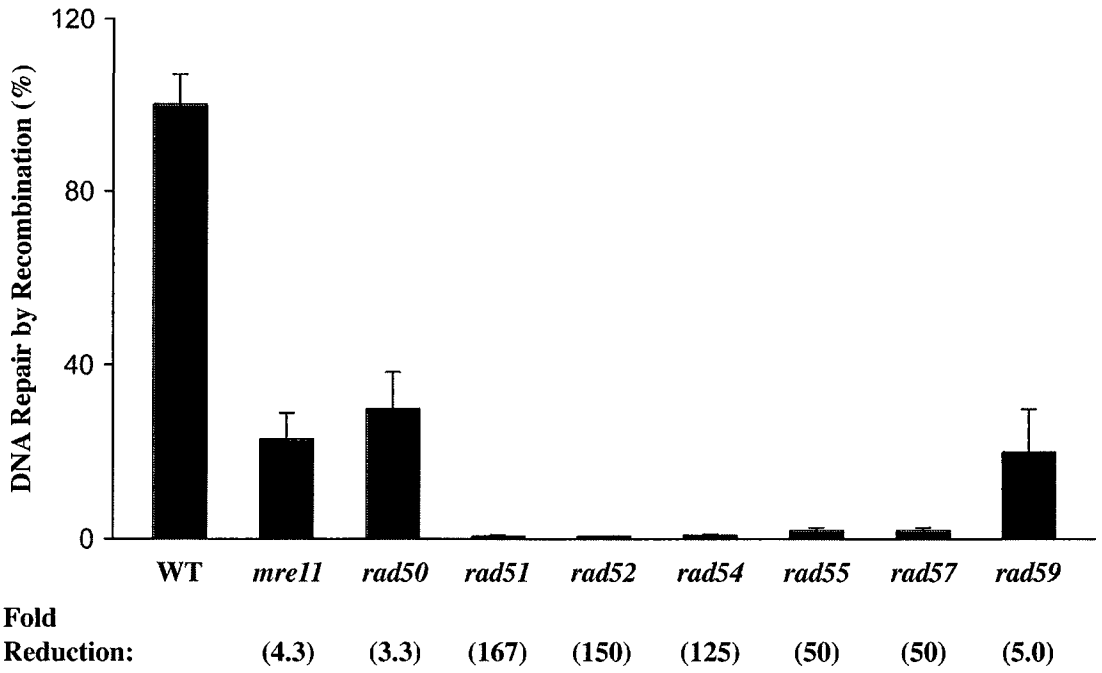

Figure 20. Inactivation of genes known to be involved in recombination caused a decrease in repair by recombination. Error bars indicate standard deviations.

As shown in Figure 21, recombination in 8 of the 10 mutants tested using the plasmid integration assay did not differ by more than 2-fold from that in WT cells. These results indicated that *dcc1*, *dun1*, *htl1*, *mms2*, *mot2*, *pat1*, *rad24* and *slx8* mutants do not have defects in plasmid:chromosome recombination. Although these mutants were not defective in general recombination, they might serve a more specialized role in mating-type switching, which is an intrachromosomal gene conversion event.

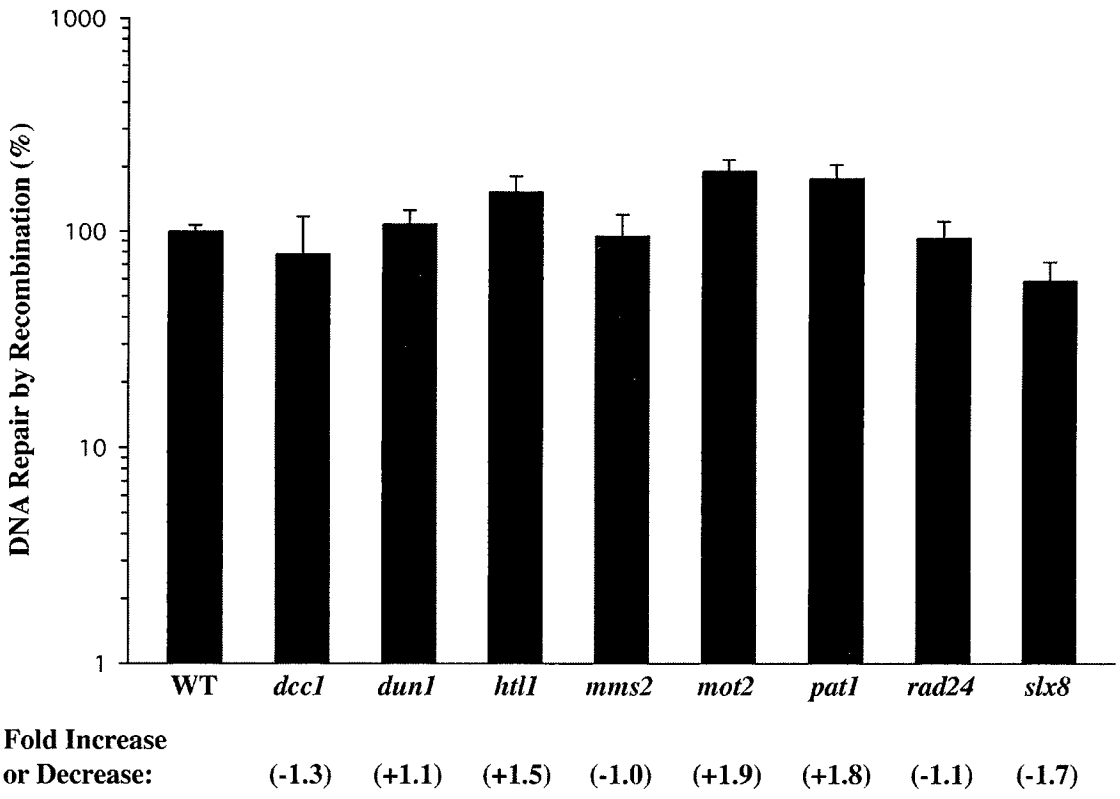

Figure 21. Eight of the HO-sensitive mutants were similar to WT cells in DSB recombinational repair. Fold increases or fold decreases for *Bse*RI-cut pLKL37Y DNA repair by integration are shown, indicated by + and -, respectively. Error bars indicate standard deviations.

Interestingly, results for the remaining 2 mutants, *grr1* and *nat3*, revealed that recombination was significantly different from WT in these mutants (see Figure 22). Upon inactivation of *NAT3*, efficiency in repair was decreased by 14.3-fold compared to that of WT cells (Figure 22A). This strong reduction suggests that *NAT3* is important in DSB repair *via* homologous recombination. Interestingly, cells with an inactivation of *GRR1* exhibited a substantial increase in recombination rather than a decrease. The 14.2-fold increase in recombination indicates that *grr1* cells are hyper active in recombination

(Figure 22B). Hyper-recombination mutants have been identified previously, e.g., *hpr1* and *hpr5* (*srs2*) (48). Interestingly, *hpr5* strains that have demonstrated hyper-recombination phenotypes in haploids displayed defects in recombination in diploids between homologous chromosomes. Currently, these defects are attributed to the mutant diploids frequently initiating recombination events between homologous chromosomes without properly completing them, resulting in lethal effects (48).

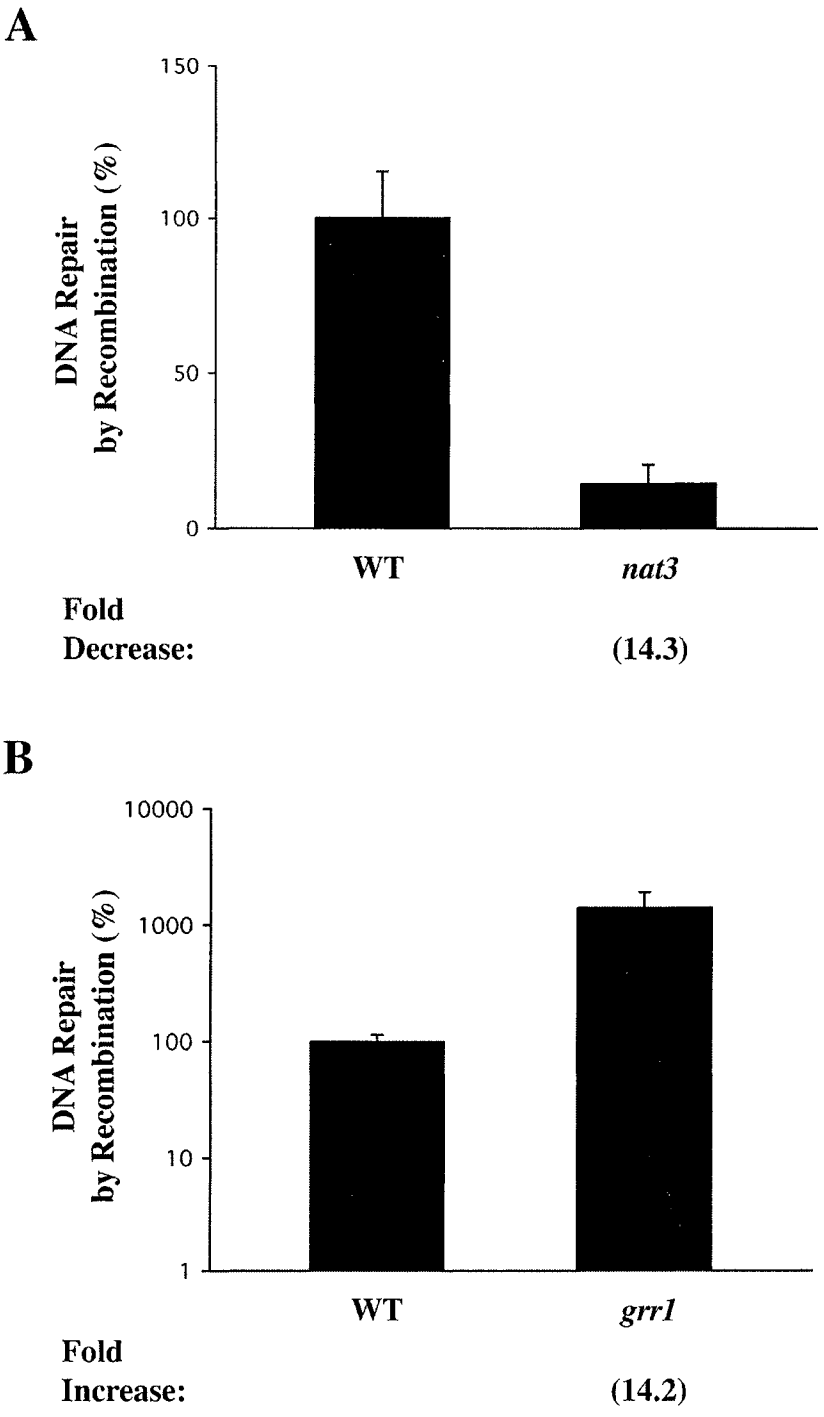

Figure 22. Inactivation of genes *nat3* and *grr1* resulted in recombinational repair phenotypes substantially different from that of WT cells. Fold increases or fold decreases for *Bse*RI-cut pLKL37Y repair by integration are shown.

Summary and Conclusions

In this project, two distinct goals were pursued and successfully accomplished. First, an assay was developed that was able to characterize previously unidentified mutant yeast strains defective in repair of HO endonuclease-induced DSBs. Secondly, a new plasmid-targeting assay to monitor recombination efficiency was created to further analyze the HO-sensitive mutants to examine whether any experienced general defects in homologous recombination.

Initial recombination assays using *Bcl*I-cut plasmids with one, two or three genes demonstrated no correlation between DNA repair efficiencies and an increasing number of selectable markers. Furthermore, all assays using *Bcl*I-cut plasmids were unable to generate a satisfactory number of recombinant colonies. The results may be attributed to the fact that digestion of *Bcl*I did not to produce sufficient homology between the broken ends of the *HIS3* plasmid and the *his3-Δ1* chromosomal DNA sequence *in vivo*, a variable that is known to be critical (43).

Conducting recombination assays using a plasmid containing a gap did increase the number of recombinants produced and created a novel approach for monitoring both homologous recombination and NHEJ in one test. However, results obtained for gap repair assays were not consistent enough to accept with confidence.

Using integrating plasmid pLKL37Y digested with *Bse*RI for conducting recombination assays increased the number of recombinants obtained. This result supports the idea that increased homology increases recombinants (43) because digesting pLKL37Y with *Bse*RI increases the homology of the plasmid to chromosomal DNA compared to *Bcl*I-cut plasmid. Additionally, the fold-reductions in recombination

efficiency in *rad51* and *rad52* mutants were consistent with values observed in earlier work using other assays (35).

Assays involving expression of HO endonuclease *in vivo* produced results which implicated 10 new genes in DSB repair by mating-type switching. Interestingly, only 2 of these new mutants, *nat3* and *grr1*, had strong effects on DNA repair by recombination in the plasmid targeting assays. It is possible that some mutants that are HO-sensitive affect recombination between or within chromosomes but do not affect plasmid:chromosome recombination. For example, *SRS2* and *RDH54* have been implicated in recombination between homologous chromosomes but are not needed for sister chromatid exchange in haploid cells (48).

Nat3 is the catalytic subunit of the complex it forms with Mdm20, commonly referred to as NatB. This complex acts to acetylate the amino-terminus of selected proteins. Interestingly, targets identified for this enzyme include proteins involved in assembly and recruitment of histones to DNA. Recent experiments in the Lewis lab have demonstrated a linkage between nucleosome assembly and homologous recombination (1), and it is possible that Nat3 also plays a role in these processes. In addition, a BLAST search was performed at the NCBI website (www.ncbi.nlm.nih.gov/sites/entrez/) that identified three similar human proteins with E scores of 10^{-19}-10^{-36}, which indicates strong homology to the yeast protein Nat3. These human proteins are also N-terminal acetyltransferases.

Grr1 protein is a ubiquitin ligase and is involved in a protease degradation of cellular proteins (49). Cells display a plethora of phenotypes upon inactivation of the *GRR1* gene, including defects in regulating cyclin (Cln1/Cln2) levels (49), resistance to

extreme levels of divalent cations, sensitivity to sulfite, deficiency in glucose repression, as well as alterations of high affinity glucose transport (www.yeastgenome.org). How Grr1 protein might influence DNA repair is unclear. However, conducting a BLAST search to find a homolog of Grr1 in humans revealed that several F-box/leucine-rich repeat proteins in humans had homology scores between 10^{-10}-10^{-25} implying potential similar functions to the yeast protein. Theoretically, both Grr1 and Nat3 could affect DNA repair proteins by either ubiquitination, which might affect protein half-lives, or N-terminal acetylation, which may regulate function.

Results from this study suggest that future experiments should include testing of intrachromosomal DNA recombination, which is more similar to HO-induced mating-type switching. This will allow any new genes implicated in chromosomal recombination to be confirmed by their response to HO endonuclease activity. In addition, the *Bse*RI-pLKL37Y integrant strains that were generated from the recombination assays used in this study might be used for new intrachromosomal recombination experiments. Because the integrants essentially contain duplicated alleles (double repeats) of *HIS3*, spontaneous intrachromosomal recombination events can occur (50). When this happens, a small circular plasmid-like piece of DNA is pinched off, so that it contains the *URA3* gene but no *CEN/ARS*. With no *CEN/ARS* region, the circular DNA will be lost due to its inability to be replicated, and the resulting cells will no longer be *URA3*$^{+}$, which can be selected for using 5-fluoroorotic acid (5-FOA).

Several human genetic disorders have been linked to defects in genes that are involved in DSB repair. The disorders Nijmegen Breakage Syndrome (NBS) and Ataxia Telangiectasia-like Disorder (ATLD) are caused by mutations in hNBS (equivalent to

yeast Xrs2) and hMre11, respectively. In addition, animals and humans with Severe Combined Immunodeficiency (SCID) have mutations in the DSB repair genes *YKU70* or *YKU80* (51-52). NHEJ genes such as *SIR2* or *KU70/KU80* have also been linked to telomere stability and aging in humans (51, 53). The ten new genes affecting DSB repair identified in the current study may also be found to affect human health, but this hypothesis awaits further tests.

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