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Thesis

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Implementation of an extrachromosomal system for the detection of novel DNA double strand break repair genes in *S.cerevisiae*

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1. ABSTRACT

The outcome of DNA damage is diverse and generally adverse. Acute effects arise from disturbed DNA metabolism, triggering cell-cycle arrest or cell death. Long-term effects result from irreversible mutation contributing to oncogenesis and genome instability. In view of the many types of lesions, several pathways were developed to repair these lesions: nucleotide-excision repair (NER), base-excision repair (BER), mismatch repair (MMR), homologous recombination (HR) and non-homologous end joining (NHEJ).

The double-strand break (DSB) is the most dangerous type of DNA lesion, it can be repaired mainly by HR (by crossover (CO), gene conversion (GC), and single-strand annealing (SSA)) or by non-homologous end-joining (NHEJ). When, after replication a second identical DNA copy is available, HR seems to be preferred, other wise cells rely on NHEJ, which is more error prone.

Up to now, all DSB repair processes have been studied separately, although it is clear that all of them can occur simultaneously in different proportions. We have created a new molecular plasmid system to simultaneously detect all four types of recombinational DBS repair. To this end, we constructed an *in vivo/in vitro* **HNS** plasmid system (**HNS**: HR, NHEJ, SSA), based upon two topologically different DNA molecules, which allows us to follow all four recombination processes at once. The plasmids, named pURRA8Δ and pRURA8Δ, contain two truncated non-functional URA3 genes in direct or inverted orientation respectively, sharing a central homologous region where a I-*Sce*I site was introduced artificially. The plasmids also carry a centromere sequence and two phenotypic markers *TRP1* and *ADE8*.

DSB can be induced *in vitro* at the homologous (*I-Sce*I) or non-homologous (*Bam*HI) region. Yeast transformation with linearized plasmids was performed in strain YPH 250 and isogenic knockout strains lacking either the *RAD52* gene involved in HR and SSA, *HDF1* (*yKU70*) and *NEJ1* involved in NHEJ. In addition *MRE11* complex and *MSH2* null mutants were studied. Distribution of DSB repair events among the various pathways was monitored by phenotypic and PCR analysis. The *rad52* knockout mutant showed lower levels of CO and SSA, while the *hdf1* mutant showed a decrease in conservative and non conservative NHEJ, as expected. These results confirm the validity of the HNS system for monitoring all 4 repair pathways simultaneously.

The HNS system has been used to identify new genes involved in DNA damage response. DBS were induced *in vivo* by the expression of the *I-Sce*I endonuclease under Gal1-promoter control. After transformation, we performed transposon mutagenesis using the mTn-lacZ/*LEU2* library system and selected cells that lost the ability to recombine.

From the initial 7000 mutants tested, we selected initially 150 that were re-screening to obtain finally 33 mutants. The identification of the locus of the transposon insertion of all of them was performed. Some known genes (*RAD50,*

SWR1, MCK1, SIN4, RSC2, SWE1 and DBP1) as well as unknown ones (*YLR238W, YLR089C, YMR278W*) were selected. Null mutants of all them were constructed, DSBR events profile as well as MMS sensitivity were determined.

Relative rates of DSB repaired by HR, NHEJ and SSA were examined in all the selected null mutants strains. By comparing the distribution of DSBR by different mechanisms, we were able to obtain a strain-specific profile in which the relative proportions of repair events occurring in the cell were characteristic of that mutation.

RSC2 (RSC complex component) and *YLR235w* (FHA containing protein) genes were selected to further characterisation. Epistatic null mutants analysis with key recombination genes (*yku70* and *rad52*) was performed. MMS sensitivity and survival was analysed, as well as DSB induction *in-vivo*. This enables us to verify the involvement of a particular gene product in DNA damage response. In particular, we showed that Ylr238w is involved in DNA damage transcription regulation.

2. ABBREVIATIONS

BER	Base-excision repair
BIR	Break-induced replication
bp	Base pair
CBP	Cruciform binding protein
CEN	Centromere
CO	Crossing over
dHJ	Double-Holliday-junction
DSB	Double-strand DNA break
DSBR	Double strand break repair
dsDNA	Double strand DNA
FHA	Forkhead-associated domain
GC	Gene conversion
HJ	Holliday-junction
HR	Homologous recombination
IR	Ionising radiation
MMR	Mismatch repair
MMS	Methyl methanesulfonate
MRX complex	RAD50-MRE11-XRS2 complex
NER	Nucleotide-excision repair
ORF	Open reading frame
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
RFC	Replication factor C
RPA	Replication protein A
SC	Synthetic complete
Sc	*Saccharomyces cereviseae*
SDSA	Synthesis-dependent strand-annealing
SMC	Structural manteinance of chromosome
SSA	Single-strand annealing
SSB	Single-strand break
ssDNA	Single strand DNA
TCR	Transcription-coupled recombination
X-DNA	Cruciform DNA

3. INTRODUCTION

3.1   DNA DAMAGE

Lesions to DNA arise from three main exogenous causes. Some environmental agents such as the ultraviolet (UV) component of sunlight, ionizing radiation and numerous genotoxic chemicals cause alterations in DNA structure, which, if left unrepaired, may lead to cell death or mutations that enhance cancer risk. In addition, some products of normal cellular metabolism can damage DNA. For example, reactive oxygen species (superoxide anions, hydroxyl radicals and hydrogen peroxide) derived from oxidative respiration and products of lipid peroxidation (CADET et al. 1997). Finally, some chemical bonds in DNA tend to spontaneously disintegrate under physiological conditions. Hydrolysis of nucleotide residues leaves abasic sites.

The outcome of DNA damage is diverse and generally dangerous. In view of the different types of lesions, no single repair process can cope with all kinds of damage. At least five main, partly overlapping, damage repair pathways operate in eukariotic cells: nucleotide-excision repair (NER), base-excision repair (BER), mismatch repair (MMR), homologous recombination (HR) and non homologous end-joining conservative (NHEJc) and non conservative (NHEJnc).

NER deals with the wide class of helix-distorting lesions that interfere with base pairing and obstruct transcription and normal replication. These lesions may or may not affect transcription and replication, although they frequently miscode. Most NER lesions arise from exogenous sources (except for some oxidative lesions). Of all

repair systems, NER is the most versatile in terms of lesion recognition. Two subpathways exist with distinct substrate specificity: global genome NER (GG-NER) which surveys the entire genome for distorting injuries, and transcription–coupled repair (TCR) which focuses on damage that blocks the elongating RNA polymerases.

The GG-NER complex XPC-hHR23B screens for disrupted base pairing. In TCR the block of the RNA polymerase at the lesion seems to be critical. The stalled polymerase is displaced by two TCR factors: CSBp and CSAp.The subsequent stages of both subpathways may be the same. The XPBp and XPDp helicases of the multi-subunit transcription factor TFIIH open the DNA around the damage (30 bp). XPAp is recruited and confirms the presence of the damage. The single-strand binding protein RPAp stabilizes the open DNA structure intermediate. The endonucleases XPGp and ERCC1/XPFp cleaves 3' and 5' borders of the open damaged strand, respectively. The regular replication machinery completes the repair by filling the gap.

Small chemical alterations of bases are targeted by BER that is therefore particularly relevant for preventing mutagenesis. Most BER lesions arise from cellular metabolism. Glycosylases flip the damaged base out of the helix structure, which is cleaved from the sugar-phosphate backbone, resulting in a abasic site. The strand incision at the abasic site is done by the Ape1p endonuclease. DNA polβ performs a one-nucleotide gap-filling and removes the 5'-terminal baseless sugar residue via its lyase activity. The nick is then ligated by XRCC1-ligase3 complex.

MMR removes nucleotides mispaired by DNA polymerases and insertion/deletion loops that results from slippage during replication of repetitive sequences or during recombination. Heterodimer MSH2/6 focus on mismatches and single –base loops, whereas MSH2/3 recognize insertion/deletion loops. A number of proteins are implicated in the excision of the new strand past the mismatch and resynthesis step, including polδ/ϵ, RPA, PCNA, RFC, exonuclease 1 and endonuclease FEN1.

Lesions for NER, BER and MMR repair processes affect only one of the DNA strands. In a 'cut-and-patch'-type reaction, the injury (with or without some flanking sequences) is taken out and the resulting single-stranded gap is filled-in using the intact complementary strand as template. DSBs are more problematic, as both strands are affected. Double-strand DNA breaks (DSBs) are induced by ionising radiation, chemicals or during replication of single-strand breaks (SSBs) and presumably during repair of interstrand crosslinks. Cells with specialized DNA recombination activities, such as B- and T-cells, induce DSBs during rearrangements of their immunoglobin or T-cell-receptor genes. During cell division, DSBs are a problem as intact chromosomes are a prerequisite for proper chromosome segregation. Thus, DSB may induce various sorts of chromosomal aberrations, including aneuploidy, deletions (loss of heterozygosity) and chromosomal translocations.

The DSB is consider to be the most lethal kind of DNA lesion, interrupting the physical continuity of the molecule. Two main pathways, homologous recombination and end-joining, and presumably additional back-up systems, have evolved to solve the DSB problem.

a Damaging agent

X-rays
Oxygen radicals
Alkylating agents
Spontaneous reactions

UV light
Polycyclic aromatic
hydrocarbons

X-rays
Anti-tumor
agents
(cis-Pt, MMC)

Replications
errors

Uracil Abasic site
8-Oxoguanine
Single-Strand Break

(6-4)PP
Bulky adducts
CPD

Interstrand cross-link
Double-strand break

A-G Mismatch
T-C Mismatch
Insertion
Deletion

Base-exision
repair (BER)

Nucleotid-excision
repair (NER)

Recombinational
repair (HR, NHEJ)

Mismatch repair
(MMR)

Repair process

b Consequences

Transient
Cell-cycle
arrest

Inhibition of:
•Transcription
•Replication
•Chromosome
 segregation

Apoptosis
(cell death)

Mutations

Chromosome
aberrations

Cancer
Aging
Inborn
disease

FIGURE 1. DNA damage, repair mechanisms and consequences. **a**, Common DNA damaging agents (top); examples of DNA lesions induced by these agents (middle); and most relevant DNA repair mechanism responsible for the removal of the lesions (bottom). **b**, Acute effects of DNA damage on cell-cycle progression, leading to transient arrest in the G1, S, G2 and M phases (top), and on DNA metabolism (middle). Long-term consequences of DNA injury (bottom) include permanent changes in the DNA sequence (point mutations affecting single genes or chromosome aberrations which may involve multiple genes) and their biological effects. Abbreviations: *cis*-Pt and MMC, cisplatin and mitomycin C, respectively (both DNA-crosslinking agents); (6–4)PP and CPD, 6–4 photoproduct and cyclobutane pyrimidine dimer, respectively (both induced by UV light); BER and NER, base- and nucleotide-excision repair, respectively; HR, homologous recombination; NHEJ, non-homologous end joining. Adapted from (HOEIJMAKERS 2001).

4

Homologous recombination seems to dominate in S and G2 cell cycle phases when the DNA is replicated, providing a pristine second copy of the sequence (the sister chromatid) for repairing the breaks. In contrast, the less-accurate end-joining is most relevant in the G1 phase of the cell cycle, when a second copy is not available (HOEIJMAKERS 2001). Figure 1 summarizes some of the most common types of DNA damage and their sources.

3.2   DNA DAMAGE RESPONSE

Cells respond to DNA DSBs through the actions of systems that detect the DNA lesion and then trigger various downstream events. These events can be viewed as classical signal-transduction cascades in which a `signal' (DNA damage) is detected by a `sensor' (DNA-damage binding protein) that then triggers the activation of a `transducer' system (protein kinase cascade), which amplifies and diversifies the signal by targeting a series of downstream `effectors' of the DNA-damage response. Clearly, such events need to be exquisitely sensitive and selective, as they must be triggered rapidly and efficiently even by a low numbers of chromosomal DNA DSB, yet must remain inactive under other conditions.

Although the repair of different types of DNA lesion relies on different sets of proteins, the various forms of DNA damage nevertheless trigger common signal transduction pathways, known as the DNA damage response. One well-established feature of the DNA damage response is the slowing or arrest of cell-cycle progression, as a result of the activation of what are termed DNA damage "checkpoints" (LOWNDES and MURGUIA 2000; MELO et al. 2001), which delay key cell-

cycle transitions until repair has occurred. Other aspects of the DNA damage response include changes in chromatin structure at the site of DNA damage and the transcriptional induction and posttranslational modification of various proteins involved in DNA repair. In addition to detecting different types of DNA lesions, the cell must also be able to recognize very low levels of DNA damage anywhere in the genome.

When damage arises in the G_1 or S cell-cycle phases, for example, entry into S-phase is prevented or progress through S-phase is slowed, respectively. This presumably gives time to allow DNA repair to occur before DNA polymerase encounters the lesions. Similarly, DNA DSBs present in G_2-phase prevent entry into mitosis, thereby preventing the mis-segregation of chromosomal fragments during cytokinesis (KHANNA and JACKSON 2001) (JACKSON 2002).

While meiotic recombination involves chromosomal homologs, it is believed that most of the recombination events during mitotic growth occur in late S and G2 phases and involve sister chromatids. However, in yeast, there is considerable capacity to carry out allelic (interchromosomal) recombination during mitotic growth, and allelic recombination appears to have somewhat different genetic requirements than sister chromatid-based recombination.

The rapidity and potency of the DNA damage response indicates that the signaling proteins involved are very sensitive and have the capacity to amplify the initial signal. These are key features of the Mec1p/Tel1p signal transduction network, a protein kinase cascade that is critical for cellular responses to many types of DNA damage (Table 1) (ROUSE and JACKSON 2002). In budding yeast the central regulator of the

pathway is Mec1p. It belongs to a family of protein kinases termed PIKKs (phosphatidylinositol 3-kinase-like protein kinases), and its functions is partially redundant with Tel1p, another PIKK family member. Orthologs of Mec1p and Tel1p have been identified in many species, including humans (ATR and ATM, respectively). Disruption of ATR or Mec1p causes cell lethality, while ATM and Tel1p are not essential proteins. Mec1p exists in a complex with another protein, Lcd1p (Ddc2p or Pie1p) that is required for all known functions of Mec1p (Table 1). For simplicity, I shall refer it for Lcd1p.

Several cellular proteins become rapidly phosphorylated in a Mec1p/Tel1p-dependent manner in response to DNA damage (MELO et al. 2001). Full Mec1p-dependent activation of downstream targets, such as Rad53p, requires several additional factors, some of which form two discrete protein complexes. The first of these resembles the pentameric replication factor C (RFC), except that the Rfc1p large subunit is replaced by Rad24p checkpoint protein forming a complex with the four small RFC subunits (Rfc2-5p). The proteins of the second complex--Rad17p, Ddc1p and Mec3p-show sequence similarity to the proliferating cell nuclear antigen (PCNA) "sliding clamp".

Both the Rad24p/Rfc2-5p and the Rad17p-Ddc1p-Mec3p complexes are recruited to sites of DNA damage (MELO et al. 2001), the translocation of the Rad17p/Ddc1p-Mec3p complex to sites of DNA damage requires Rad24p but not Mec1p-Lcd1p.

It has been shown that the Mec1p-Lcd1p complex is also recruited to sites of DNA damage, independently of the PCNA-like and RFC-like complexes (MELO et al. 2001). Direct DNA binding by Lcd1p may contribute to recruitment of Mec1p-Lcd1p

Table 1. DNA damage-detection and signalling proteins.

Process	Protein	Human/*S.pombe* homologue	Interactions	Properties
Sensors	Ddc1	Rad9/Rad9	Mec3:**Rad17** Rad27, Rfc4	PCNA-like dCMP deaminase
	Mec3	Hus1/Hus1	Ddc1:**Rad17** Srl3, **Spo12**, Lif1, **Cdc33**, **TF**	PCNA-like DNA binding
	Rad17	Rad1/Rad1	**Mec3:Ddc1** Rad27	PCNA-like 3'-5' exonuclease
	Rad24	Rad17/Rad17	**Rad24**, RFC, Pol1, Cdc2, Dun1	RFC-like
	RFC	RFC	RFC, **Rad24**	RFC-like DNA binding, ATPase
	Mec1	ATR/Rad3	Rfa1, Msh6, Lcd1	PIKK
	Lcd1	ATRIP/Rad26	Mec1, Dna2, Ddc1, H4, Msh6, Sml1	DNA binding
	Tel1	ATM/Tel1	H4	PIKK
Adaptors	Rad9	BRCA1?/Crb2	**Rad53**, Rad27, **Rrm3**, **Chk1**, Dun1	DNA replication and repair, NER
	Mrc1	Claspin/Mrc1	Bim1	Silencing at HML, HMR and telomeres
Transducer Kinases	Rad53	Chk2/Cds1	**CBF**, H2A2, **Rad9**, Tbf1, Dun1, Ptc2, Mus81, Swi4, **Fob1**, Cdc13 Rnr4	Kinase
	Chk1	Chk1/Chk1	**Rad3**, **Rad9**	Kinase

Protein-protein interactions were defined by GRID analysis. Interactions defined by two hybrid are in bold letters, by affinity precipitation or purified complex are underline. Rad27 and Sgs1 interactions were defined by synthetic lethality.

to sites of DNA damage, and indicate that this is likely to be essential for the DNA damage response (ROUSE and JACKSON 2002).

DNA damage-induced Mec1 dependent phosphorylation of H2Ap and Lcd1p. This phosphorylation is RFC-like and PCNA-like complexes independent (ROUSE and JACKSON 2002). Sequentially, Mec1p phosphorylates Rad9p. Phosphorylated Rad9p binds to Rad53p through the FHA domain, leading to cell cycle arrest (LOWNDES and MURGUIA 2000) (Figure 2).

The Rad53p protein kinase is phosphorylated and activated in a *MEC1*-dependent manner. Downstream of Mec1p and Rad53p is the Dun1p protein kinase that is also activated in response to DNA damage and is required for the transcriptional response.

In normal conditions, repression of transcription of some repair genes involves Ssn6p, Tup1p, and Crt1p (negative regulator of RNR transcription). In response to DNA damage and replication blocks, Crt1p becomes hyperphosphorylated in a Mec1p-Rad53p-Dun1p dependent manner, and no longer binds DNA, resulting in transcriptional induction of *RNR* genes; as well as certain other repair genes. *CRT1* is auto regulated and is itself induced by DNA damage, indicating the existence of a negative feedback pathway that facilitates return to the repressed state ensuring a transient peak of expression (HUANG *et al.* 1998).

3.2.1 Steps for sensing to repair. Several proteins have been proposed as DNA-damage sensors but there are numerous details that are not fully understood. For example, how does the recognition of damage by a sensor actually lead to activation

9
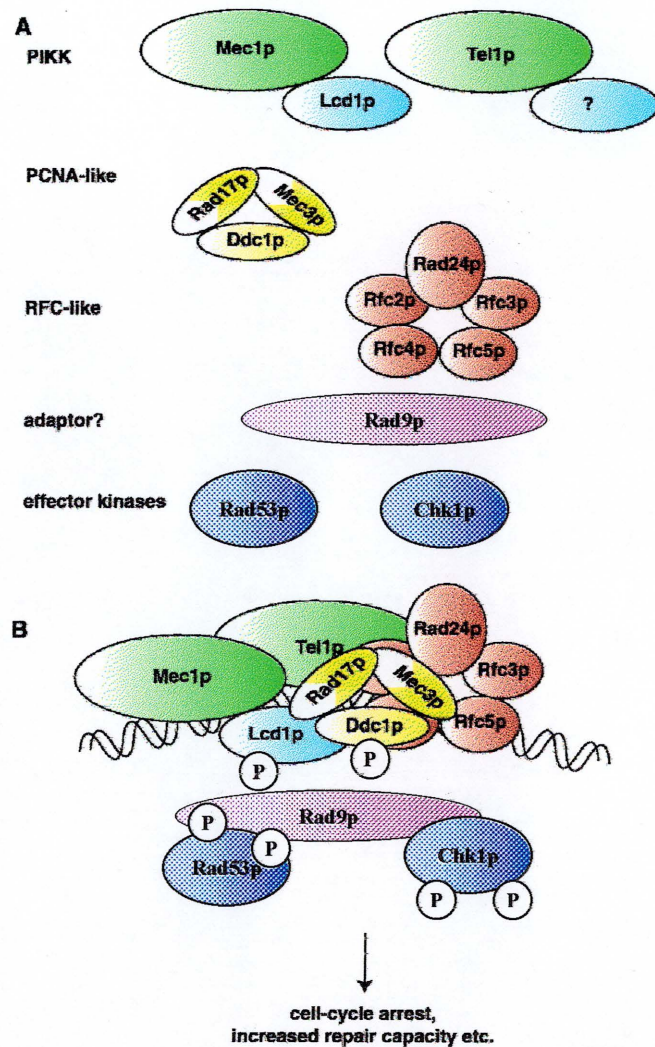
FIGURE 2. An integrated model for DNA damage sensing. A. DNA Damage response components. **B**. After detection by lesion-specific repair factors, DNA damage is either quickly repaired or it persists, depending on the nature of the lesion and/or the genomic context. If the lesion is not repaired sufficiently quickly, then Mec1p-Lcd1p is recruited to sites of DNA damage, which have probably been modified by the action of the lesion-specific repair factors. Mec1p now phosphorylates targets that are in the vicinity of the lesion, such as H2Ap and Lcd1p (which may be considered a "local" response), and if full repair occurs, the global DNA damage response is averted. However, if DNA repair still cannot be completed, the RFC-like (Rad24p/Rfc2-5) and PCNA-like (Rad17p-Ddc1p-Mec3p) complexes are recruited to sites of damage that have probably been modified further, which allows Mec1p-dependent activation of Rad53p and Chk1p. This triggers a global DNA damage response including cell-cycle arrest, further chromatin modulation, and up-regulation of the repair capacity of the cell, all of which combine to facilitate repair of recalcitrant lesions and to prevent key cell-cycle transitions. Adapted from (ROUSE and JACKSON 2002).

of the repair or signaling pathway? Are there different sensors for different types of damage or is all damage processed into one or a few common intermediates? An immediate challenge is to understand how Mec1p and Tel1p are regulated and what their individual and overlapping roles are.

The identification of the full range of physiological targets and the understanding of what effects these phosphorylation events have on the activity of the target, are the subject of much ongoing research. Further areas of interest include the downstream signal transduction pathways that are activated by DSBs, how they are integrated with one another and what their cellular consequences are in the context of the whole organism.

3.3   DSB REPAIR MECHANISMS

3.3.1 Homologous Recombination (HR)

The process of homologous recombination plays an essential role in the mitotic and meiotic cell cycle of most eukaryotic organisms. In meiosis, meiotic recombination contributes to diversity by creating new linkage arrangements between genes, or parts of genes. In addition, recombination establishes a physical connection between homologous chromosomes to ensure their correct disjunction at the first meiotic division. It is now widely recognized that the primary function of homologous recombination in mitotic cells is to repair double-strand breaks (DSBs).

Recombination is also required to repair the DSBs that initiate programmed chromosomal rearrangements, such as mating-type switching in *Saccharomyces cerevisiae.*

Most of the genes in the *RAD52* epistasis group (*RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RDH54 (TID1), MRE11 (RAD58),* and *XRS2*) were identified by their requirement in the repair of ionizing-radiation (IR)-induced DNA damage (Table 2). Mutations in these genes lead to defects in meiotic and/or mitotic recombination, providing evidence for a link between DSB repair (DSBR) and homologous recombination.

Much of our understanding of the mechanisms of recombination is based on organisms, such as *S. cerevisiae,* in which all of the products of an individual meiosis can be recovered for analysis in the form of asci containing four haploid spores. Two types of recombination events have been identified based on the segregation of heterozygous markers during meiosis: crossing over (CO) and gene conversion (GC). A crossover between linked heterozygous markers results in new linkage arrangements for two spore products, but the markers are still recovered in Mendelian ratios. Gene conversion represents the nonreciprocal transfer of information between two homologous sequences to duplicate one of the alleles, with the corresponding loss of the other, resulting in a non-Mendelian segregation (SYMINGTON 2002).

3.3.1.1 DSBR Models

Several models have been proposed to explain the molecular mechanisms of recombination, and of these the DSBR and synthesis-dependent strand-annealing (SDSA) models are most consistent with the available genetic data (SZOSTAK *et al.* 1983). The observation that IR stimulates recombination suggested that recombination is initiated by DSBs.

These observations formed the basis for the DSBR model of recombination (SZOSTAK *et al.* 1983) (Figure 3A). In this model, the ends of the break are resected to form 3' single-stranded tails that are active in strand invasion with a homologous DNA duplex. Following strand invasion, the 3' end is extended by DNA synthesis. The D-loop formed by strand invasion is able to pair with the other side of the DSB, and the 3' end of the non-invading strand is also extended by DNA synthesis, forming a double-Holliday-junction (dHJ) intermediate. Random resolution of the two Holliday junctions is expected to yield equal numbers of crossover and non-crossover products.

To explain the low levels of associated crossing over observed for some DSB-induced gene conversion events, the SDSA model was proposed (FERGUSON and HOLLOMAN 1996) (Figure 3B). In this model, strand invasion occurs as proposed in the DSBR model, but after extensive DNA synthesis primed from the invading strand, the elongated invading strand is displaced and pairs again with the other side of the break. DNA synthesis can then be primed from the non-invading 3' end to

A. The double-strand-break repair model   B. Synthesis-dependent strand-annealing   C. Break-induced replication   D. Single-strand annealing

End processing to generate
3' single-stranded tails

Two-ended strand invasion

One-ended strand invasion

DNA synthesis from the
invading 3' ends to resynthesize
degraded DNA

Replication from the invading
3' strand proceeds to the end of
the chromosome

2   3

1   4

Resolution to generate non-crossover
products (1, 4 or 2, 3 cleavage)

OR   Resolution to generate crossover
products (1, 3 or 2, 4 cleavage)

Displacement of the invading 3' strand
providing a template for the repair from
the other 3' end resulting in non-
crossover products

Sequences distal to the break are lost and
replaced with sequences from the donor

Trimming of 3' heterologous tails and
ligation to generate a deletion

FIGURE 3. Models for the repair of DSBs. A. In the DSBR model, the ends are processed to yield 3' single-stranded tails. One of the 3' ends invade the homologous duplex, priming DNA synthesis. After ligation, a dHJ intermediate is formed, which can subsequently be resolved by endonucleolytic cleavage of the two Holliday junctions to generate crossover or noncrossover products. **B.** In the SDSA model, the ends are processed to yield 3' single-stranded tails, one of which invades the homologous duplex, priming DNA synthesis. The displacement loop (D-loop) formed by strand invasion could be extended by DNA synthesis or could migrate with the newly synthesized DNA. After displacement from the donor duplex, the nascent strand pairs with the other 3' single-stranded tail and DNA synthesis completes repair. (C) The initial steps in the BIR model are the same as in the SDSA model, but DNA synthesis from the invading strand continues to the end of the DNA molecule. (D) In the SSA model, a DSB made between direct repeats is subject to resection to generate 3' single-stranded tails. When complementary sequences are revealed due to extensive resection, the single-stranded DNA anneals, resulting in deletion of one of the repeats and the intervening DNA. The 3' tails are endonucleolytically removed, and the nicks are ligated. The 3' ends are indicated by arrowheads. From (SYMINGTON 2002).

14

repair the DSB or gap. An alternative scenario for gap repair involves coupling of

lagging-strand DNA synthesis to leading-strand synthesis from the invading strand.

Further evidence in support of the SDSA model comes from the observation that the

donor sequences are generally unchanged during DSB-induced gene conversion

(PAQUES et al. 1998).

Allers and Lichten propose that non-crossovers are generated by SDSA and

crossovers are generated from the resolution of dHJ intermediates that are formed

as proposed by the DSBR model (ALLERS and LICHTEN 2001).

In certain genetic backgrounds (rad51 null mutant), gene conversion is eliminated

and repair occurs by invasion of the donor duplex by the broken chromosome

followed by replication to the end of the donor chromosome (MALKOVA et al. 1996)

(Figure 3C). This process is known as break-induced replication (BIR). This

nonreciprocal process is likely to be important for telomere maintenance in the

absence of telomerase (LE et al. 1999).

Homology-dependent duplication of an entire chromosome arm to the end of a

transformed linearized plasmid has also been detected in yeast and is presumed to

occur by BIR (MORROW et al. 1997).

Single-strand annealing (SSA), another pathway of homology-dependent repair, is

restricted to DSBs that occur between direct repeats (Figure 3D). These events have

been detected in yeast and animal cells by using artificial direct repeats and could

be important for repair in genomes of higher eukaryotes that contain many repeated

sequences. After formation of the DSB, the ends are resected to produce 3' single-

stranded tails, which can anneal when resection is sufficient to reveal

complementary single-stranded regions. Nucleases remove single-stranded tails, and the resulting gaps and nicks are filled in by DNA repair synthesis and ligation. This process is considered to be mutagenic because it results in the deletion of one of the repeats and the DNA between the direct repeats.

3.3.1.2 Genetic and Biochemical Properties of the *RAD52* Group Genes and Proteins

The genes of the *RAD52* group can be broadly grouped into the *MRE11, RAD50, XRS2* (*NBS1*) subgroup and the *RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RDH54/TID1* subgroup. *MRE11, RAD50,* and *XRS2* are implicated in the formation and processing of DSBs during meiotic recombination and also function in the end-joining pathway of repair, telomere maintenance, in DNA replication-associated repair, and in the DNA damage checkpoint in mitotic cells. While, the *RAD51* subgroup appears to function only in homologous recombination.

3.3.1.3 *RAD51, RAD52, RAD54 RDH4/TID1, RAD55, RAD57, RAD59* and *RFA1* subgroup

Genetic studies showed that the *RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RDH54,* and *RFA1* genes are involved in the homologous recombination pathway. Within this group, the *RAD51, RAD52, RAD54, RAD55,* and *RAD57* genes are essential for conservative DSB repair, resulting in gene conversion (and associated crossing over), and *RAD52* and *RAD59* have additional functions in the non conservative BIR and SSA pathways. The requirement for replication protein A (RPA) has been more difficult to study *in vivo* because of the essential function of

this complex in DNA replication. However, several non-null alleles of *RFA1* exhibit recombination and repair deficiencies. *RDH54/TID1* is discussed with *RAD54* because it encodes a protein with homology to Rad54p and shows redundancy with *RAD54* in some assays. The biochemical activities of each of the proteins are summarized in Table 2.

2.3.3.1.3.1 Rad51p. *S.cerevisiae RAD51* encodes a 43-kDa protein with 30% identity to bacterial RecAp; the highest homology is with the catalytic domain of RecAp, encompassing the Walker A and B motifs for nucleotide binding and/or hydrolysis. Rad51p is conserved in all eukaryotes for which sequence information is available. The mouse and human proteins are 59% identical to ScRad51p (BASILE *et al.* 1992).

Yeast *rad51* null mutants are viable but show high sensitivity to IR and meiotic inviability. Surprisingly, deletion of *RAD51* in vertebrates results in cell inviability and early embryonic death in mice.

The *RAD51* transcript is highly induced during meiosis and following treatment of cells with DNA-damaging agents such as methyl methanesulfonate (MMS) (BASILE *et al.* 1992). Rad51p foci are detected during meiosis coincident with the timing of meiotic recombination and colocalize with the meiosis-specific RecA homologue, Dmc1p. The formation of Rad51p foci is dependent on Spo11p (endonuclease essential for DSB formation during meiosis recombination) and can be induced in *spo11* mutants by treatment of meiotic cells with IR (GASIOR *et al.* 2001). Meiosis-induced Rad51p foci are not formed in *rad52, rad55,* or *rad57* mutants, consistent with biochemical studies implicating these factors in assembly of the Rad51p

Table 2. HR repair proteins.

Protein	Human homologue	Interactions	Properties	Cellular localization
Rad51	Rad51	**Rad51**, Rad52, **Rad55**, Rad27, Rpa1, Mlh1	ATP-dependent homologous DNA pairing and strand exchange	Forms nucleoprotein filaments
Rad52	Rad52	Rad51, Rad59, Rad27, **Rfa1**, **Rfa2**, **Rfa3**, Msh6	ssDNA binding and annealing	Mediator of strand exchange, required for SSA and BIR
Rad54	Rad54	Rad27	DNA-dependent ATPase DNA supercoiling	Member of Snf2 family: promotes homologous DNA pairing by Rad51
Rad55	Xrcc2, Xrcc3 Rad51B Rad51C Rad51D	**Rad51**, Rad27, Rad57	ssDNA binding	Forms heterodimer with Rad57: Rad55-57 functions as mediator in strand exchange BIR, SSA, SDSA
Rad57	Xrcc2, Xrcc3 Rad51B Rad51C Rad51D	Rad55, Rad27	ssDNA binding	Forms heterodimer with Rad55: Rad55-57 functions as mediator in strand exchange BIR, SSA, SDSA
Rad59		Rad52, Scr1 (BER)	ssDNA binding and annealing	Homology to Rad52; required for SSA and BIR
Dmc1	Dmc1	**Dmc1**, **Swi5**, **Ris1**, Rvb2, **Rdh54**	ATP-dependent homologous DNA pairing	Meiotic recombination
Rdh54	Rad54	**Dmc1**, **Rif2** (telomere)		Member of Snf2
RPA	Rpa	Mec1, Dna2, **Rad52**, Rad51, Msh6, Msh2, Rvb1	ssDNA binding	Removes secondary structure in ssDNA during the presynaptic phase of strand exchange
Rad1	Ercc4	Rad14, **Swr1**, Dun1, Rad10, **Orc1**	SS Specific endonuclease	Removal of non-homologous ends.
Rad10	Ercc1	**Rad1**		NER, SSA
Rad27	DNase IV	MRX, Ddc1, Sae2 Rad9, 17, 24, Sgs1, Rad51,52,54,55,57, Mus81	Flap endonuclease	
Crp1				Cruciform DNA binding protein
Sae2		Sgs1, **Sae2**, Rad27		Meiotic DNA DSB processing

Protein-protein interactions were defined by GRID analysis. Interactions defined by two hybrid are in bold letters, by affinity precipitation or purified complex are underline. Rad27 and Sgs1 interactions were defined by synthetic lethality.

presynaptic filament (GASIOR *et al.* 2001). Radiation-induced Rad51p foci are detected at reduced levels in *rad55* and *rad52* mutants but are absent in *rad52 rad55* double mutants.

Purified Rad51p forms right-handed helical filaments on double-stranded DNA with structural similarity to those formed by RecAp. Rad51p binds with higher affinity to DNA duplexes with single-stranded tails than to duplex or single-stranded oligonucleotides (MAZIN *et al.* 2000a). Rad51p DNA binding is ATP dependent.

These observations suggest that the role of RPA in presynapsis is to remove secondary structures from ssDNA to allow the formation of a continuous Rad51p nucleoprotein filament.

The Rad51p nucleoprotein filament is able to interact with a second DNA molecule, either ssDNA or dsDNA, and to initiate strand exchange. The polarity of strand exchange is 5' to 3' with respect to the complementary strand of the DNA duplex, opposite to the polarity observed for RecAp.

Both Rad52p and Rad55p-Rad57p are thought to mediate the assembly of the Rad51 presynaptic filament, although most biochemical studies have focused on the effects of these proteins in the strand exchange assay.

Comparison of the sequences of RecAp and Rad51p shows that several residues are conserved, in particular in the regions assigned to ATP binding or hydrolysis. Mutation of the conserved lysine residue within the Walker A motif to alanine (Rad51-K191A) abolishes DNA binding and ATPase activities of ScRad51p (SUNG and STRATTON 1996). When the same lysine residue is replaced by arginine (Rad51-

K191R), the protein retains ATP-dependent DNA binding, strand exchange, but no significant hydrolysis of ATP (SUNG and STRATTON 1996).

When the *rad51-K191R* allele is expressed in haploid cells, the phenotype conferred is quite similar to that conferred by the null allele with respect to radiation sensitivity, mating-type switching and spontaneous mitotic recombination (MORGAN *et al.* 2002). However, diploids homozygous for the *rad51-K191R* allele show normal levels of sporulation and high spore viability and are much more resistant to IR than is the *rad51-K191R* haploid. These results suggest that the suppression of the DNA repair defect conferred by the *rad51-K191R* allele in diploids is due to differential expression of genes regulated by *MAT* heterozygosity-regulated promoters.

When *RAD54* is present at high copy-number suppresses the radiation sensitivity of the *rad51-K191R* strain (MORGAN *et al.* 2002). Rad54p could function to stabilize the Rad51p-DNA interaction by binding to the Rad51p nucleoprotein filament or could function to displace Rad51p from DNA by translocation activity (MAZIN *et al.* 2000b).

Rad51p self-association, as well as Rad51p-Rad52p interaction, is mediated via a domain in the N-terminus of Rad51p. Single-amino-acid substitutions within Rad51p that disrupt the interaction with Rad52p map to the C terminus of Rad51p (KREJCI *et al.* 2001). Some of the mutations that disrupt the Rad51p-Rad52p interaction also disrupt the interaction between Rad51p and Rad54p, suggesting that interactions between these proteins and Rad51p are likely to be dynamic. Mutations within the N terminus of Rad51p that reduce Rad51p homotypic interactions also disrupt the interaction with Rad55p.

The *rad51* mutations that disrupt interaction with Rad52p and Rad54p confer sensitivity to MMS, confirming the importance of these interactions (KREJCI *et al.* 2001). Most of the Rad52p present in protein extracts is associated with Rad51p, suggesting that this interaction is quite stable (SUNG 1997).

3.3.1.3.2 Rad51p paralogs. The *RAD55* and *RAD57* genes of *S. cerevisiae* are considered to be Rad51p paralogs because they encode proteins with sequence similarity to RecAp and Rad51p. Null mutations of either *RAD55* or *RAD57* cause cold sensitivity for DNA repair (JOHNSON and SYMINGTON 1995). Cold sensitivity is usually indicative of proteins that act as components, or stabilizers, of protein complexes. Rad55p and Rad57p form a stable heterodimer and Rad55p also interacts with Rad51p (SUNG 1997). Unlike Rad51p, neither Rad55p nor Rad57p exhibits self-interaction in the two-hybrid system. Over expression of Rad51p partially suppressed the radiation sensitivity of *rad55* and *rad57* mutants, and further suppression occurred when Rad52p was also over expressed. Mutation of the invariant lysine residue of the Walker A box of Rad57p confers no defect in DNA repair or sporulation, but mutation of the corresponding residue in Rad55p does cause sensitivity to IR and prevents sporulation (JOHNSON and SYMINGTON 1995). Diploids homozygous for *rad55* or *rad57* mutations are less sensitive to IR than are haploids. Because overexpression of *RAD51* also suppresses the radiation sensitivity of *rad55* and *rad57* mutants, it seemed possible that *MAT*- heterozygosity promoters might regulate *RAD51*.

Rad55p is phosphorylated in response to DNA-damaging agents. This phosphorylation is dependent on Mec1p and partially dependent on Rad53p but not on other checkpoint functions (BASHKIROV et al. 2000). The rad55 mutants show normal responses to DNA damage, indicating that Rad55p is not required for the damage checkpoint activation. Interestingly, mec1 mutants are defective in both spontaneous and MMS-induced heteroallelic recombination (BASHKIROV et al. 2000). This reduction is greater than reported for rad55 mutants, suggesting that there might be RAD55-dependent and independent pathways for recombination, both of which require MEC1.

3.3.1.3.3 Dmc1p. DMC1 is not considered to be a member of the RAD52 epistasis group because mutants are resistant to IR, but DMC1 is essential for the repair of DSBs during meiotic recombination. DMC1 was identified in a screen for meiosis-specific prophase-induced genes that, when disrupted, resulted in meiotic prophase arrest. Dmc1p is 45% identical to Rad51p. Biochemical studies indicate a higher functional conservation than the other Rad51p paralogs to RecAp and Rad51p. Both Dmc1p and Rad51p form foci during meiosis (BISHOP 1994). RAD51 and DMC1 are both required for high levels of meiotic recombination.

3.3.1.3.4 Rad52p. Deletion of RAD52 in S.cerevisiae results in severe defects in homology-dependent DSBR and meiosis. rad52 mutants are defective in BIR and SSA in addition to the RAD51-dependent gene conversion pathway; consequently, they show the most severe recombination defects of all the rad52 group mutants.

Rad55p is phosphorylated in response to DNA-damaging agents. This phosphorylation is dependent on Mec1p and partially dependent on Rad53p but not on other checkpoint functions (BASHKIROV *et al.* 2000). The *rad55* mutants show normal responses to DNA damage, indicating that Rad55p is not required for the damage checkpoint activation. Interestingly, *mec1* mutants are defective in both spontaneous and MMS-induced heteroallelic recombination (BASHKIROV *et al.* 2000). This reduction is greater than reported for *rad55* mutants, suggesting that there might be *RAD55*-dependent and independent pathways for recombination, both of which require *MEC1*.

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3.3.1.3.4 Rad52p. Deletion of *RAD52* in *S.cerevisiae* results in severe defects in homology-dependent DSBR and meiosis. *rad52* mutants are defective in BIR and SSA in addition to the *RAD51*-dependent gene conversion pathway; consequently, they show the most severe recombination defects of all the *rad52* group mutants.

However, chicken and human cell lines lacking *RAD52* are viable, and, in contrast to *RAD51,* the deletion of *RAD52* does not cause embryonic death in mice (RIJKERS *et al.* 1998). Furthermore, mutant cell lines show no increase in sensitivity to DNA-damaging agents and the efficiency of gene targeting is only marginally reduced.

Rad52p of budding yeast is expressed throughout the cell cycle and is induced 2- to 3-fold by DNA-damaging agents and about 10-fold during meiosis. Rad52p is nuclear and forms discrete foci in response to IR and during S phase of unirradiated cells (LISBY *et al.* 2001). Surprisingly, only one or two foci are observed using a Rad52p-GFP fusion, independent of the dose of IR, suggesting that damaged DNA is lead to be processed at one or two sites within the nucleus. Rad52p foci are also observed during meiosis, are dependent on *SPO11,* and show extensive co-localization with RPA (GASIOR *et al.* 2001; LISBY *et al.* 2001).

Both yeast and human Rad52p are multimeric and form ring structures that can be visualized by electron microscopy (RANATUNGA *et al.* 2001; SHINOHARA *et al.* 1998). It is generally accepted that DNA is bound within the central channel, but to date there is no evidence that DNA lies within the central channel of the Rad52p heptamer. Rad52p appears to have two modes of self-association. Assembly of monomers into rings requires sequences in the conserved N-terminal domain of Rad52p, whereas the formation of higher-order multimers is mediated by the C-terminus (RANATUNGA *et al.* 2001).

The purified Rad52p protein binds preferentially to ssDNA and promotes annealing of complementary ssDNA. Rad52-promoted annealing of long molecules is stimulated by RPA, whereas Rad52p efficiently anneals oligonucleotides in the absence of RPA (SHINOHARA et al. 1998). The probable role of RPA in strand annealing is to remove secondary structures from ssDNA to allow annealing by Rad52p, but the stimulation of annealing also requires a species-specific interaction between Rad52p and RPA.

Because Rad52p interacts with both Rad51p and RPA, the contemporary models shows that Rad52p replaces RPA from ssDNA with Rad51p or that Rad52p provides a seeding site within the RPA-bound ssDNA for subsequent cooperative binding by Rad51p. Rad52p forms a complex with RPA-coated ssDNA, but does not displace RPA (SUGIYAMA and KOWALCZYKOWSKI 2002). Rad51p can displace RPA from ssDNA following interaction with Rad52p bound to RPA-coated DNA (SUGIYAMA and KOWALCZYKOWSKI 2002).

Rad52p self-associates to form a ring structure. It also interacts with Rad51p via the C-terminal domain of Rad52p, and deletion of residues 409 to 412 eliminates Rad51p binding (KREJCI et al. 2002). The Rad51p interaction domain of Rad52p is necessary for overcoming the RPA inhibition to strand exchange *in vitro*, consistent with the model that the mediator function of Rad52p requires interaction between Rad52p and Rad51p.

Rad52p also interacts with Rad59p, raising the possibility of formation of a heteromeric Rad52p-Rad59p ring (DAVIS and SYMINGTON 2001).

The *rad52* null alleles, and most inactivating point mutations within the N-terminal region of *RAD52*, cannot be suppressed by Rad51p over expression. No extragenic or high-copy-number suppressors of the *rad52Δ* allele have been identified. These observations suggest that the N-terminal region of Rad52p comprises a core domain with discrete functions in DSB repair that are independent of Rad51p.

A non-null allele of *RAD52, rad52-R70K,* was identified in a screen for mutants defective in *RAD51*-independent recombination of inverted repeats (BAI *et al.* 1999). The *rad52-R70K* strain showed only a 4-fold reduction in inverted-repeat recombination, compared with a 3,000-fold decrease observed for the *rad52* null strain and a 1,300-fold decrease found for the *rad51 rad52-R70K* strain. The *rad52-R70K* mutation conferred partial sensitivity to γ irradiation and was synergistic with a *rad59* null mutation for inverted-repeat recombination, SSA, mating-type switching, and sporulation, suggesting that some weak alleles of *rad52* are highly dependent on *RAD59* function (BAI *et al.* 1999).

In a systematic analysis, positively charged, aromatic, and hydrophobic residues within the N-terminal region of Rad52p were replaced by alanine and tested for radiation resistance and spontaneous mitotic recombination (MORTENSEN *et al.* 2002). From this analysis, mutations that conferred a phenotype similar to that for a null mutation were identified, along with several classes of separation-of-function mutations. One class, defined by a single allele, conferred intermediate sensitivity to IR but was completely defective for heteroallelic recombination. Another class, represented by several alleles, showed intermediate sensitivity to IR but wild-type or

higher rates of heteroallelic recombination. The last class was partially defective for heteroallelic recombination but showed only mild sensitivity to IR. To date, there is no information on the biochemical activities of these Rad52p mutants (MORTENSEN et al. 2002).

Model of action. A weak sequence similarity between Rad52p and RecO (*E.coli*) proteins was found (KANTAKE et al. 2002), but not with UvsY (T4 phage) protein. Despite the lack of strong sequence similarity, RecO and UvsY proteins display biochemical and genetic characteristics that justify their description as counterparts of eukaryotic Rad52 protein; they all possess ssDNA- and dsDNA-binding abilities, facilitation of ssDNA-binding protein displacement by the cognate DNA strand-exchange protein, annealing of simple DNA, and annealing of complex DNA in the presence of a cognate ssDNA-binding protein.

The conservation of these properties suggests that these activities comprise important biological functions. Kowalczykowski et al, propose that the ability to anneal DNA that is complexed with a homologous ssDNA-binding protein is necessary for two biochemical steps of double-strand DNA break (DSB) repair: these are (*i*) to serve as the mediator protein that accelerates the displacement of ssDNA-binding protein by the RecA-like protein and (*ii*) to anneal the ssDNA within the D-loop made by strand invasion and the ssDNA of the processed dsDNA end that did not participate in DNA strand invasion (KANTAKE et al. 2002). The model of this process is shown in Figure 4.

According to this model, both displacement of the ssDNA-binding protein and annealing between the ssDNA produced at the second processed end of DSB and

the DNA strand displaced by strand invasion are mediated by the same protein: RecO protein, Rad52 protein, or UvsY protein or their functional counterparts.

3.3.1.3.5 Rad59p. The *RAD59* gene was identified in a screen for mutants defective for *RAD51*-independent spontaneous mitotic recombination between inverted repeats (BAI and SYMINGTON 1996). The *rad59* mutation was shown to cause a moderate defect in several mitotic recombination assays and moderate sensitivity to IR (BAI *et al.* 1999). In the chromosomal inverted-repeat recombination assay, *rad52* was epistatic to *rad51* and *rad59* while *rad59* was synergistic with *rad51*. The level of recombination in a *rad51 rad59* double mutant is higher than in a *rad52* mutant, suggesting either that there is an additional pathway or that Rad52p is able to carry out some recombination functions by itself. *RAD59* is important for SSA between chromosomal direct repeats, and the requirement for *RAD59* increases as the repeat length decreases.

The *RAD59* gene encodes a 238-residue protein with significant homology to the N-terminal half of Rad52p (BAI and SYMINGTON 1996). The *RAD52* gene, when present on a *CEN*-based plasmid, partially suppressed the radiation sensitivity of the *rad59* strain, suggesting that Rad52p and Rad59p have overlapping functions and/or that they interact (DAVIS and SYMINGTON 2001). A complex of Rad51p, Rad52p, and Rad59p can be immunoprecipitated from yeast extracts, but Rad51p and Rad59p fail to interact in the absence of Rad52p.

Rad59p binds to DNA, preferentially to ssDNA, and anneals complementary ssDNA. Despite the similarity of their biochemical activities, *RAD59* cannot substitute for
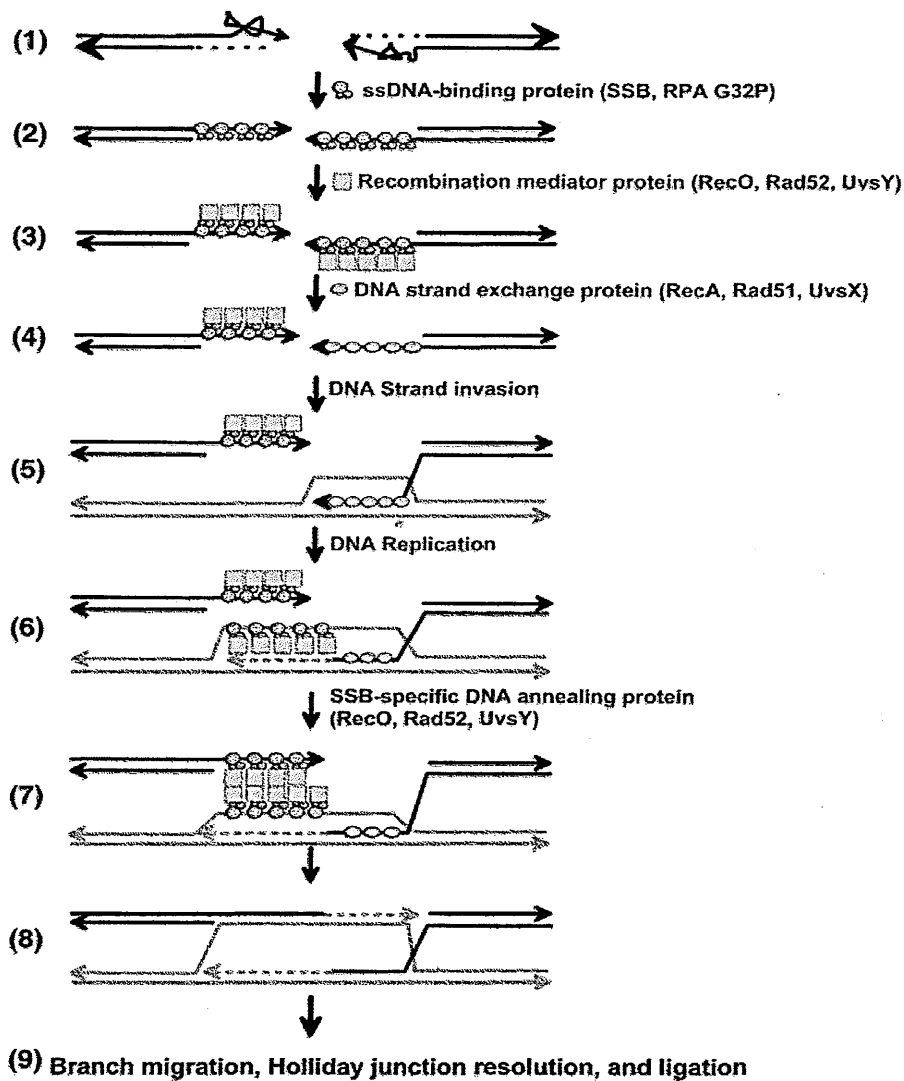
FIGURE 4. A model for DSB repair. This model illustrate the proposed role for annealing of SSB-ssDNA complexes by RecO, Rad52, or UvsY proteins. (1) The DSB is processed to expose ssDNA with 3' overhangs. (2) The ssDNA is coated by an ssDNA-binding protein (SSB/RPA/gp32). (3) The recombination mediator protein (RMP: RecO/Rad52/UvsY) binds to the SSB-ssDNA complex. (4) The DNA strand-exchange protein (RecA/Rad51/UvsX) is recruited by the RMP (RecO/Rad52/UvsY) and replaces the ssDNA-binding protein at one of the processed ssDNA tails. (5) The presynaptic complex (DNA strand-exchange protein-ssDNA complex) invades homologous DNA, displacing one strand of homologous dsDNA. (6) DNA replication initiates from the invaded 3' end within the D-loop. SSB (SSB/RPA/gp32) and RMP (RecO/Rad52p/UvsY) bind the displaced strand produced by DNA-strand invasion and DNA synthesis. (7) The complex of displaced ssDNA-SSB-RMP anneals with the ssDNA-SSB-RMP complex containing the other 3' overhang. In the case of phage T4, gp32, UvsY protein, or both may actually anneal these strands. (8) Further DNA synthesis (9), ligation, branch migration, and resolution of double Holliday junction result in two intact homologous DNA molecules. From (KANTAKE et al. 2002).

28

RAD52 in vivo, even in *RAD51*-independent recombination events such as SSA (DAVIS and SYMINGTON 2001).

3.3.1.3.6 Rad54p and Rdh54p (Tid1p). Rad54p and the Rad54p homologue Rdh54p have sequence motifs characteristic of DNA helicases and are members of the SNF2/SW12 family of DNA-dependent ATPases. As with the other *RAD52* group genes, *RAD54* is not essential for viability in yeast but is required for resistance to IR.

Diploid yeast cells disrupted for *RDH54/TID1* show slight sensitivity to high levels of MMS, but haploid mutants show no significant sensitivity to DNA-damaging agents (KLEIN 1997). The *rad54 rdh54* haploid strains have similar growth rates and MMS sensitivities to *rad54* haploids, but homozygous *rad54 rdh54* diploids grow slowly and are more sensitive to MMS than are *rad54* diploids.

Both Rad54p and Rdh54p/Tid1p proteins show dsDNA-dependent ATPase activity and promote a conformational change of closed-circular duplex due to the creation of positive and negative writhe (MAZIN *et al.* 2000a; VAN KOMEN *et al.* 2000). However, neither protein shows helicase activity in the standard strand displacement assay. ATP-dependent translocation of Rad54p along duplex DNA generates both negative and positive supercoiled domains and is stimulated by Rad51p-ssDNA filaments (MAZIN *et al.* 2000a; VAN KOMEN *et al.* 2000). DNA remodeled by Rad54p becomes more sensitive to the ssDNA-specific nuclease, P1, indicating transient strand separation (VAN KOMEN *et al.* 2000).

Presumably, the change in wriggle by Rad54p facilitates the invasion of duplex DNA by the Rad51p nucleoprotein filament by creating transiently unwound DNA. All of the biochemical activities of Rad54p and Rdh54p, except binding to dsDNA, are dependent on ATP hydrolysis and are eliminated by mutation of the invariant lysine residue within the Walker A motif to either arginine or alanine (PETUKHOVA *et al.* 1999).

3.3.1.3.7 Replication protein A. The RPA complex consists of three subunits of 70, 34, and 14 kDa, encoded by the *RFA1, RFA2,* and *RFA3* genes, respectively. All three subunits of the heterotrimeric complex are essential for viability in yeast, confirming the requirement for RPA in DNA replication (HEYER *et al.* 1990).

A *RFA1* allele, *rfa1-D228Y,* was identified as a suppressor of the spontaneous direct-repeat recombination defect of a *rad1 rad52* strain (SMITH and ROTHSTEIN 1995). The *rfa1-228Y* strain by itself conferred a hyper-recombination phenotype for deletions between direct repeats that was independent of *RAD52* and partially dependent on *RAD1*. Physical studies showed an increased level of deletion product by SSA in the *rad52 rfa1-D228Y* strain and the disappearance of long single-stranded intermediates that are characteristic of *rad52* strains (SMITH and ROTHSTEIN 1999). These results suggest that Rad52p is required to displace RPA from ssDNA to promote strand annealing; in the absence of Rad52p, RPA is inhibitory to spontaneous annealing or to another factor that is unable to displace RPA. These studies confirm an important role for RPA in homologous recombination, presumably to remove secondary structure from ssDNA in order to allow more efficient binding by Rad51p.

3.3.1.3.8 NER and MSH Proteins. The Rad1p/10p heterodimer is a structure-specific nuclease that cleaves at the 5' side of UV-induced photoproducts and bulky lesions during nucleotide excision repair. The Rad1p/10p nuclease is thought to remove 3' flaps during SSA. In addition, it is required to remove heterologies from the 3' ends of DSB breaks to allow initiation of DNA synthesis during gene conversion. Thus, the requirement for *RAD1* and *RAD10* in homologous recombination is specific for events that require removal of heterologies, either during strand invasion or during SSA (IVANOV and HABER 1995).

The mismatch repair proteins Msh2p and Msh3p are also required for SSA and for removal of heterologies of more than 30 nucleotides from DSBs during gene conversion (SUGAWARA *et al.* 1997). Mutation of *MSH2* or *MSH3* is epistatic to *rad1* in these repair processes. These results have led to the suggestion that branched intermediates are stabilized by Msh2p/Msh3p binding in preparation for cleavage by Rad1p/Rad10p.

3.3.1.3.9 Holliday Junction Resolution Activities. Molecular and biochemical studies of the Rad52p group proteins have shown that most are required at early steps during recombinational repair. The absence of a yeast X-ray-sensitive mutant defective for Holliday junction resolution is surprising and suggests that (i) redundant activities exist in eukaryotes, (ii) it is an essential activity, or (iii) Holliday junction resolution is not obligatory for recombinational repair in yeast.

However, some mitotic recombination events do result in reciprocal exchange, and integration of linearized plasmids into the genome is thought to occur by resolution of Holliday junctions as predicted by the DSBR model (Figure 3A). The X-forms are readily detected, indicating that they form at high frequency

In *E. coli,* the RuvCp nuclease promotes the resolution of Holliday junctions and works in concert with the RuvAp/RuvBp branch migration complex (WEST 1997). Biochemical approaches have identified three Holliday junction-resolving activities from fractionated extracts of mitotic yeast cells (KLEFF *et al.* 1992). Of these, Cce1p (Mgt1p) is mitochondrial and is important for the segregation of mitochondrial genomes (SYMINGTON and KOLODNER 1985). The identity of the other two yeast resolvase activities is still unknown. Recent studies have identified the Mus81p-Mms4p (Eme1p) heterodimer as a putative Holliday junction resolvase (HABER and HEYER 2001).

Studies with *S. cerevisiae* are not fully compatible with the hypothesis of Mus81p-Eme1p as a HJ resolvase. First, *mus81* mutants are resistant to IR. Although one could argue that IR-induced damage is repaired by mechanisms that do not require Holliday junction resolution, *mus81* mutants also do not show any defect in

integration of plasmids (SYMINGTON 2002). Second, *mms4* and *mus81* diploids show quite high spore viability in some strain backgrounds and crossover products are readily detected by both genetic and physical analyses. Third, Holliday junctions cleaved by Mus81p-Mms4p/Eme1p cannot be ligated, indicating that the cleavage sites are not symmetrical (CONSTANTINOU *et al.* 2002). The preferred substrate for Mus81p-Mms4p/Eme1p is structurally related to a stalled replication fork, leading to the hypothesis Mus81p-Mms4p function can be processing stalled replication forks (DOE *et al.* 2002).

Holliday junction resolvases have also been identified from fractionated extracts of mammalian cells (CONSTANTINOU *et al.* 2001). Fractionation of HeLa cell extracts revealed two discrete Holliday junction resolvase activities, one corresponding to Mus81p and the other corresponding to the previously described resolvase that cofractionates with a branch migration activity, referred to as resolvase A (CONSTANTINOU *et al.* 2002). Resolvase A shows high specificity for Holliday junctions, whereas Mus81p cleaves 3' flap and Y-shaped molecules more efficiently than Holliday junctions. Resolvase A gene identification is still under study (CONSTANTINOU *et al.* 2002; DOE *et al.* 2002).

Very recently, a synthetic cruciform DNA (X-DNA) binding protein (CBP) Crp1p was identified in yeast (RASS and KEMPER 2002). The DNA-binding domain of Crp1p was mapped to positions 120-141of its protein sequence. This domain can act autonomously as an X-DNA-binding peptide and provides a new, lysine-rich DNA-binding domain. As reported earlier for several other CBPs, Crp1p exerts an

enhancing effect on the cleavage of X-DNA by endonuclease VII from bacteriophage T4 (RASS and KEMPER 2002).

3.3.1.4 Mre11-Rad50-Xrs2 (MRX) complex. Yeast strains with null mutations of *MRE11, RAD50,* or *XRS2* have very similar phenotypes. All of the mutants show poor vegetative growth, high sensitivity to IR, and defects in meiosis. The three proteins interact in the two-hybrid system, and co-immunoprecipitation studies have confirmed that they form a stable complex (JOHZUKA and OGAWA 1995). Although all three proteins can be co-immunoprecipitated from wild-type cells with antibodies directed against any one of the components, Rad50p and Xrs2p fail to interact in the absence of Mre11p (USUI *et al.* 1998). Although *mre11, rad50,* and *xrs2* null mutants of yeast are viable, *MRE11, RAD50,* and *NBS1* are all essential for the viability of vertebrate cell lines and for mouse early embryonic development.

The 83-kDa Mre11 protein is highly conserved among eukaryotes, and the N-terminal region has several sequence motifs shared by a large family of phosphodiesterases, including the *E.coli* SbcDp and bacteriophage T4 gp46p nucleases and protein phosphatases (Figure 5A).

Yeast and human Mre11 proteins have single-stranded DNA (ssDNA) endonuclease and weak 3'-to-5' exonuclease activities, the Mre11p nuclease activities are dependent on manganese as a cofactor. Two-hybrid and size exclusion chromatography analyses indicate that Mre11p forms a dimer (D'AMOURS and JACKSON 2002).

Rad50p, like Mre11p, is conserved in all kingdoms of life. In bacteria, the Rad50p homologue, SbcCp, forms a complex with SbcDp, the homologue of Mre11p. The SbcCD complex has ATP-dependent 3'-to-5' exonuclease and ATP-independent single-stranded endonuclease activity. The 150-kDa Rad50 protein is related to the SMC (Structural maintenance of chromosome) proteins, which have the Walker A and B motifs characteristic of nucleotide triphosphate (NTP)-binding proteins separated by a long coiled-coil region. The SMC proteins, including the Rad50p subgroup, have a conserved hinge region within the coiled region. The hinge region of the Rad50p subfamily is distinct from that of other SMC proteins and contains a conserved Cys-X-X-Cys motif. Electron microscopy studies of Rad50p suggest a dimeric structure to bring together the Walker A and B motifs, forming two catalytic sites. The dimer could result from two protomers in an antiparallel configuration or by hinge-mediated interactions between two intramolecularly coiled protomers (Figure 5A).

The Mre11p-Rad50p complex could function in sister chromatid interactions during DSBR, as suggested by genetic studies (Figure 5Bc). Electron microscopy studies have provided direct evidence for end binding by the human and yeast Mre11p-Rad50p complex and bridging of different DNA molecules (CHEN et al. 2001).

The DNA binding activity of yeast Rad50p is stimulated by ATP, but no ATPase activity has been observed for the purified protein. The yeast Mre11p-Rad50p complex cleaves hairpin structures in the absence of Xrs2p (TRUJILLO and SUNG 2001). In mammals, Nbs1p (p95) appears to be the functional homologue of Xrs2p in
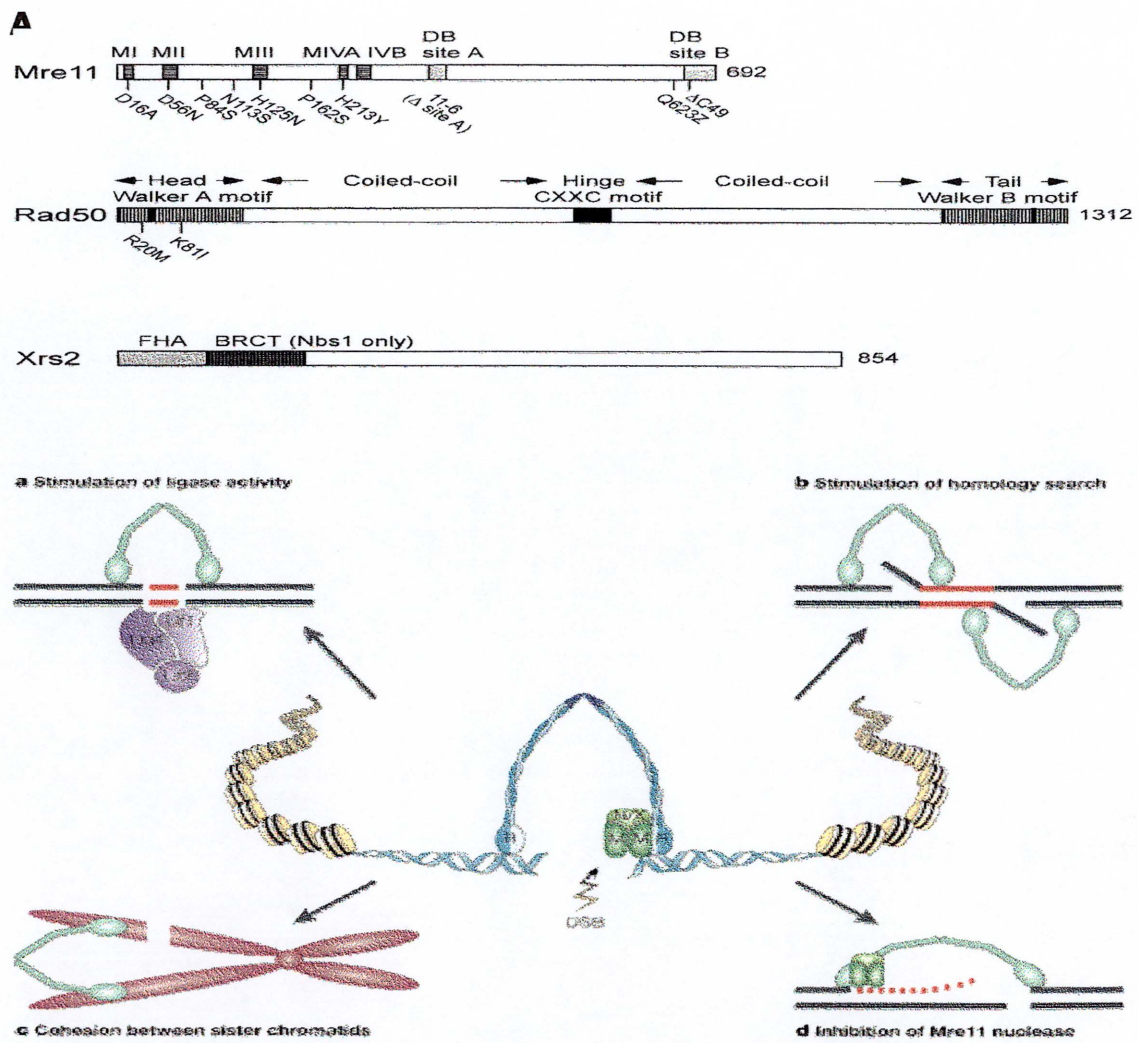
FIGURE 5. MRX complex. **A**. Schematic representation of Mre11p, Rad50p, and Xrs2p (Nbs1). The phosphodiesterase motifs of Mre11p are labeled MI through MIV, and DNA binding sites are labeled DB site A and B. The Mre11p-D16A, Mre11p-D56N, Mre11p-H125N, Mre11p-H213Y, and Mre11p-6 mutants are nuclease defective in vitro. Residue Pro84 is mutated in the *mre11-S* allele, and Pro162 is mutated in the *mre11-1* temperature-sensitive allele. The *mre11-N113S* and *mre11-Q623Z* alleles correspond to the mutations in the A-TLD patients. Rad50p contains two coiled-coil domains separating the Walker A and B motifs for NTP binding and hydrolysis. The hook domain, containing the conserved CXXC motif, is located between the two coiled-coil domains. The positions of the *rad50S* alleles, *rad50-R20M* and *rad50-K81I*, are shown. **B**. The DNA end-bridging activity of Rad50p. This function of Rad50p could potentially stimulate the cellular response to DNA damage in several ways. **a** | The maintenance of two DNA extremities in close proximity might stimulate ligation by increasing the local concentration of substrate for the Lig4p complex DNA. **b** | A higher concentration of DNA ends could stimulate homologous recombination by increasing the probability that homologous sequences meet in two DNA templates. **c** | Binding of Rad50p to damaged and undamaged chromatids in G2 could stabilize the severed chromosome arm (a role similar to that of cohesins, which maintain cohesion between sister chromatids), and hence stimulate the processing of the DNA double-strand break (DSB). **d** | Rad50p could inhibit the nuclease activity of Mre11p through structural constraints that are associated with the length of the Rad50p arms. The limited range of DNA resection imposed might be optimal for checkpoint signalling and for the subsequent DSB repair. Adapted from (SYMINGTON 2002) and (D'AMOURS and JACKSON 2002).

that it is tightly associated with Mre11p and Rad50p, but sequence similarity to Xrs2p

is limited to the N-terminal. A forkhead-associated (FHA) domain (Figure 5A), which

is thought to be important for interactions between phosphorylated proteins, is found

at the N terminus of Xrs2p and Nbs1p.

3.3.1.4.1 Role of the MRX Complex in Mitotic Recombination. Although the

functions of Rad50p-Mre11p-Xrs2p complex are not yet fully understood, its

possession of exonuclease activity suggest that it may help to process IR-induce

DNA damage before it is repaired by NHEJ or HR.

The complex phenotype of *mre11, rad50,* and *xrs2* mutants is even more apparent in

vegetative cells. Although it is generally assumed that yeast cells repair IR-induced

DNA damage by homologous recombination, *mre11, rad50,* and *xrs2* mutants show

little or no defect in spontaneous mitotic recombination. It has been suggested that

MRE11, RAD50, and *XRS2* are specifically involved in sister chromatid

recombination. The hyper-recombination phenotype exhibited by *mre11, rad50,* and

xrs2 mutants for heteroallelic recombination in diploids could be due to channeling

lesions from the normal sister chromatid repair pathway into interactions between

homologues (Figure 5B).

The most striking finding that emerged from studies of mating-type switching is the

decreased extent of processing of the 5' strand at the HO-cut site in the null mutants.

This result, in combination with the defect in processing of meiosis-specific breaks in

certain *mre11* and *rad50* mutants, led to the suggestion that the MRX complex

resects ends to produce 3' single-stranded tails. However, even in *mre11, rad50,* or

xrs2 null mutants, processing does occur, and while there is a delay in mating-type switching, most cells are able to complete the process with fairly high efficiency (IVANOV *et al.* 1994; TSUBOUCHI and OGAWA 1998).

The DSBs resection activity is a 5' to 3' end, the exonuclease activity of Mre11p is target to the 3' strand, reason why Mre11p is unlikely to be the unique DSB endonuclease. The role of the Mre11p nuclease in resection has been investigated by generating nuclease-defective alleles of *MRE11* (mre11-H125N) for analysis of end processing *in vivo* (TSUBOUCHI and OGAWA 1998). These observations suggest that the Mre11p nuclease may not be involved in the resection of mitotic DSBs or is redundant with another nuclease.

A redundant endonuclease would be expected to substitute for Mre11p in both mitotic and meiotic cells, suggesting that it is a 5'-to-3' exonuclease that processes ends in the absence of Mre11p in mitotic cells. The *EXO1* gene, which encodes a 5'-to-3' exonuclease with a twofold preference for double-stranded over ssDNA, was found to suppress the mitotic DNA repair defect of *mre11* strains when present at high copy number (LEWIS *et al.* 2002). The *exo1* null mutation alone confers no significant sensitivity to IR or mating-type switching defects. This contrasts with the severe defect observed for the *exo1 mre11* double mutant.

One attractive model is for the endonuclease activity of Mre11p to remove damaged nucleotides or protein-DNA covalent adducts (such as Spo11p) from ends to provide the substrate for the resection nuclease. Further support for this model comes from

recent studies suggesting that Mre11p removes the terminal protein of adenovirus during infection (STRACKER *et al.* 2002).

Recent studies suggest the Mre11p nuclease is important for processing unusual DNA structures, such as hairpins. Insertion of a 323-bp quasipalindrome derived from human Alu elements into the yeast *LYS2* gene stimulates the rate of ectopic recombination. This stimulation is dependent on *MRE11, RAD50,* and *XRS2* (LOBACHEV *et al.* 2002).

An allele of *SAE2* was isolated in a screen for mutants that aberrantly process DSBs within an inverted repeat (RATTRAY *et al.* 2001). The *mre11-H125N* and *rad50S* mutations conferred the same phenotype as did the *sae2* mutation in this assay, again suggesting that the Mre11p nuclease and Sae2p resolve palindromes.

The *RAD27* gene encodes a flap endonuclease that removes RNA primers from Okazaki fragments during DNA synthesis. *rad27* mutants are viable but depend on homologous recombination functions (*RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, MRE11,* and *XRS2*) and *SAE2* for viability (SYMINGTON 1998). The Mre11p nuclease could be partially redundant with the Rad27p nuclease or could be required to process aberrant DNA structures that accumulate in *rad27* mutants (DEBRAUWERE *et al.* 2001). An alternative explanation is that the large number of lesions generated in a *rad27* mutant overloads the homologous recombination system so that mutants with subtle DNA repair defects are unable to repair all of them. An attractive model is for Sae2p to interact with the MRX complex to activate

the Mre11p nuclease and for the Sae2p-MRX complex to be disrupted by *rad50S* and *mre11S* mutations.

It has been suggested that one of the functions of the MRX complex at telomere is recruit telomerases (TSUKAMOTO *et al.* 2001). The biochemical activities of the proteins are shown in Table 3.

3.3.2 NON HOMOLOGOUS END-JOINING (NHEJ)

Early work in the yeast *S.cerevisiae* analyzing the rejoining of DSBs induced by ionizing radiation suggested that repair could occur via homologous recombination. More recent studies have revealed that recombination-independent end-joining mechanisms of DSB repair are also efficient in yeast cells and are also present in bacteria (WELLER *et al.* 2002).

During NHEJ, the two broken DNA ends are directly joined with no overlap (end-to-end) or with minimal overlap and the use of short fortuitous homologies near the two ends. Thus, the term `non homologous' refers to the absence of extended segments of homologies between the two recombined DNA molecules. The simple re-joining of two ends with cohesive protruding single strands or two blunt ends, a process that conceivably can be achieved by a DNA ligase alone, can also be classified as a form of NHEJ.

Genes required for NHEJ have been identified and characterized by assessing DSB repair under conditions where homologous recombination is not possible. This has been accomplished by analyzing the rejoining of broken DNA ends that do not share

significant homology with any other DNA in the cell and through the study of DSB repair under conditions where the recombination pathway is inactive, i.e. in *rad52* mutants. Such studies have revealed that at least 10 genes are required for efficient and accurate repair by NHEJ: *YKU70* (*HDF1*), *YKU80* (*HDF2*), *DNL4* (*LIG4*), *LIF1*, *SIR2*, *SIR3*, *SIR4*, *RAD50*, *MRE11*, and *XRS2*. An additional factor, Nej1p/Lif2p, which interacts with Lif1p and is regulated by mating type, is required for end joining in yeast (FRANK-VAILLANT and MARCAND 2001);(KEGEL *et al.* 2001; OOI *et al.* 2001). Interestingly, three of the genes (*RAD50*, *MRE11*, and *XRS2*) are also members of the *RAD52* epistasis group and are required for meiotic recombination and for some classes of recombination events occurring in vegetative cells (LEWIS and RESNICK 2000).

All NHEJ mutants are deficient in re-circularization of a linear, centromeric plasmid containing complementary overhangs after the plasmid DNA has been introduced into cells by transformation.

The end-joining pathway of repair requires yKu70p and yKu80p, encoded by the *YKU70* (*HDF1*) and *YKU80* (*HDF2*) genes, respectively, a specialized DNA ligase encoded by the *DNL4* gene, and a ligase stimulatory factor, Lif1p (XRCC4 in mammals) (PAQUES and HABER 1999). In mammals, the Ku heterodimer associates with a kinase (DNA-PKcs) to form the DNA-dependent protein kinase (DNA-PK), but to date a similar kinase has not been identified in yeast (GOTTLIEB and JACKSON 1993). Defects in any of the components of this pathway, with the exception of *MRE11, RAD50,* and *XRS2,* do not cause IR sensitivity but do increase the IR sensitivity of a *rad52* strain in stationary phase. This suggests that the homologous
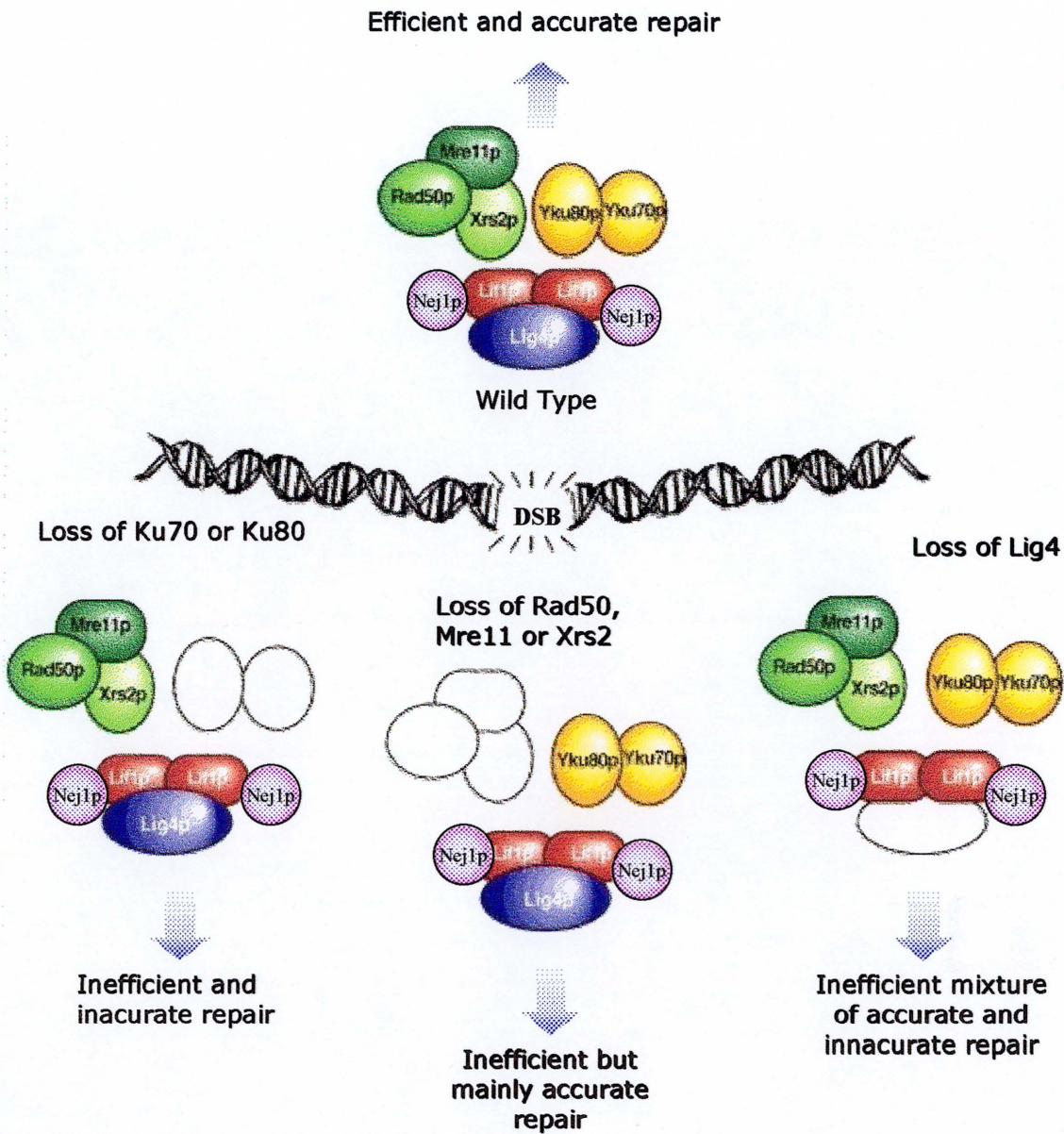
FIGURE 6. **Plasmid-repair products in non-homologous-end-joining (NHEJ)-mutant strains**. Schematic representation of the relative accuracy and efficiency of plasmid rejoining in *S.cerevisiae* strains in which various components of the NHEJ apparatus are disrupted. DSB: double-strand break. Adapted from (CRITCHLOW and JACKSON 1998).

pathway is the primary means of repairing IR-induced damage and that end joining can be used as a backup pathway.

Several assays have been used to measure end joining in yeast. The transformation efficiency of autonomously replicating plasmid DNA that has been linearized with a restriction enzyme to produce cohesive ends is used to measure precise rejoining (Figure 6) (BOULTON and JACKSON 1996). A similar assay to monitor the repair of chromosomal breaks measures cell survival in response to induction of EcoRI endonuclease in a strain containing a GAL1-regulated EcoRI gene (LEWIS et al. 1999). Imprecise end joining can be assayed by survival in response to HO

endonuclease induction of a strain that cannot repair the break at the MAT locus by homologous recombination (either by deletion of the donor cassettes or by deletion of RAD52) (MOORE and HABER 1996). In all these assays, mre11, rad50, xrs2, and yKu70 strains have similar phenotypes and appear to be epistatic (BOULTON and JACKSON 1998). Although mre11 and yKu70 mutations cause a similar reduction in

the efficiency of joining cohesive ends of plasmids, the types of products recovered are different. Repaired plasmids recovered from yKu70 and yKu80 strains have large deletions flanking the break site and rejoin through short sequence homologies, whereas plasmids recovered from mre11, rad50, and xrs2 strains show mainly faithful repair (BOULTON and JACKSON 1998). In the HO assay, faithful repair restores the HO-cut site, which can then be re-cut by HO. Survivors of continuous HO expression have small deletions or insertions at the HO cut site, which prevent further cutting by HO. The frequency of survivors is reduced in mre11, rad50, and

xrs2 mutants, but the survivors have large deletions (MOORE and HABER 1996). Although it has been suggested that the Mre11p nuclease could function in processing ends for the end-joining pathway, the characterization of junctions produced in *mre11* mutants in yeast is inconsistent with this hypothesis because some accurate repair products were found in this background.

Assessments of the sensitivities of known NHEJ mutants to the common ionizing radiation, MMS, and bleomycin have revealed significant differences. *rad50*, *mre11* and *xrs2* strains, which are defective in both NHEJ and some types of recombination, are extremely sensitive to each agent. In contrast, all other NHEJ mutants exhibit near-wild-type sensitivity to ionizing radiation. Furthermore, *yKu70*, *yKu80*, *sir2*, *sir3*, and *sir4* strains display variable sensitivities to MMS and bleomycin that appear to be strain-specific. Cell survival after treatment with radiation has been reported to be dependent on *yKU70* and/or *DNL4* when the recombination pathway is inactivated (i.e. in *rad52* mutants), but this is not observed in all strains (SIEDE *et al.* 1996), (TEO and JACKSON 1997), (WILSON *et al.* 1997).

A recent study noted that *sir4 yKu70* double mutants display a synergistic increase in sensitivity to MMS compared to either single mutant (MARTIN *et al.* 1999). This result suggests that the function(s) of each protein in NHEJ repair are partially redundant.

The efficiency of rejoining of blunt ends in the plasmid NHEJ assay is very low in wild-type cells and is not decreased in most end-joining defective strains (the exceptions are *DNL4* - and *LIF1*-deficient strains, which displayed a further reduction

44

in efficiency of approximately 3–5-fold) (HERRMANN *et al.* 1998). These data suggest that blunt ends and damaged ends produced by ionizing radiation (which have typically sustained additional base and sugar damage at the DSB termini) or bleomycin (which retain phosphoglycolate moieties at the ends of many induced SSBs and DSBs) are poor substrates for the NHEJ repair pathway in yeast.

Results obtained using genetic and molecular approaches have suggested that proteins involved in NHEJ can be placed into five groups: yKu70p/yKu80p, Dnl4p/Lif1p, Nej1p, Sir2p/Sir3p/Sir4p, and Rad50p/Mre11p/Xrs2p (Table 3).

3.3.2.1 yKu70p/yKu80p. The yKu70p/yKu80p complex is essential for recombination-independent, precise rejoining of the ends of DSBs *in vivo* and has been observed to relocate to the sites of DNA breakage inside cells (MARTIN *et al.* 1999).

Hypotheses about the function(s) of the Ku proteins based on studies in yeast and mammalian cells have included the possibilities that they may (i) protect the ends of broken DNA from cellular nucleases, and/or (ii) promote the joining of DNA ends directly or through stimulation of DNA ligase activity, and/or (iii) recruit DNA end-modifying enzymes such as exo or endonucleases to the sites of DSBs to promote processing and subsequent ligation of the ends (FEATHERSTONE and JACKSON 1999).

3.3.2.2 Dnl4p and Lif1p. *DNL4* and *LIF1* encode yeast homologues of mammalian DNA ligase IV and XRCC4, respectively (HERRMANN *et al.* 1998; TEO and JACKSON 1997; WILSON *et al.* 1997). Dnl4p is an ATP-dependent DNA ligase that physically interacts with Lif1p *in vivo. dnl4* and *lif1* strains differ from other NHEJ mutants in

45

Table 3. NHEJ repair proteins.

Protein	Human homologue	Interactions	Properties	Site of Action
yKu70	Ku70	**yKu80**, **Sir4**, Cdc13	Non-specific ssDNA binding dsDNA end-binding	Sites of induced DNA damage Telomere ends and subtelomeric chromatin DNA replication origins
yKu80	Ku80	**yKu70**, Cdc13		
Dnl4	Lig4	**Lif1**, Histone H2A, HTA2	ATP-dependent DNA ligase	
Lif1	Xrcc4	**Dnl4**, **Nej1**, Mec3, Cka2, **Cdc50**	Ligase interaction factor	Nucleous-cytoplasm
Nej1		**Lif1**	Repressed by MAT heterozygosity	Nucleous
Sir3		Sir2, Sir4	Transcriptional silencing at mating loci, rDNA and telomeres	Sites of induced DNA damage Telomeric chromatin HML and HMR Nucleolar rDNA
Sir4		Sir2, **yKu70**		
Sir2	Sir2A	Sir3:Sir4 + others components of silenced chromatin	Acetylation/deacetylation of histones	
Rad50	Rad50	Rad50, Mre11: Xrs2, Sgs1, Dun1, Rad27	ATP binding	Nucleous.
Mre11	Mer11	Rad50:Xrs2, Msh5, Rad27	3'-5'ssDNA exonuclease, ssDNA endonuclease, strand dissociation, strand-annealing	
Xrs2	Nbs1	**Rad50:2Mre11**, Rad27, Cdc16	FHA domain	

Protein-protein interactions were defined by GRID analysis. Interactions defined by two hybrid are in bold letters, by affinity precipitation or purified complex are underline. Rad27 and Sgs1 interactions were defined by synthetic lethality.

that they have not been reported to have appreciable sensitivity to MMS, bleomycin, or radiation (in dividing cells; the two mutants are slightly more sensitive to X-rays than wild-type cells when in stationary phase) (HERRMANN *et al.* 1998). Also, in contrast to other NHEJ genes, these two genes do not appear to be required for maintenance of telomere stability.

3.3.2.3 Nej1p. Nej1p is involved in regulation of NHEJ in a cell-type-dependent manner (FRANK-VAILLANT and MARCAND 2001; KEGEL *et al.* 2001; VALENCIA *et al.* 2001). Nej1p interacts with Lif1p. In the diploid state, the NHEJ machinery is down-regulated through suppression of Nej1p expression. This is achieved by the *Mata*1-*Mat*α2 transcriptional repressor, which is expressed only in diploid cells. When *NEJ1* expression is repressed in diploid cells, this appears to result in the loss of nuclear localization of Lig4p (VALENCIA *et al.* 2001). It will be interesting to see whether Nej1p is also regulated during cell cycle.

Database searches have failed to identify homologs of Nej1p in other organisms at the sequence level, but the low level of primary sequence similarity between other NHEJ factors indicates that there might be a functional counterpart of Nej1p in mammals (JAZAYERI and JACKSON 2002).

3.3.2.4 Sir2p, Sir3p, Sir4p. *SIR2, SIR3* and *SIR4* encode proteins that are required for transcriptional silencing at the mating type loci *HML* and *HMR*, within the rDNA repeat cluster and at telomeres (LUSTIG 1998). The Sir proteins differ from other NHEJ proteins in that they are not believed to interact directly with chromosomal DNA. These proteins have been found to associate with each other and with several

other proteins involved in establishing chromatin structure and initiation of DNA replication. A recent report has provided evidence that the deficiency of Sir-strains to recircularize plasmid DNA after transformation is due to derepression of the a1/ α2 repressor in these cells (ASTROM et al. 1999), this repressor could be the recently discovered NHEJ regulator Nej1p (FRANK-VAILLANT and MARCAND 2001; KEGEL et al. 2001; VALENCIA et al. 2001).

A more direct role for the Sir proteins was indicated by the finding of a Sir4p/yKu70p association in vivo and that the Sir proteins become redistributed to the sites of DNA damage after treatment with DNA-damage inducing chemicals or after cleavage of the chromosome by EcoRI or HO endonuclease (MARTIN et al. 1999). The established role of these proteins in chromatin remodeling in conjunction with these latter results suggests a structural role for Sir2p, Sir3p and Sir4p in NHEJ. For example, the proteins might influence the accessibility of the broken DNA ends to DNA processing enzymes and/or to the yKu70p/yKu80p complex. The synergistic defect in repair of MMS-induced lesions in sir4 yKu70 mutants compared to either single mutant suggests that these complexes have overlapping functions in NHEJ.

3.3.2.5 Mre11p-Rad50p-Xrs2p. The purified MRX complex stimulates intermolecular DNA joining by the Dnl4p-Lif1p complex (CHEN et al. 2001). Atomic force microscopy analysis revealed juxtaposition of DNA ends by the MRX complex to form linear concatemers, suggesting that the MRX complex can align DNA molecules for ligation. Interaction between the MRX complex and Dnl4p-Lif1p appears to be mediated by Xrs2p, suggesting that Xrs2p might function to recruit Dnl4p to ends held together by Mre11p and Rad50p.

3.3.3 DSBR in Cellular Context. Coordination of DSBR with other Cellular Processes.

DSBs may be introduced by several different routes. These include direct introduction by agents such as ionizing radiation or inhibitors of DNA topoisomerase II. DNA replication itself carries an intrinsic probability of DSB formation. It is possible that the type and timing of DSBs influences the choice of repair pathway. There may perhaps be subpathways, which involve slightly different constellations of, for example, HR participants that are more likely to function on DSBs from a particular source.

A related issue concerns the selection of repair pathway. Is there simply a direct competition between the Ku heterodimer, Rad50p/Mre11p or members of the Rad52 pathway. Is there some signaling to the appropriate pathway? It would make sense if repair by HR was activated in late S or G_2 phases of the cell cycle when a homologous copy is present (Figure 7).

Understanding the intimate relationship between damage sensing, replication, and the several mechanisms for DSB repair, is an unsolved issue as well as the understanding of how the cell coordinates the activities of the multiple systems that respond to DNA DSBs and how the relative importance of these different pathways is modulated during the cell cycle and in different cell ploidy.

Finally, it will be of interest to define the roles of DSB response proteins in other cellular functions, including telomere maintenance and programmed genome changes such as, class-switch recombination and meiotic recombination.
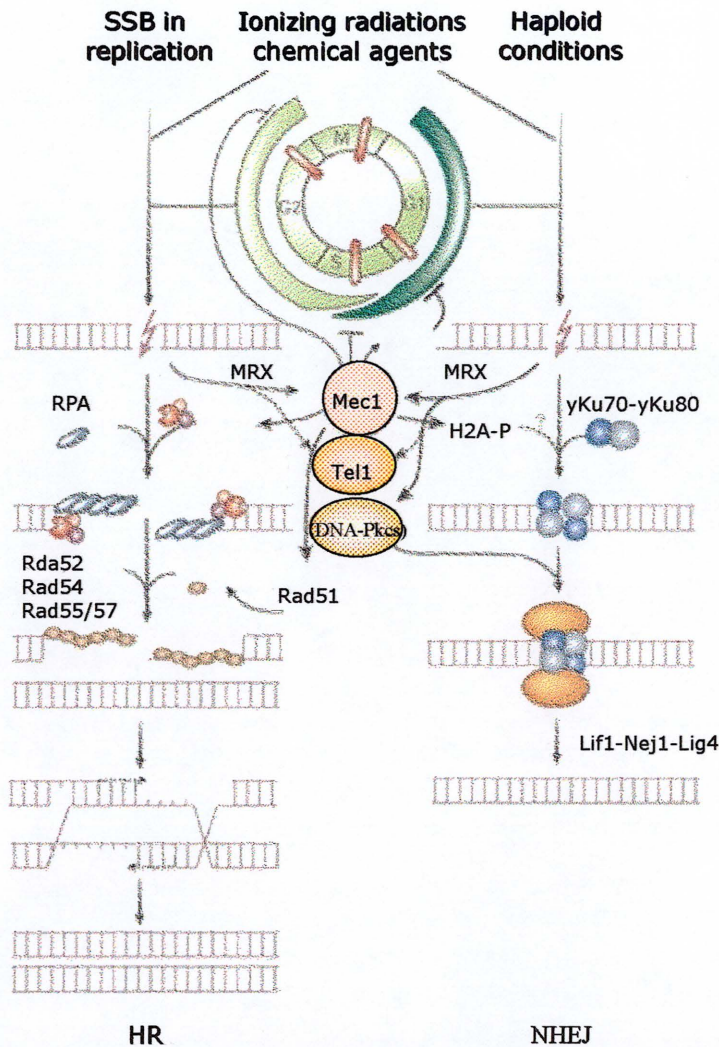
FIGURE 7. DNA damage-detection, and repair model. A tentative scenario for the homologous-recombination reaction is depicted in the left panel of the figure. To promote strand invasion into homologous sequences, the 5'–3' exonuclease activity. RPA facilitates assembly of a Rad51p nucleoprotein filament that probably includes RAD51p-related proteins Rad55p, Rad57p. Rad52p stimulates filament assembly. Rad51p has, like its *Escherichia coli* RecAp counterpart, the ability to exchange the single strand with the same sequence from a double-stranded DNA molecule. Correct positioning of the sister chromatids by cohesins probably facilitates the identification of a homologous sequence. A candidate for the complex chromatin transactions associated with these DNA gymnastics is Rad54p. After identification of the identical sister chromatid sequence, the intact double-stranded copy is used as a template to properly heal the broken ends by DNA synthesis. Finally, the Holliday-junctions are resolved by resolvases. Homologous recombination involves the simultaneous action of large numbers of the same molecules, which are found to be concentrated in radiation-induced nuclear foci.

In haploid conditions, a favored alternative is the end-joining reaction that simply links ends of a DSB together, without any template, using the end-binding yKu70p/80p complex, followed by ligation by Nej1p-Lif1p-Lig4p. The function of yKu70p/80p might involve end protection and approximating the ends. End joining may be further facilitated when the ends are still held together through nucleosomes or other structures. End joining is sometimes associated with gain or loss of a few nucleotides if internal microhomologies are used for annealing before sealing. This implies the involvement of DNA polymerases and/or nucleases. Adapted from (HOEIJMAKERS 2001).

50

3.4 Transcription Associated Recombination (TAR[1]). DNA replication, repair and recombination occur in a DNA substrate that is simultaneously undergoing transcription. Thus, transcription will at times take place on a DNA segment that is simultaneously being replicated or contains lesions that need to be repaired. A connection between transcription and other DNA metabolic processes has emerged over the last few years. If transcription is blocked by a particular DNA lesion, the blocked RNA polymerase is used to sense the damage and to load the DNA repair machinery at the site of the lesion via a mechanism termed transcription-coupled repair (TCR). In addition, as the transcriptional elongation apparatus advances together with proteins bound to the nascent RNA, it causes transient changes in DNA topology and chromatin structure and it can encounter the replication machinery. As a consequence, genomic stability can be compromised by increasing mutation and recombination rates (AGUILERA 2002).

Evidence for TAR in eukaryotes was shown with the *HOT1* DNA sequence of *Saccharomyces cerevisiae*. *HOT1* contains the initiation site (I) of the 35S rRNA precursor plus the enhancer (E) of transcription by RNA polymerase I (RNAPI). *HOT1*-dependent hyper-recombination can be abolished when either the I or E element is deleted, when a mutant RNAPI incapable of transcribing the 35S rRNA is used, or when a transcription terminator is inserted between *HOT1* and adjacent

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TAR is also Transformation Associated Recombination (LARIONOV, V., N. KOUPRINA, J. GRAVES, X. N. CHEN, J. R. KORENBERG *et al.*, 1996 Specific cloning of human DNA as yeast artificial chromosomes by transformation-associated recombination. Proc Natl Acad Sci U S A **93:** 491-496.

sequences. These results indicate that transcription through the recombining sequences is required for stimulation of recombination.

In yeast, RNA polymerase II (RNAPII)-mediated TAR was first shown in direct repeats transcribed under the control of the regulable *GAL1* promoter. Induction of transcription increased deletions by 10-fold. TAR has also been reported in other recombination assays in *S.cerevisiae* (SAXE *et al.* 2000).

Different studies of the yeast hyper-recombination mutations *hpr1* and *tho2* have led to the proposal that transcription-associated recombination may be caused by transcription-elongation failures leading to recombinogenic substrates (CHAVEZ and AGUILERA 1997; CHAVEZ *et al.* 2000).

3.5 Chromatin structure. Aguilera et al found that *spt4* and *spt6* mutations confer hyper-recombination of particular DNA repeat constructs. Spt6p is an essential protein involved in chromatin structure and transcription elongation. The *spt6-140* mutation has been shown to alter chromatin structure of yeast cells as well as transcription. Spt4p is a nonessential protein also involved in chromatin structure and transcription (MALAGON and AGUILERA 2001). The *spt6-140* and *spt4-3* mutations stimulate recombination between inverted repeats primarily by a Rad52-dependent mechanism that is partially dependent on Rad1p and Rad59p and very efficient both in the absence and the presence of Rad51p, Rad54p, Rad55p, and Rad57p. These results are consistent with the idea that inversions occur primarily by BIR-SSA (KANG and SYMINGTON 2000).

3.6 Recent Strategies to look for new DNA Damage repair genes.

To date, there are still some unidentified DSBR proteins. Figure 8, shows a schematic summary of the proteins involved in the DSBR pathways.

The recent completion of the deletion of essentially all of the ORFs in yeast is an important new resource for identifying the phenotypes of unknown genes. Each ORF is replaced with a cassette containing unique tag sequences that allow rapid parallel analysis of strains in a pool by using hybridization to a high-density oligonucleotide array. This system was used for example, to identify genes conferring resistance to UV irradiation (BIRRELL et al. 2001), or that are associated with tolerance to ionizing radiation damage (BENNETT et al. 2001).

Brown et al., compared the genome-wide expression patterns of wild-type cells and mutants defective in Mec1p signaling, including *mec1, dun1*, and *crt1* mutants, under normal growth conditions and in response to MMS and ionizing radiation (GASCH et al. 2001). They present a comparative analysis of wild-type and mutant cells responding to these DNA-damaging agents, and identify specific features of the gene expression responses that are dependent on the Mec1p pathway (GASCH et al. 2001).

A systematic screen of the set of approximately 5,000 viable *S.cerevisiae* haploid gene deletion mutants has identified 103 genes whose deletion causes sensitivity to MMS. Comparison with the set of genes known to be transcriptionally induced in response to MMS revealed surprisingly little overlap with those required for MMS resistance, indicating that transcriptional regulation plays little, if any, role in the response to MMS damage. Clustering of the MMS response genes on the basis of
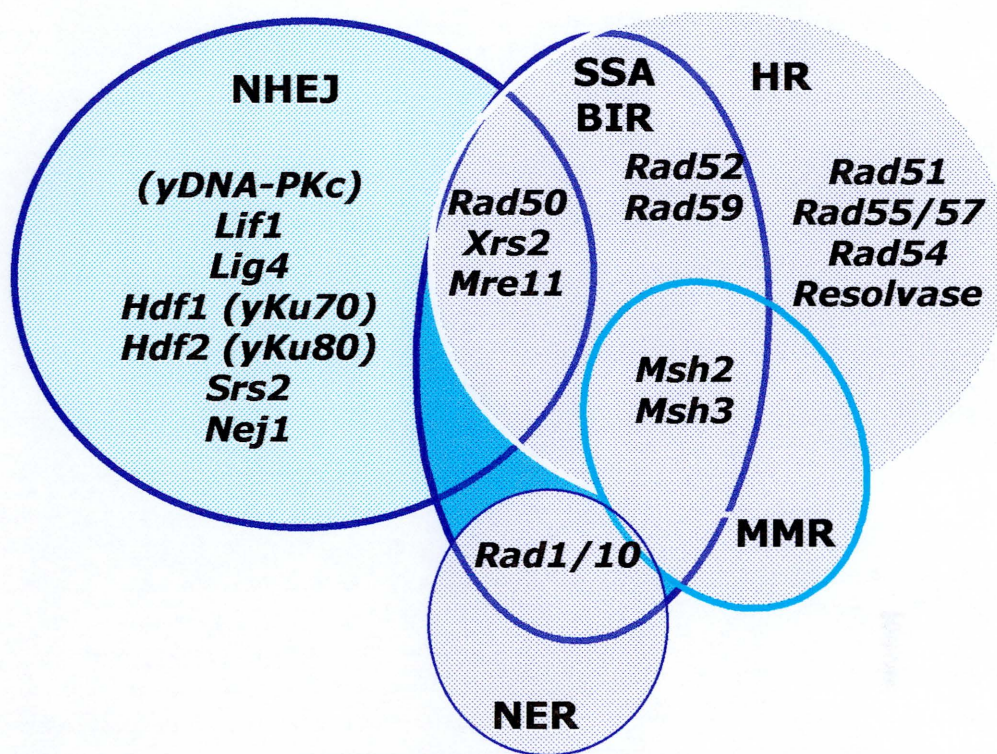
FIGURE 8. Proteins involved in DSB repair. The circle on the left contains a list of the known participants in NHEJ. Gene products involved in DSB repair by homology directed recombination (HR) are indicated in the large circle to the right. The SSA and BIR pathways are shown as a subpathway of HR. Adapted from (KARRAN 2000).

their cross-sensitivities to hydroxyurea, UV radiation, and ionizing radiation revealed a DNA damage core of genes required for responses to a broad range of DNA-damaging agents (CHANG *et al.* 2002).

Using a null mutant genome-wide microarray-based plasmid religation screen, genes involved in NHEJ pathway were identified. Known components of the pathway were identified, as well as genes not previously known to be involved in NHEJ (OOI *et al.* 2001).

Very recently, Wilson described a yeast assay suitable for genetic screening in which NHEJ and SSA compete for repair of an *I-Sce* I-created double-strand.

This study perform a comprehensive yeast genetic screen that had the ability to find those mutants deficient in the SSA and NHEJ repair pathways, but also those that changed the relative NHEJ/SSA repair ratio. The screen revealed all known, but no novel, genes required for catalysis of NHEJ, as well as several novel genes that proved to serve two separable regulatory roles promoting NHEJ in the haploid and postdiauxic/stationary growth stages (WILSON 2002).

3.7 Summary and Unsolved Questions

The *RAD51* pathway is clearly important for gene conversion unassociated with crossing over and for true reciprocal-crossover recombination. These events probably occur as envisioned in the DSBR or SDSA models, with the Rad51 protein playing a central role in homologous pairing and strand invasion with the assistance of the mediator proteins. In the absence of *RAD51, RAD54, RAD55,* and *RAD57,* some types of recombinational repair can still occur but are dependent on the context of the recombining sequences. SSA is only a viable option if direct repeats

are available flanking the break site, and *RAD51*-independent BIR may be restricted because certain sequences are required for strand invasion. *RAD52* is the most important recombination gene in *S.cerevisiae,* and this is presumably due to the important role of Rad52p as a mediator for Rad51p and in strand annealing for *RAD51*-independent recombination. While many advances have been made in this field, we still know little about the nucleases that function in early and late stages of recombination, the roles of the Rad51p accessory proteins, and coordination of homologous recombination with other cellular processes.

3.8 Competition-Complementation-Cooperation. How is the decision made at the cellular level for channeling a DNA DSB into a certain recombination pathway or into non-homologous DNA end-joining? Is it possible that this decision is influenced by the cell cycle stage (BRUSCHI and ESPOSITO 1983), the DSB characteristics or is dependent on post-translational modifications of key protein components?

It has been suggested that Rad52p and Ku compete for binding to DSBs and channel the repair of DSBs towards HR or NHEJ, respectively. Given that the trio of Rad50p, Mre11p, and Xrs2p are involved in both homologous recombination and non-homologous DNA end-joining, could they have a role in executing the cellular command to conduct either recombination or end-joining? These are interesting questions that again can be addressed genetically and biochemically.

3.8.1 Coupled Processes. It seems plausible that DNA end-processing, heteroduplex joint formation, and DNA synthesis are not distinct steps that occur independently, but rather that they are linked to one another. This idea predicts a

hierarchy of functional and physical interactions among factors traditionally thought of being required only in one or the other step of recombination.

3.8.2 Chromatin structure. The initiating ssDNA substrate that triggers recombination can be as long as 1Kb or more. Assuming that all of this ssDNA is utilized for heteroduplex DNA formation, then an extensive region of chromatin probably needs to be remodeled to allow strand invasion, branch migration, and subsequent reactions to occur. How is chromatin remodeling mediated during recombination and repair? Do Rad54p and Rdh54p/Tid1p play a role in chromatin remodeling?, There are likely to be other components that function in this process of chromatin remodeling.

3.8.3 Timing. Marcand et al showed that the stability of DNA ends generated by the HO endonuclease in yeast is surprisingly high with a half-life of more than an hour. This transient stability is unaffected by mutations that abolish non homologous end-joining (NHEJ). The unprocessed ends interact with yku70p and yku80p, but not significantly with Rad52p. Repair of a double- strand break by NHEJ is unaffected by the possibility of HR, although the use of HR is increased in NHEJ-defective cells. Partial in vitro 5' strand processing suppresses NHEJ but not HR. These results show that NHEJ precedes HR temporally, and that the availability of a suitable substrate dictates the particular pathway used (FRANK-VAILLANT and MARCAND 2002).

3.9 Transposon mutagenesis. The use of transposable elements as insertional mutagens is an extremely powerful technology for genetic analysis. A bacterial transposon containing a selectable yeast gene can be transposed into a cloned fragment of yeast DNA in *E.coli*. The transposon insertion can be returned to the

yeast genome by homologous recombination. The efficiency of yeast in integrating linear DNA into homologous region of the genome ensures that each integrant becomes a mutant having a transposon somewhere in the genome (SEIFERT *et al.* 1986). This method permits the generation and analysis of a large number of independent insertional-mutants. This approach offers several advantages over the traditional methods; it frequently creates loss-of-function mutations by insertional mutagenesis and it allows rapid sequencing of candidate genes.

3.10 Thesis aim

We have showed that additional HR/NHEJ/SSA, DSB detection and regulation genes may still await identification. In this thesis, I will approach the identification and genetic and functional characterization of new DNA damage repair gene(s) in *S.cerevisiae*.

To this end, several steps have been to be followed:

I.   Implementation of a *in vivo/in vitro* HNS (HR/NHEJ/SSA) DSBR system.
II.   Transposon mutagenesis and HNS screen to detect DSBR deficient mutants.
III.   Characterization of selected mutant(s).

4. MATERIALS AND METHODS

4.1 Primers. All yeast gene sequences were taken from SGD Web page (http://genome-www.stanford.edu/Saccharomyces). All primers used in this work are listed in Table 4.

4.2 Media and Techniques. Yeast cells were routinely grown at 30 C in either complete (YPD) or synthetic complete (SC) media. YPD medium contained 1% yeast extract, 2% bacto-peptone and 2% glucose. SD medium contained 0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulphate, 2% glucose and appropriate amino acids drop out mix and (SAMBROOK *et al.* 1989). Solid medium contained 2% agar.

The yeast transformation protocol and yeast colony PCR were performed as described in the Guidelines for EUROFAN B0 Program (http://www.mips.biochem.mpg.de/proj/eurofan/eurofan_1/b0/home_requisites/ guideline/exp-transformation.html).

All restriction enzymes used were used as described by the producer (New England Biolabs Hitchin, England UK).

4.3 Strains. All strains were isogenic derivatives of YPH250 and YPH252 (Table 5). Gene knockout was performed using the *FRT-KanMx4*-based cassette system for multiple gene disruption (STORICI *et al.* 1999). The *E. coli* strain used in this study was DH5α. It was grown at 37°C in LB medium (1% yeast extract, 2% bacto-peptone, 2% sodium chloride) or on plates (LB medium plus 2% agar), with or without ampicillin (75μg/ml).

Table 4. Primer sequences.

	Primer name	Sequence (5'-3')
I Sce I ORF	SceOR	GGATCCATGCATATGAAAAACATC
	SceOR	CAGGAAAGTTTCGGAGGAG
ADE8	Ade8-F	GAATTCAGAAAGTTTCTGTACCGC
	Ade8-R	TCTTTATAAACAGGCAAAGG
ADE8::HIS3	Ade-HisF	ATGGCCAGAATTGTCGTATTAATTTCGGGCTCAGGTTCCAAC ACAGTCCCTTTCCCGCAA
	Ade-HisR	TTATTTGTGAAGCTGCTGTAAAACCTTATATGTAGCTTCTAATA TGAAATGCTTTTCTTG
URA3 ORF	URA3F	GCAGGAAACGAAGATAAATC
	URA3R	TTTACTTATAATACAGTTTT
Sce I site	Sce I F	GGATCCATGCATATGAAAAACATC
	Sce I R	TCAGGAAAGTTTCGGAGGAG
UR-Aat II	UR-F (a)	ATATAGACGTCAGATGCTAAGAGATAGTGAT .
	UR-R(b)	ATATAGACGTCTGTCATAATCAACCAATCGT
Tel	TelF	TACACTCCGCTATCGCTACG
	TelR	GCATCAAGTGACCAAACAGG
KO cassette	Kan F head	AAAAATAGGCGTATCACGAG
heads	Kan R head	TCGATGATAAGCTGTCAAAC
Δmsh2	Δ msh2 F	GTATGTCCTCCACTAGGCCAGAGCTAAAATTCTCTGATGT
	Δ msh2 R	CCTTTTCTGGTTCATTTGCTATAGCACGCAATAGCTCTTG
	msh2 F1	CTCCATCAAGTGAACCTCAA
	msh2 R1	CGGAGGCATCCAACTTCATA
	msh2 F2	CGGAAAGAAGAACTCACCTA
	msh2 R2	GTGACAGTGGAATAAAGGAA
Δhdf1	Δhdf1 F	ATGCGCTCAGTCACTAATGCATTTGGCAATAGTGGAGAAC
	Δhdf1 R	TTATATATTGAATTTCGGCTTTTTATCAAAGGGCTTCTTT
	hdf F1	AGATCGGGCGTTCGACTCGC
	hdf R1	CTTCCTTGGCATCCTCTCTG
	hdf F2	CAGAATGATGCGTGAGGACG
	hdf R2	CAAGTGATCATTTGTCTATG
Δrad52	Δrad52 F	CGAATGGCGTTTTTAAGCTATTTTGCCACTGAGAATCAAC
	Δ rad52 R	TCAAGTAGGCTTGCGTGCATGCAGGGGATTGATCTTTGGT
	rad52 F1	ACGTGTACCGTGGATTCAAC
	rad52 R1	TGCTAAACTTTCCCTGTCGC
	rad52 F2	CCACCGCGAGGGATTCTGTC
	rad52 R2	TGAACCTAAGGATTCCGCTG
Δxrs2	Δ xrs2 F	ATGTGGGTAGTACGATACCAGAATACATTGGAAGATGGCT
	Δ xrs2 R	TTATCCTTTTCTTCTTTTGAACGTAAACTTCGGACCGTCG
	xrs2 F1	ATTTGGAATGTAGAGACGTG
	xrs2 R1	GACTTTAAAACGTCCGAGGC
	xrs2 F2	TTCAGGTACTGCAGCCAGCG
	xrs2 R2	GTCGTTGATATGAAGTGGGG
Δmre11	Δ mre11 F	ATGGACTATCCTGATCCAGACACAATAAGGATTTTAATTA
	Δ mre11 R	AAGTACAACTATTTTCTTTTCTTAGCAAGGAGACTTCCAA
	mre11 F1	AGAGTTCACAAGCAAGCCTG
	mre11 R1	TAGTGAAAAACTTGTGAGGG
	mre11 F2	AACGAGTGCGAACTGCAACG
	mre11 R2	AACAAAAGAGCAAAGGCTGG
Δrad50	Δ rad50 F	ATAGCGCTATCTATAAATTATCTATTCAGGGCATACGGTA
	Δ rad50 R	TCAATAAGTGACTCTGTTAATATCGACCCACTCAATTTGT
	rad50 F1	AACTGCAGTAGAACCCACCC

	rad50 R1	TTTCACCAGTTATCTTCGGG
	rad50 F2	CACTAGACGAACCTACCACC
	rad50 R2	TACGTGCTTGCTAAGTCTGC
Δnej1	Δ nej1 F	ATGGATTCTGAGTTGAAAGGGCAGCAGCTAAGTGATGCAG
	Δ nej1 R	TTAGTTTTTTATTCTCACCTTTCCAAATTTTCTTTTTTTC
	nej1 F1	TTTCCAAAGACCTTTGGTCC
	nej1 R1	TGGTTAATTTCTTGAGGAGC
	nej1 F2	GAGCGTCAGATTTCCTGCGC
	nej1 R2	TGATGGCGAGCTTGCGCGGC
Δmsh2	Δ msh2 F	GTATGTCCTCCACTAGGCCAGAGCTAAAATTCTCTGATGT
	Δ msh2 R	CCTTTTCTGGTTCATTTGCTATAGCACGCAATAGCTCTTG
	msh2 F1	CTCCATCAAGTGAACCTCAA
	msh2 R1	CGGAGGCATCCAACTTCATA
	msh2 F2	CGGAAAGAAGAACTCACCTA
	msh2 R2	GTGACAGTGGAATAAAGGAA
Δmed1	Δ med1 F	ATGGTAGAAGGAGACTCTTATGTGGAGACTTTAGACTCC
	Δ med1 R	GCTAACGATATCCTGAAATTTTTCAATAAATTTACTCCAG
	med1 F1	CCTGTACTTACGAGTAGGTATTTG
	med1 R1	CATGCTCTCCATCTCGTTGG
	med1 F2	TTCGAATCAAGAAAATGCAGG
	med1 R2	CTCACAATGATAGAGATGTCC
Δrsc2	Δ rsc2 F	ATGATGCCTGATGACAATTCAAACTCGTCCACTCAAAACT
	Δ rsc2 R	TATTTCATTTTTCCTGCTTTGTTTGAAGTTTGACTCTGCTC
	rsc2 F1	TAGGTGCTCTTGCACTTGGT
	rsc2 R1	ATTTCTCAAGGACTAAGGCG
	rsc2 F2	GCCTGCCACTGAATAGGTGG
	rsc2 R2	CATATCTAGAAGACAATGGC
Δswe1	Δswe1 F	ATGAGTTCTTTGGACGAGGATGAAGAGGACTTCGAAATGC
	Δ swe1 R	AAAAAGTATGTAAATAAAACAAGGTTTTTTTGTTCCATTTA
	swe1 F1	TGAACATTGGCGTGCCCCTG
	swe1 R1	ACGCTTTCCTCATCCTTGCC
	swe1 F2	GTAACAACGCTGGCACCTCC
	swe1 R2	AAAAATGCTTGAAGCGGCTG
Δswr1	Δswr1 F	ATGCCACATCTCGTAAATCGCATGCGAAAGACAAAAAGG
	Δ swr1 R	TCAATAATAATAACCGTTGGCAATAAACCTGATCATGTAC
	swr1 F1	GAGTGCAAAGGGATAGCGGG
	swr1 R1	CAACTGTCGATGGTCCTGAGG
	swr1 F2	ATAGCAGATGCAGATGTGGC
	swr1 R2	AAAACCAACCTTGATCGCGC
Δsin4	Δ sin4 F	ATGATGCTTGGAGAGCATTTAATGAGCTGGTCTAAGACTG
	Δ sin4 R	AGCCGTCCATCTCAAAAAGCATACCTGAACATATGCACAG
	sin4 F1	ATACTCTAAATGCGGAACTG
	sin4 R1	TGGAGTGCTTCCAACCCCGG
	sin4 F2	TTGCAACCATTAGAAGAGGG
	sin4 R2	GATGCCCCTTTTGCCTACGG
Δsli15	Δ sli15 F	ATGGACTGGGCAATCAAAGCAGCTAGGAAGAAAACTCAAA
	Δ sli15 R	AATTCAAGACCTTTTGGGCACAATTTGACGCGGTTTCAAC
	sli15 F1	TACGCTCTTTCGTTATAGCG
	sli15 R1	GTTATGCGTATTTTCGGGGC
	sli15 F2	GACCCCAAAGATAGCCTCTG
	sli15 R2	GTACCATTGTTCGATAACGC
Δdbp1	Δ dbp1 F	ATGGCAGACTTGCCACAGAAGGTATCTAATTTAAGCATCA
	Δ dbp1 R	AAGGAGTTCTATATTTGGATTAGTCTTTTATTCTTTCTGC
	dbp1 F1	CGTAAATATTACCCCGCGAG

	dbp1 R1	TTCGCGTTCTTAGGCCCCGG
	dbp1 F2	AGAACTAGGGGAGGAGGAGG
	dbp1 R2	CACCCATTTTATGATTGGGAC
Δkic1	Δ kic1 F	ATGACGACGAAGCCACAAAATAGTAAGCAGGGTTTAGCCG
	Δ kic1 R	GAATATGTACATAAACAAAAGAATAAACTAGACGATGCTT
	kic1 F1	AGTCAAAGAAGAACAGAGGC
	kic1 R1	TTCCATTATGATCCAAAGGC
	kic1 F2	CATATTCTGAGTTCCGCCGC
	kic1 R2	GGAAAAAACAAATGGCGAGG
Δbud16	Δ bud16 F	ATGCCTCGTCTCTTGGCCACGCAGTCTCATGTTGTACATG
	Δ bud16 R	ATCACAACCTTGCGTAAATATAATCTGTTTGATGAATGTT
	bud16 F1	ATGTGAATTCTATTTCCGCC
	bud16 R1	ATCATTTCTGGATTCGCCTC
	bud16 F2	ACTAGGAGCTATGCTTCAGG
	bud16 R2	CCTGAAACTAAGAACGGGAG
Δydr200	Δ ydr F	ATGGTAGAACTGGAAAAAAGAAGACGGCCCCCTCCGCAA
	Δ ydr R	GCTTTGTAGATTATGCTTGTAGTATTTTTCTTCTTTCGAG
	ydr F1	TCCCTCCAACGCAAGGACGC
	ydr R1	GGAAGGTGGAGATGAAGCGG
	ydr F2	CAAATAGAAGAAGAGAGGGG
	ydr R2	CAACCTTTCCTTCCATTCGC
Δylr238	Δ ylr F	ATGACTGGTCCTGGACCTGAAATAAATAAGGAGGAGCACC
	Δ ylr R	GACGTGAAAAGCGGATCGTGTGTGTCTTGTATTTACGATG
	ylr F1	TTATTCCGTAATCGTGGTGC
	ylr R1	TATTAGCAACAGGCCTTCCC
	ylr F2	CGAACATGACACTAGAGGCG
	ylr R2	AATTATGGAACAACGACCCG
Δshe4	Δ she4 F	ATGCCACTGTGTGAGAAAGGGAATGATCCAATCGATAGCT
	Δ she4 R	TTAGACTTTAATTTTAGCAAGGATAACGGGAATTGCCGCTG
	she4 F1	ACGGCTTCATTATCACCACC
	she4 R1	TTTAGATAAATGTACGCGGG
	she4 F2	TGCTATTCAGGTATTTGCGG
	she4 R2	CGAAGCCATCGGACCGAGGC
KanMX4	K1	CAATCGATAGATTGTCGCAC
	K2	AGTCGTCACTCATGGTGATT
P224		CGAATCGTAACCGTTCGTACGAGAATCGCT
Anchor	Anchor F	GACTCTCCCTTCTCGAATCGTAACCGTTCGTACGAGAATCGC TGTCCTCTCCTTC
	Anchor R	GAAGGAGAGGACGCTGTCTGTCGAAGGTAAGGAACGGACGA GAGAAGGGAGAG
RT-PCR	RNR2 FI	CGATGCATTGTCCGACTTGG
	RNR2 RI	ATCTTAAAGCCCATTCGGCC
	YLR238 FI	CGCCTCTAGTGTCATGTTCGG
	YLR238 RI	CCATCGAAAAATAAGTGCCAC
	YDR200 FI	CCCCTCTCTTCTTCTATTTG
	YDR200 RI	CAAGTTAGACCAGATAACGG
	HIS3IF	ATGACAGAGCAGAAAGCCCTAG
	HIS3IR	CAACCGCAAGAGCCTTGAACGC

Table 5. Yeast Strains.

Strain	Genotype
YPH250	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3-52*
YPH252	*MATα, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3-52*
Y	*MATα, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3-52, leu2:: leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H)*
Y L	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1- pGal1-Sce I-leu2Δ, ura3Δ::FRT (G), ade8::HIS3, leu2:: pGal1-Sce I*
Y0	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H)*
Y0yku70	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H), hdr1Δ::KanMX4 (X)*
Y0rad52	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H), rad52Δ::KanMX4 (X)*
Y0nej1	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H), nej1Δ::KanMX4 (X)*
Y0mre11	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H), mre11Δ::KanMX4 (X)*
Y0rad50	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H), rad50Δ::KanMX4 (X)*
Y0msh2	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H), msh21Δ::KanMX4 (X)*
Y0xsr2	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H), xrs2Δ::KanMX4 (X)*
Y0rsc2	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H), rsc2Δ::KanMX4 (X)*
Y0ydr200	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H), ydr200cΔ::KanMX4 (X)*
Y0ylr238	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H), ylr2385wΔ::KanMX4 (X)*
Y0swe1	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H), swe1Δ::KanMX4 (X)*
Y0sin4	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H),sin4Δ::KanMX4 (X)*
Y0mck1	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H), mck1Δ::KanMX4 (X)*
Y0kic1	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H), kic1Δ::KanMX4 (X)*
Y0med1	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H), med1Δ::KanMX4 (X)*
Y0bud16	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H), bud16Δ::KanMX4 (X)*
Y0swr1	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H), swr1Δ::KanMX4 (X)*
Y0sli15	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H), sli15Δ::KanMX4 (X)*
Y0she4	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H), she4Δ::KanMX4 (X)*
Y0dbp1	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H), dbp1Δ::KanMX4 (X)*
YLyku70	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G),*

ydr200	*ade8::HIS3, leu2Δ1- pGal1-Sce I-leu2Δ::FRT(H), hdr1Δ::FRT (X)*
YL*rad52* *ydr200*	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2:: pGal1-Sce I, ydr200Δ::KanMX4(H), rad52Δ::FRT (X)*
YL*yku70* *ylr238*	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2:: pGal1-Sce I, ylr238Δ::KanMX4(H), hdr1Δ::FRT (X)*
YL*rad52* *ylr238*	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2:: pGal1-Sce I, ylr238::KanMX4(H), rad52Δ::FRT (X)*
YL*ylr238* *ydr200*	*MATa, lys2-801a, ade2-101o, trp1Δ1, his3Δ200, leu2Δ1, ura3Δ::FRT (G), ade8::HIS3, leu2Δ1- pGal1-Sce I-ydr238::FRT(H), ydr200cΔ::KanMX4 (X)*

*I-Sce*I endonuclease gene was obtained from the pPEX7 plasmid (RICCHETTI *et al.* 1999) (kindly provided by B. Dujon) and subcloned in to YPL128 plasmid to obtain YPL I-SceI plasmid. Stable integration at the *Leu2* locus was obtained by transforming the Y0 strain with the YPL *I-Sce*I plasmid linearized with *Bst* XI. Transformants were selected on synthetic complete SC-Leu plates. Integration was confirmed by PCR using the SceOF and SceOR primers. The *ADE8* gene was disrupted with an ade8::HIS3 cassette obtained by PCR using primers Ade-HisF and Ade-HisR. Transformants were selected on SC-His plates. *URA3* gene knockout was performed in YPH250 and YPH252 strains generating Y0 and Y2 respectively. The pop out of the *KanMX4* gene was performed and tested as described in (STORICI et al. 1999).

4.4 Plasmid Construction. Plasmid DNA was extracted from *E.coli* using the Promega (Promega, Madison WI, USA) mini-preparation kit. Restriction and PCR fragments were separated by agarose gel-electrophoresis and purified using the Quiagen gel extraction kit (QIAGEN GmbH, Hilden Carlsbad, CA) as specified.

4.4.1 PCR-URA3-*I SceI*. The *URA3* gene was amplified by PCR, using primers URA3F and URA3R. The PCR product was cloned into the PCR II vector, using a TA Cloning Kit (Invitrogen, Carlsbad, CA). Plasmid PCR-URA3 was digested at the unique *Stu*I site in the *URA3* ORF filled in with Klenow polymerase and ligated with the *I-Sce*I site oligo obtained by the annealing of the primers SceI F and SceI R (Table 4).

Partially 3' truncated *ura3-SceI* (URR-*SceI*) sequence was amplified by PCR using the primers UR-F(**a**) and UR-R(**b**). Both primers contain the *AatII* site on the 5' site.

4.4.2 pURRA8Δ and pRURA8Δ. For the 5' truncation of the *URA3* gene (RRA), pYAC3 was digested with the *SnaBI* and *XcmI*, treated by the Mung Bean Nuclease and religated. To invert the RRA fragment, the plasmid was digested with *PpMuI* and religated. The URR-SceI fragment was cloned at the *AatII* site to obtain pURRA or pRURA depending on its direct or indirect orientation to the RRA sequence, respectively.

HIS3 from pURRA and pRURA was excised from both plasmids by *BamHI* digestion, and ligated with *ADE8* gene obtained by PCR using primers Ade8-F and Ade8-R containing *BamHI* sites at the head, to obtain pURRA8 and pRURA8 respectively (Figure 9).

HIS3 was excised from pURRA and pRURA by *XhoI* digestion, filled in with Klenow polymerase and ligated with *ADE8* gene obtained by PCR using primers Ade8-F and Ade8-R to obtain pURRA8Δ and pRURA8Δ respectively (Figure 9).

4.5 HNS *in-vitro* Assay. To assess DSB repair events, yeast strains were transformed with the circular plasmid and *I-SceI* and *BamHI* or *KpnI* linearized plasmids. Transformants were selected on SC-Trp medium, and grown for three days at 30°C. The red color was allowed to developed by storing the plates at 4°C for 16 hours. The number of red, white and red-white (sectored) colonies was

determined. Colonies from SC-Trp plates were replica plated into SC-Ura, and into SC-His only when plasmids pURRA and pRURA were used. The results were express in frequency of repair events, considering the total number of transformants as 100% of repair events. Probability of repair was calculated as the ration between the number of transformants obtained with linear plasmid versus circular plasmid, when same concentration of DNA and number of cells were used.

4.5.1 Molecular Assay. Ura⁻ red colonies obtained in the transformation with *I-Sce*I linear plasmid were used as a substrate for colony-PCR analysis using primers UR-R (**a**) and UR-R (**b**). After amplification, the PCR products were digested by *I-Sce*I or *Stu*I.

4.6 Plasmid Recovery. To better characterize the repair events, plasmid DNA was purified from the yeast using the Y-DER Yeast DNA Extraction Reagent Kit (PIERCE, Rockford IL) and transformed to electro-competent DH5α *E.coli*. DNA restriction analysis was carried out to characterize the plasmids.

4.7 *In-vivo* DSB Induction. Strains were transformed with circular pURRA8Δ and pRURA8Δ, transformants were selected on SC-Trp plates. Exponential phase cultures were washed twice and resuspended in the same volume of SC-Trp containing 2% galactose as a unique carbon source. After 12 hours, cells were counted and appropriate dilution were plated on SC-Trp plates, and grown for three days at 30°C. The red color was allowed to developed by storing the plates at 4°C for 16 hours, after which the number of red, white and sectored colonies was determined. Colonies from SC-Trp plates were replica plated into SC-Ura.

4.8 Transposon Mutagenesis. Yeast genomic libraries with random transposon insertions were kindly provided by Yale University (http://ygac.med.yale.edu/). The library contained genomic DNA fragments cloned into vector pHSS6. This library was then mutagenised, using transposon Tn3::*LEU2*::*lacZ* (BURNS *et al.* 1994). This mutagenesis treatment was repeated in 15 independent experiments, resulting in 15 different pools, which we used in independent yeast transformations.

The mutated yeast DNA was released from vector DNA by digestion with *Not* I. The linear DNA mix was used to transformed yeast cells caring pURRA, or pRURA8 plasmid. Transformants were selected by plating into SC-Leu-Trp. Plates were incubated at 30°C for 3 to 4 days (SEIFERT *et al.* 1986).

4.8.1 Screening of DSBR Deficient Mutant. Subsequently, Leu-Trp-positive transformants were transferred to microtitre plates with 100μl of SC-Trp medium and grown overnight at 30°C. Then, 5μl of each culture was transferred to microtitre plates containing 100μl of SC-Trp–galactose (2%) and grown 24 hours at 30° C. Then, they were transferred to YPD, SC-Trp, SC-Ura and SC-His (only when the strain carried pURRA plasmid) plates. Plates were incubated at 30° C for four days.

Mutants that were not able to growth after galactose DSB induction were selected. The mutants selected were screened in the same way, in a second round of selection.

4.8.2 Identification of Genes Carring Insertions.

4.8.2.1 Vectorette-PCR. Yeast genomic DNA of selected mutants, was cut of overnight with 8-10U of blunt cutting enzyme (*Alu*I or *Dra*I) in a 20μl final volume

reactions. The reaction was heat inactivated. Ligation with the linker was performed by adding:

3µl 10x NEBuffer used in digest, 1µl annealed anchor bubble, 1µl (400U) ligase, 0.5µl of 5mM ATP (50µM ATP final) and water to 50µl. The reaction was incubated at 16°C for 9-24 hours.

4.8.2.2 Anchor Bubble Preparation. To anneal the anchor bubble, primers (Anchor F and Anchor R) were mixed a 2-4µM (in ddWater) and heated to 65°C for 5 minutes, then MgCl$_2$ to 1-2mM was added and the mix was allowed to cool to room temperature.

4.8.2.3 PCR Amplification. The reaction mix was composed by: 2.5µl of 20µM specific primer (M13 (-47) for mTn3 library), 2.5µl of 20µM 224 primer, 8µl of 2.5mM dNTPs, 10µl of Taq 10X PCR buffer, 71µl water and 1µl of Taq DNA polymerase (PROMEGA).

The PCR program used was:

Denature 92°C, 2 minutes

35 Cycles (92°C, 20sec; 67°C, 30sec; 72°C, 60sec (>1 min/1 kb))

72°C, 90sec

The amplified PCR product was gel extracted (Qiagen), and resuspended in 40µl of water. Sequence was performed by BMR CRIBI (Padova University Italy), using mTn3 specific primer M13 (-47) (RILEY et al. 1990).

Sequence analysis was performed with BLAST program from SGD WEB page.

4.9 Plasmid Loss. Yeast strains containing plasmids pURRA8, pRURA8, pURRA8Δ, pRURA8Δ, pYAC3 and pRAP-TRP, were grown on SC-Trp over night.

Cells were washed and re-inoculated in YPD at 3×10^{-6} cell/mL. Samples were taken at 0, 2.5, 5, 9 and 23 hours and dilutions were plated on YPD and SC-Trp plates. Ratio between colonies in SC-Trp and YPD is used as a % of plasmid maintenance.

4.10 MMS Sensitivity. Exponential phase yeast cultures (wild-type, transposon mutants and null mutants) were spotted in serial dilution in YPD plates containing 0, 0.001, 0.005, 0.01, 0.02 and 0.05 % of MMS. Cells were let grown at 30°C for three to five days.

4.11 MMS Survival. MMS was added to 0.1% to logarithmic cell culture. Cells were incubated at 30°C for 0, 30, 60 and 120 minutes. For each time point, sample was pelleted and washed twice. Appropriate dilutions were plated in YPD plates. Control samples were take from cultures without MMS. Ratio of number of colonies at each time point with/without MMS was take as % of survival.

4.12 Western blot analysis. The preparation of yeast protein extracts from TCA-treated cells was performed. The antibodies against Rad53p and Rad9p were a gift from D. Stern (SCHWARTZ et al. 2002).

4.13 RT-PCR. RNA was isolated using the SV Total RNA isolation System (Promega, Madison WI, USA). Logarithmically growing cells were induced with 0.1% MMS for 45 and 90 min at 30°C in liquid YPD. Control cells were grown without MMS. RT reaction was performed using AMV Reverse Transcriptase (Promega, Madison WI, USA), 1µg of RT product was used as a template for the PCR. Primers for the detection of *YLR238W*, *YDR200C*, *RNR2*, and *HIS3* transcripts were used (Table 1).

4.14 Bioinformatic analysis

SGD (http://genome-www.stanford.edu/Saccharomyces). Yeast genes and proteins general information (function, sequences), was take from SGD web page.

BLASTp (http://www.ncbi.nlm.nih.gov/blast/Blast/). Protein homologs were defined using BLASTp server. Only low score values ($< 10^{-20}$) were consider.

GRID (http://biodata.mshri.on.ca/grid/). Protein-protein interactions were searched using GRID server. In the tables only some of the interactions are mentioned.

SBASE (http://hydra.icgeb.trieste.it/~kristian/SBASE/). Protein domains were searched using SBASE program.

5. RESULTS

5.1. HNS PLASMID SYSTEM.

5.1.1 HNS Plasmid System. To monitor the overall dynamics of DSB repair processes in yeast, we constructed six centromeric plasmids (pURRA, pRURA, pURRA8, pRURA8, pURRA8Δ and pRURA8Δ) that were used as a substrate that could be repaired by HR, NHEJ or SSA. All plasmids contain two non-functional *URA3* copies (URR and RRA), truncated at their 3' or 5' ends respectively, which share a central homologous region. A rare *I-Sce*I endonuclease recognition sequence (Table 1) was inserted at the natural *Stu* I site of the URR fragment in the shared region. The pURRA, pURRA8 and pURRA8Δ (Figure 9A, 9C and 9E), have truncated *URA3* fragments in direct orientation (head to tail), while the pRURA, pRURA8 and pRURA8Δ (Figure 9B, 9D and 9F) plasmids have them in inverted (head to head) orientation.

They all carry the *TRP1* gene and either the *HIS3* (pURRA and pRURA) or *ADE8* (pURRA8, pRURA8, pURRA8Δ and pRURA8Δ) genes. *TRP1* allows selection of all possible repair events while *HIS3* and *ADE8* allow discrimination between different repair processes. The *HIS3* gene allows discrimination on the basis of histidine prototrophy, while *ADE8* on the basis of colony colour. The genetic background that is used in each case is *trp1/his3* or *trp1/ade8/ade2* (*ade1*), when plasmids carring *HIS3* or *ADE8* gene markers are used, respectively (Table 6). The presence of the *ADE8* in the *ade8/ade2* background (white cells), results in the accumulation of red pigment in the cell. However, if the *ADE8* gene is lost by plasmid resolution during
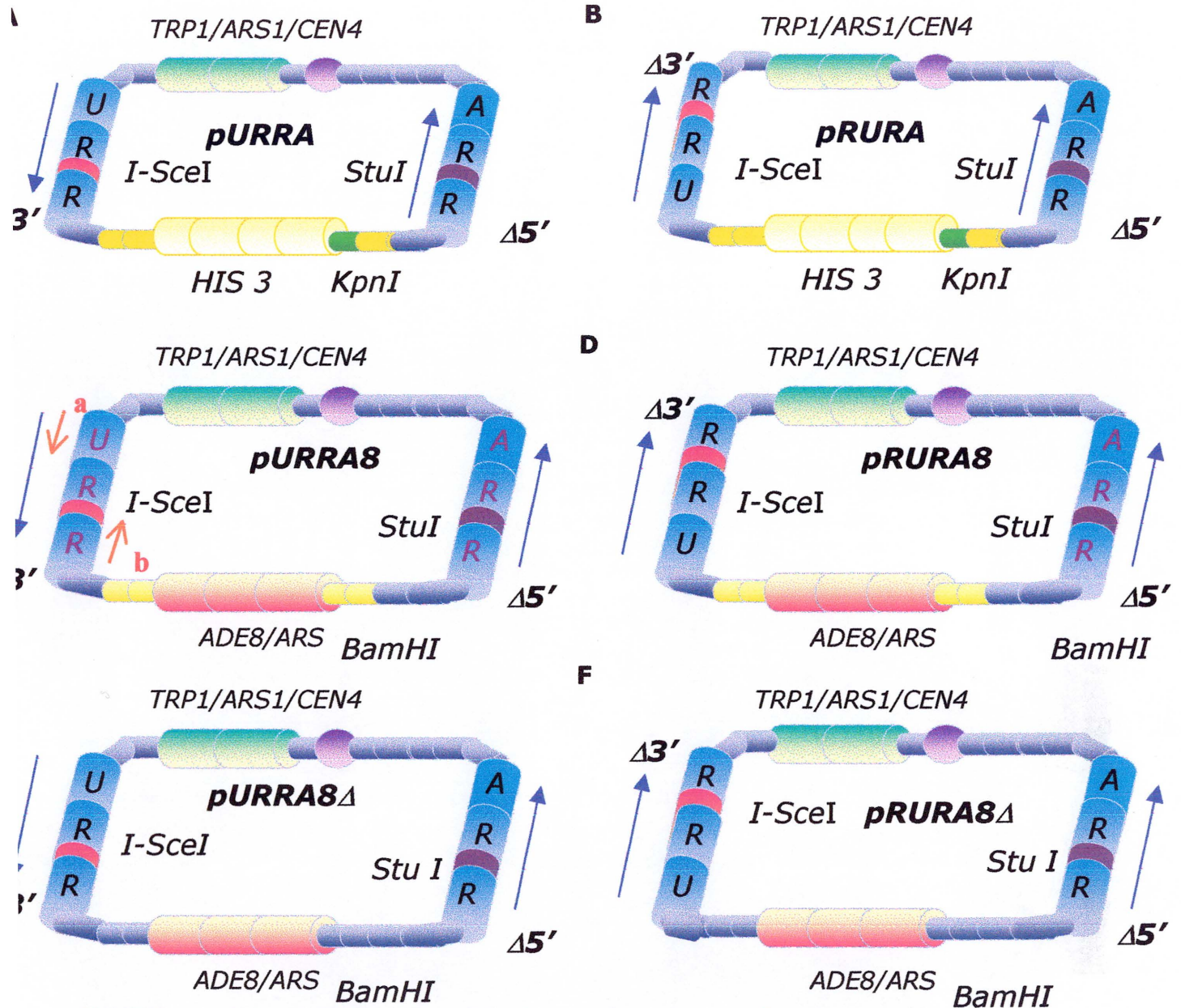
FIGURE 9. HNS plasmids. The plasmid contains two non-functional *URA3* truncated copies in direct orientation (URR and RRA). A *I-Sce*I site (in red) was inserted in 3'truncated copy at the original *Stu*I site (in purple). Phenotypic markers *TRP1*, *HIS3* and *ADE8* are shown in green, yellow and red, respectively. *ARS1* and *CEN IV* sequences are shown in green-violet. pURRA plasmid. **B**. pRURA plasmid The unique Kpn I site is shown in green. **C** pURRA8 plasmid. **D**. pRURA8 plasmid. The *Tethrahymena* sequences flanking *ADE8* gene, are shown in yellow and the primers UR-F (**a**) and UR-R (**b**) are shown in the URR sequence (orange). **E** pURRA8Δ plasmid. **F** pRURA8Δ plasmid. Orientation of the repeats are shown with blue arrows. The unique *Bam* HI site is shown in blue (C to D).

Table 6. HNS plasmids features.

Plasmid	URR-RRA repeats orientation	Markers	Genetic background	DSB unique sites	Tet repeats
pURRA	Direct	**TRP1-HIS3**	*trp1 his3*	*I Sce* I, *Kpn* I	+
pRURA	Inverted	*TRP1-HIS3*	*trp1 his3*	*I Sce* I, *Kpn* I	+
pURRA8	Direct	**TRP1-ADE8**	*trp1, ade8, ade2*	*I Sce* I, *Bam* HI	+
pRURA8	Inverted	**TRP1-ADE8**	*trp1, ade8, ade2*	*I Sce* I, *Bam* HI	+
pURRA8Δ	Direct	**TRP1-ADE8**	*trp1, ade8, ade2*	*I Sce* I, *Bam* HI	-
pRURA8Δ	Inverted	**TRP1-ADE8**	*trp1, ade8, ade2*	*I Sce* I, *Bam* HI	-

the recombinational repair process, the colour of the cell turns back to white (Figure 10A).

To introduce a DNA DSB *in vitro*, plasmids were digested at the unique *I-Sce*I, *Kpn*I or *Bam* HI site. The *I-Sce*I endonuclease creates the break inside the URR copy that has the homologous sequence on the same plasmid (RRA fragment). *Kpn*I and *Bam*HI endonucleases create a unique break in the sequence between the *HIS3* or *ADE8* genes and the plasmid backbone, respectively (Figure 9). *In vivo,* the DSB was introduced only in the URR fragment by galactose-inducible expression of the *I-Sce*I endonuclease (RICCHETTI *et al.* 1999).

5.1.1 Simultaneous detection of DSB repair pathways. To assess the proportion of each DSB repair mechanism in overall DSB repair, we transformed haploid (*Mata*) Y or Y0 strains, with the *in vitro* linearized pURRA, or pURRA8 and pURRA8Δ plasmids. Only the cells that repair DNA DSBs in the plasmid can give rise to colonies on SC-Trp plates after transformation. Colonies from SC-Trp plates were replica-plated onto SC-Ura, to assess the reconstitution of the functional *URA3* gene. When pURRA plasmid was used, replica-plating onto SC-His plates was also performed.

The relative frequency of each DSB repair event was determined using the HNS (HR/NHEJ/SSA) system with the appropriate combination of phenotype/repair pathway, as shown in Table 7 and Figure 11.

The possible outcomes of DSB repair by CO, GC, SSA and NHEJ (in this case only for pURRA8Δ and pRURA8Δ plasmids) are schematically represented in Figure 11.
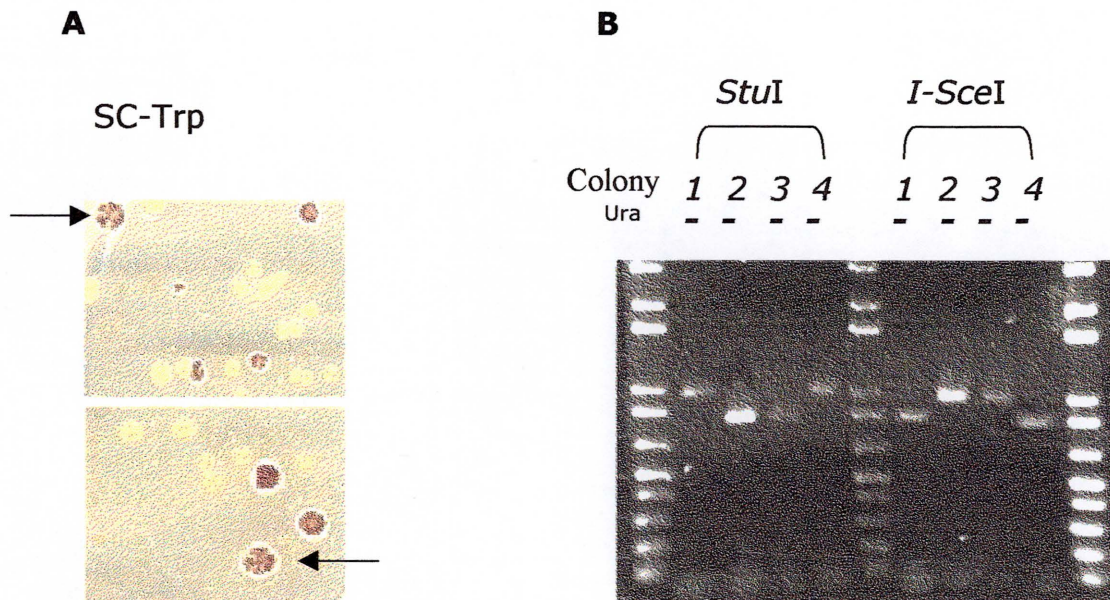
FIGURE 10. HNS plasmid system phenotypic and molecular analysis. **A**. SC-Trp plate showing transformants obtained after Y0 transformation with *I Sce* I-linearized pURRA8Δ. Red and white colonies are shown. The arrows indicate two sectored (red-white) colonies. **B**. Discrimination between NHEJ and GC process. Red Ura⁻ transformants obtained after Y0 transformation with *I-Sce* I-linearized pURRA8Δ served as a template for the PCR amplification, using primers **a** and **b**. PCR products were digested by the *Stu* I or *I-Sce* I. Colonies number 1 and 4 repair DNA DSB by NHEJ while colonies 2 and 3 repair the break by GC.
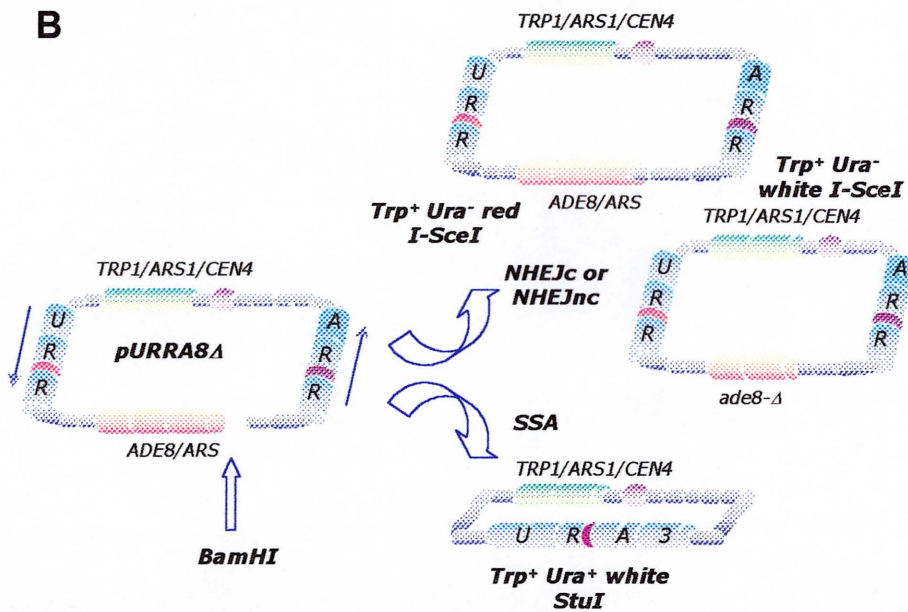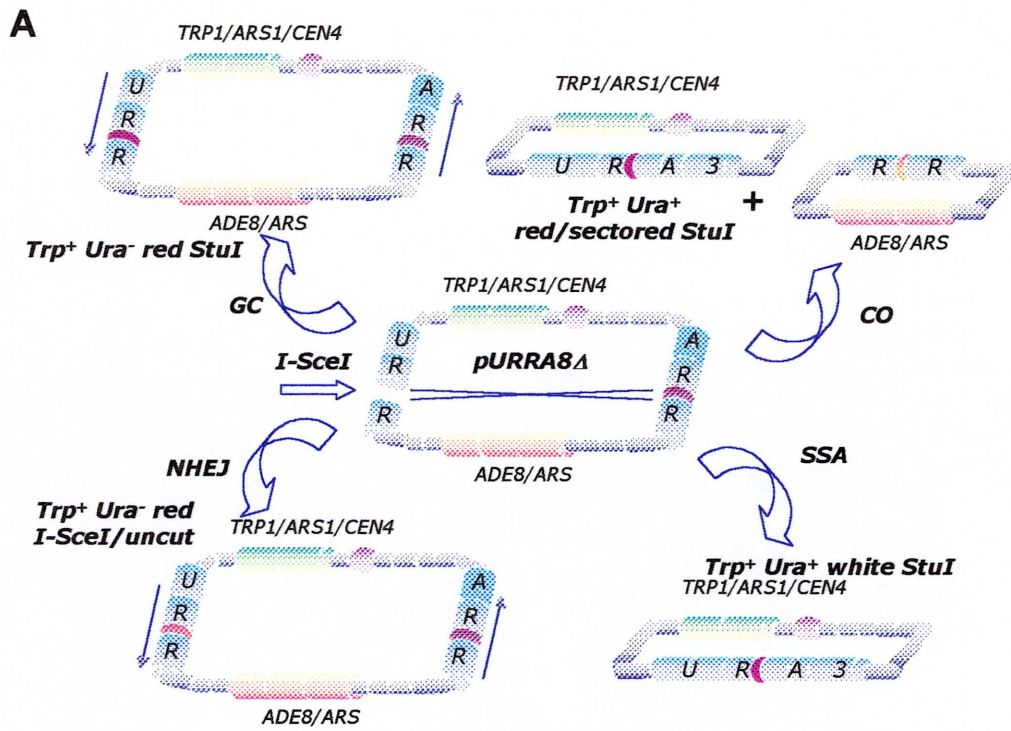
FIGURE11. Graphic representation of HNS DSB repair of pURRA8Δ plasmid. **A**. *I-Sce* I-linearized plasmid. **B**. *Bam* HI linearized plasmid. The plasmid contains two non-functional *URA3* truncated copies in direct orientation (URR and RRA). A *I-Sce* I was inserted in 3' truncated copy at the original *Stu* I site (showed on the RRA region, in purple). Two phenotypical markers *TRP1* and *ADE8* are shown in green and red respectively. *ARS1* and *CEN IV* sequences are shown in green. Possible outcomes of DSBR can be discriminated by phenotype analysis: Ura⁻, Ura⁺, red, white or sectored colonies. In addition, phenotypically similar processes can be further distinguished by *Stu* I or *I-Sce* I digestion of ab amplified PCR products.

FIGURE 11C. Graphic representation of repair events of *I-Sce* I-linearized pRURA8Δ plasmid. The plasmid contains two non-functional *URA3* truncated copies in inverted orientation (URR and RRA). A *I-Sce* I was inserted in 3' truncated copy at the original *Stu* I site (showed on the RRA region, in purple). Two phenotypic markers *TRP1* and *ADE8* are shown in green and red respectively. *ARS1* and *CEN IV* sequences are shown in green. Possible products of DSBR when the break is introduced in the non-homologous region can be discriminated by phenotype analysis: Ura⁻ or Ura⁺ colonies. In addition, phenotypically similar processes can be further distinguished by *Stu* I or *I-Sce* I digestion of **ab** amplified PCR products.
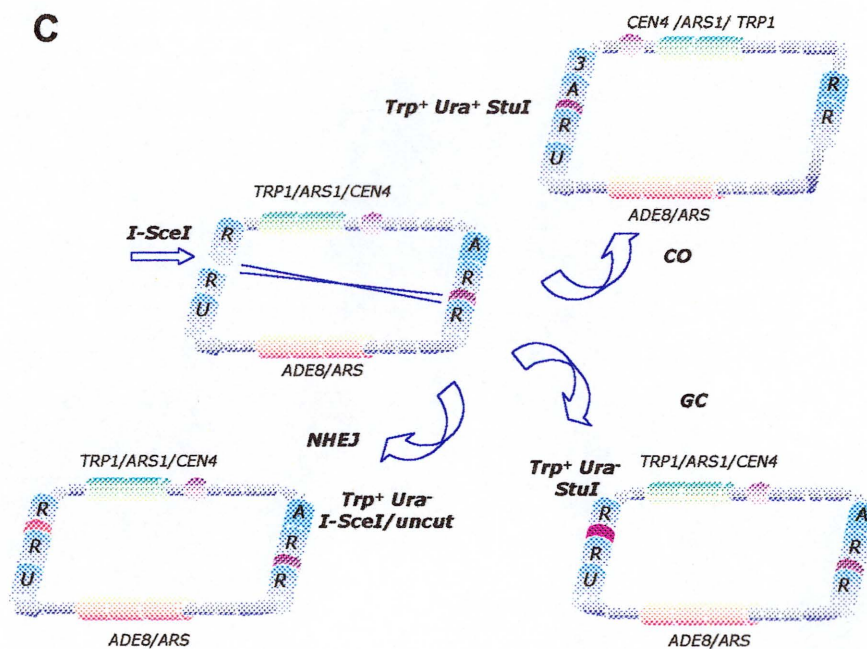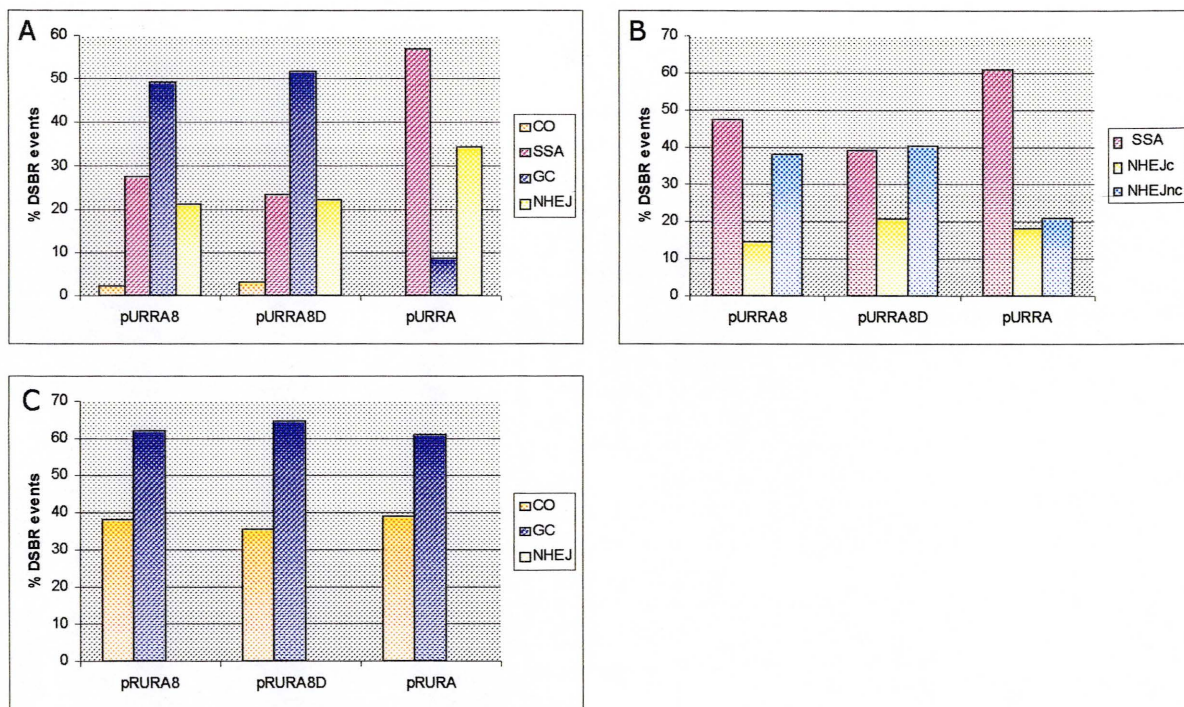
FIGURE 12. Relative frequencies of DSB repair events. DSBR events distribution of Y0 and Y strains. Cells were transformed with linear plasmids and selected on SC-Trp plates. Relative frequencies of each event were assessed by phenotype and molecular assay. **A.** Cells transformed with pURRA8, pURRA8Δ or pURRA linearized with *I-Sce*I. **B.** Cells transformed with pURRA8, pURRA8Δ or pURRA, linearized with *Bam*HI or *Kpn*I. **C.** Cells transformed with pRURA8, pRURA8Δ or pRURA linearized with *I-Sce*I. CO: crossing-over, GC: gene conversion, NHEJ: non homologous end-joining (C: conservative, NC: non conservative), and SSA: single strand annealing.

Breaks introduced in the URR copy by *I-Sce*I can be repaired by several mechanisms that can be distinguished by phenotypic and molecular analysis (Figure 10).

At the site of the break, the DNA can be simply re-ligated by NHEJ generating Trp$^+$ red colonies that cannot grow on SC-Ura plates. If re-ligation is accurate (NHEJc), the PCR products amplified using primers **a** and **b** (Figure 10B), would be digested by *I-Sce*I (NHEJc, Figure10B). If re-ligation is inaccurate with partial degradation at the break site, the *I-Sce*I site will be destroyed and the PCR products will remain uncut (NHEJnc). On the other hand, due to the presence of the homologous sequence on the plasmid, the break inside the URR can be repaired by CO generating Trp$^+$ Ura$^-$ red colonies. Although repair by NHEJ results in the same phenotype, the two processes can be distinguished after restriction analysis of the **ab**-amplified PCR products since in the case of GC, the plasmids will be digested by the *Stu*I (Figure 10B). Repair by CO leads to the formation of a functional *URA3* gene and the consequent resolution of the pURRA8Δ plasmid into two molecules. Since the *ADE8* fragment amplified by primers Ade8F and Ade8R contains an *ARS*-like sequence permitting the maintenance of the plasmid moiety carrying it after resolution, CO will be characterised by the presence of Trp$^+$ Ura$^+$ sectored colonies (see arrows in Figure 10A). Finally, repair can also occur by SSA since the two truncated *URA3* copies are in direct orientation. In this case, SSA will result in the loss of sequence information between repeats giving raise to Trp$^+$ Ura$^+$ white colonies (Figure 10A and 11A).

The combined data from the analysis of Trp⁺ colonies after transformation of Y0 and Y strains with the test plasmids are reported in Figure 12.

We detect that in Y0 strain, in approximately 50% of the cases, GC was the major mechanism that repaired the *I-Sce*I linearized pURRA8Δ and pURRA8 plasmids (Figure 12A). SSA repaired 23-28 % of the breaks, while NHEJ repaired 21 % of the breaks. Frequency of breaks repair by CO was 2-3 %.

In Y strain, CO and SSA are phenotypic indistinguishable events and they represent more than 50% of the repair events, while repair by GC was 8.6% and NHEJ 35 %.

By linearizing the plasmids with the *Kpn*I or *Bam*HI we introduced a DSB in the unique region that has no sequence homology in the cell. Following the repair of *Bam*HI-linearized pURRA8Δ plasmid (Figure 11B). Repair by SSA would result in the Trp⁺ Ura⁺ white colonies. Conservative NHEJ would give rise to Trp⁺ Ura⁻ red phenotype, while the degradation of the *ADE8* gene would produce Trp⁺ Ura⁻ white colonies. Most of the breaks were repaired by SSA (>40 %), followed by non-conservative NHEJ and conservative NHEJ. The distribution of repair events was similar for all 3 plasmids used (Figure 12B).

The *I-Sce*I DSB in the indirect repeat substrate, pRURA8Δ, can be repaired by NHEJ, CO and GC (Figure 11C). Once again, repair by NHEJ and GC result in the same Trp⁺ Ura⁻ red phenotype, that can be discriminated by *I-Sce*I or *Stu*I digestion of the **ab**-amplified PCR products. CO produces Ura⁺ colonies that are stably red since the substrate did not allowed plasmid resolution.

TABLE 7. Summary of the DSBR processes that can be assessed by each plasmid depending on the region in which the DSB is introduced.

Plasmid	Homology (*I Sce* I)	Phenotype	Heterology (*Bam* HI)	Phenotype
pURRA	CO+SSA NHEJ GC	Ura $^+$ His$^-$- Stu I Ura$^-$ His$^+$- Sce I/N Ura$^-$ His$^+$ Stu I	SSA NHEJnc NHEJc	His$^-$- Ura $^+$ Ura$^-$ His$^-$ Ura$^-$ His$^+$
pRURA	CO NHEJ GC	Ura $^+$ His$^+$- Stu I Ura$^-$ His$^+$- Sce I/N Ura$^-$ His$^+$- Stu I	NHEJnc NHEJc	Ura$^-$ His$^-$ Ura$^-$ His$^+$
pURRA8	CO SSA NHEJ GC	Ura $^+$ S- Stu I Ura $^+$ W- Stu I Ura$^-$ R- Sce I/N Ura$^-$ R- Stu I	SSA NHEJnc NHEJc	Ura $^+$ W Ura$^-$ W Ura$^-$ R
pRURA8	CO NHEJ GC	Ura $^+$ R- Stu I Ura$^-$ R- Sce I/N Ura$^-$ R- Stu I	NHEJnc NHEJc	Ura$^-$ W Ura$^-$ R
pURRA8Δ	CO SSA NHEJ GC	Ura $^+$ S- Stu I Ura $^+$ W- Stu I Ura$^-$ R- Sce I/N Ura$^-$ R- Stu I	SSA NHEJnc NHEJc	Ura $^+$ W Ura$^-$ W Ura$^-$ R
pRURA8Δ	CO NHEJ GC	Ura $^+$ R- Stu I Ura$^-$ R- Sce I/N Ura$^-$ R- Stu I	NHEJnc NHEJc	Ura$^-$ W Ura$^-$ R

*Stu*I: **ab** PCR *Stu*I sensitive, *I-Sce*I: **ab** PCR *I-Sce*I sensitive, N: **ab** PCR no *I-Sce*I no *Stu*I. W: white colonies, R: red colonies and S: red/white (sectored) colonies.

As shown in Figure 12C, the repair of the breaks was distributed between two HR process. CO was performed in 36-39% in pURRA, pURRA8 and pURRA8Δ. Repair by NHEJ was not detected. The BamHI DSBs in pRURA8Δ plasmid can be repaired only by NHEJ. Non-conservative NHEJ was rejoining most of these breaks (> 80%). The results presented demonstrated that our system was proficient in detecting all DSB repair pathways in the wild-type haploid Y and Y0 strains, using different substrates. From the six plasmids constructed, we decided to use pURRA8Δ and pRURA8Δ plasmids for further experiments only. This decision was based upon the fact that the *ADE8* marker allows the use of the colour system to characterise the transformants in a faster and easier way. These plasmids do not contain the *Tethrahymena* repeats (derived from the original pYAC3 plasmid) flanking the *ADE8* marker. These direct repeats, acting as a telomers, could have some effect on the DSBR processes we were studying.

5.1.2 System Validation: *rad52* and *hdf1* Show Differential Distribution of DSBR Events.

Validation of our system supposes that under conditions where either HR or NHEJ cannot take place due to lack of an essential component, differences in the distribution of repair events among the other pathways should be detectable. To this end, Y0 *rad52* and *hdf1* (*yku70*) mutants were transformed with the four linearized plasmids. Absence of *RAD52*, encoding a key protein of HR and SSA (SUNG *et al.* 2000; VAN DYCK *et al.* 2001), decreased primarily the number of SSA events (Figure 13A and 13B) and slightly decreased CO events when inverted repeat substrates
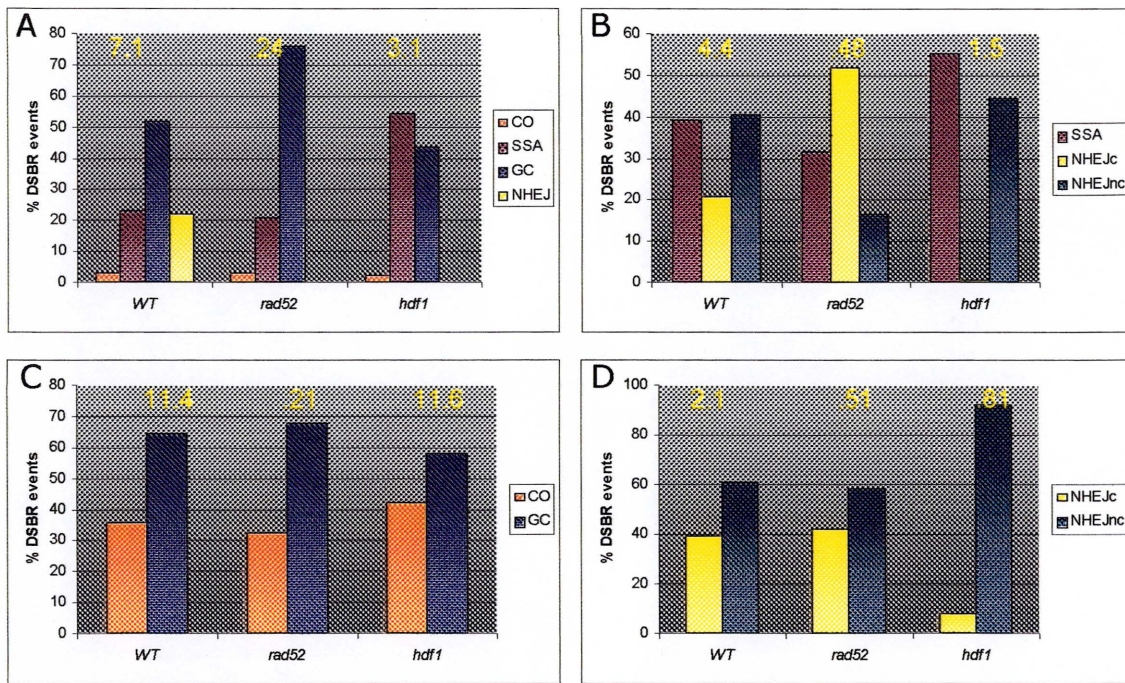
FIGURE 13. **DSBR profile of WT, *rad52* and *hdf1* strains**. DSBR events distribution of Y0 (*WT), hdf1*, and *rad52* mutants. Cells were transformed with linear plasmids and selected on SC-Trp plates. Relative frequencies of each event were assessed by phenotype and molecular assay. Probability of repair is shown in over each histogram **A**. Cells transformed with pURRA8Δ linearized with *I-Sce*I. **B**. Cells transformed with pURRA8Δ linearized with *Bam*HI. **C**. Cells transformed with pRURA8Δ linearized with *I-Sce*I. **D**. Cells transformed with pRURA8Δ linearized with *Bam*HI. CO: crossing-over, GC: gene conversion, NHEJ: non homologous end-joining (C: conservative, NC: non conservative), and SSA: single strand annealing. Probability of repair was calculates as the ration between the number of transformants obtained with linear plasmid versus circular plasmid, when same concentration of DNA and number of cells were used.

were used (Figure 13C), whilst deletion of *HDF1* resulted in the expected decrease of conservative NHEJ events (Figure 13B and 13D). NHEJ repair pathway was not detected when cells where transformed with *I-SceI*- linearized plasmids (Figure 13A and 13C). In this strain, the lack of Rad52p increased GC (Figure 13A) while not affecting NHEJ (Figure 13B) during *I-SceI* break repair.

The system had revealed the expected differences in the distribution of DSB repair pathways when the null mutants of known repair genes were used in both haploid (*Mata* and *Matα*) and diploids cells (Data not shown).

5.1.3 HNS analysis of known recombination genes.

In an attempt to test the usefulness of the system for assessing the function of DNA DSB repair genes that have not been well characterised, we used *nej1*, *mre11*, *rad50*, *xrs2* and *msh2* mutants in the Y0 background strain. HNS DSBR profiling (Figure 14) as well as MMS sensitivity tests were performed for all these null mutants (Figure 17A).

5.1.3.1 *NEJ1* null mutant. Nej1p regulates NHEJ. In haploid cells its expression facilitates the transport of Lif1p into the nucleus enabling the cell to carry out NHEJ. In diploid cells, its expression is repressed (VALENCIA *et al.* 2001).

The *nej1* deletant strain transformed with pURRA8Δ linearized with *I-SceI* did not perform NHEJ as expected and it showed an increase in GC (Figure 14A). In the absence of sequence homology, breaks were repaired by SSA and non-conservative NHEJ, almost abolishing NHEJc (Figure 14B). In this case the distribution of events correlates with *hdf1* Y0 and diploid strains (Data not shown).

CO levels were not affected in repair of the direct repeat substrate, but when inverted repeat substrate was used instead, CO repair events were 2- 3 times less frequent than in the Y0 and Y0 *hdf1* mutant strains (Figure 14C). Moreover the *in vivo-* induced DSBR of pRURA8Δ, showed 4 times less frequent CO (Data not shown), and *in vivo* spontaneous CO by inversion event of pRURA8Δ was 5 times less frequent than in the Y0 strain (Data not shown). The MMS sensitivity was low (Figure 17A).

5.1.3.2 *MRE11* null mutant. The *mre11* deletant transformed with pURRA8Δ linearized with *I-Sce*I showed an increase in NHEJnc (particular phenotype saw only in this case as white colonies that were Ura⁻) and SSA, while GC was decreased (Figure 14A). In the absence of sequence homology, breaks were repaired by SSA and non-conservative NHEJ, abolishing NHEJc (Figure 14B and 14D). CO levels were increased when the inverted repeat substrate was used (Figure 14C). MMS sensitivity was extremely high (Figure 17A).

5.1.3.3 *RAD50* null mutant. The *rad50* deletant transformed with pURRA8Δ- linearized with *I-Sce*I, was not able to perform repair by CO or NHEJ, while it showed an increase in SSA (Figure 14A). In the absence of sequence homology, breaks were repaired by SSA and non-conservative NHEJ, almost abolishing NHEJc (Figure 14B and 14D). CO levels were almost not affected when *I-Sce*I linearized pRURA8Δ was used (Figure 14C). MMS sensitivity was extremely high (Figure 17).
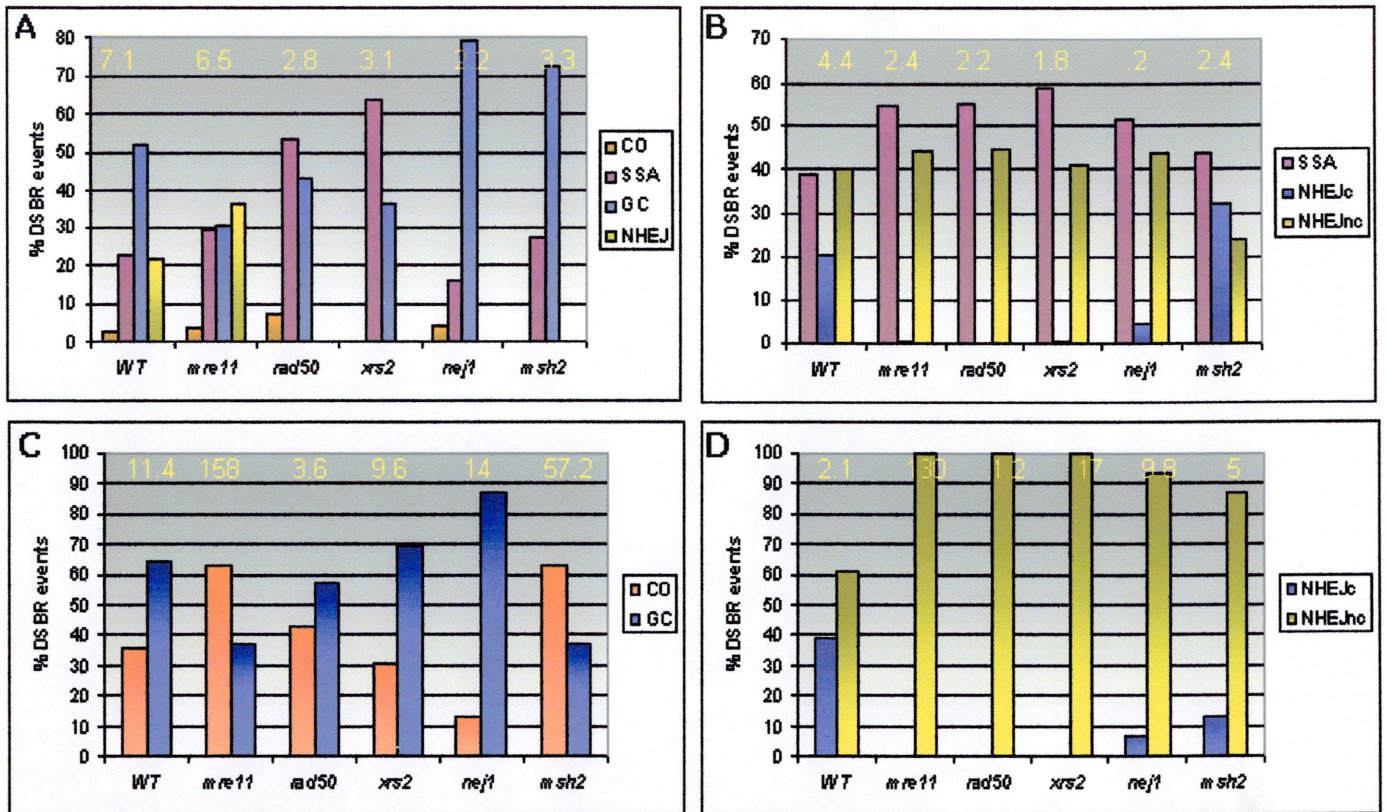
FIGURE 14. DSBR profile of WT, *mre11, rad50, xrs2, nej1* and *msh2* strains. DSBR events distribution of Y0 (WT*, mre11, rad50, xrs2, nej1* and *msh2)*. Cells were transformed with linear plasmids and selected on SC-Trp plates. Relative frequencies of each event were assessed by phenotype and molecular assay. Probability of repair is shown in over each histogram **A**. Cells transformed with pURRA8Δ linearized with *I-Sce*I. **B**. Cells transformed with pURRA8Δ linearized with *Bam*HI. **C**. Cells transformed with pRURA8Δ linearized with *I-Sce*I. **D**. Cells transformed with pRURA8Δ linearized with *Bam*HI. CO: crossing-over, GC: gene conversion, NHEJ: non homologous end-joining (C: conservative, NC: non conservative), and SSA: single strand annealing. Probability of repair was calculated as the ratio between the number of transformants obtained with linear plasmid and circular plasmid, when the same concentration of DNA and number of cells were used.

In the case of *mre11* and *msh2* mutants, the number of transformants obtained with pRURA8Δ circular plasmid was extremely low, making the probability of repair extremely high.

5.1.3.4 *XRS2* null mutant The *xrs2* deletant transformed with pURRA8Δ-linearized with *I-SceI*, was not able to perform repair by CO or NHEJ, while it showed an increase in SSA (Figure 14A). In the absence of sequence homology, breaks were repaired by SSA and non-conservative NHEJ, almost abolishing NHEJc (Figure 14B and 14D). CO levels were almost not affected when the substrate was pRURA8Δ (Figure 14C). When pRURA8Δ plasmid was used to test for spontaneous CO by inversion, *xrs2* null mutant showed a hyper-recombinogenic phenotype (Data not shown). MMS sensitivity was extremely high (Figure 17A).

5.1.3.5 *MSH2* null mutant. The *msh2* deletant transformed with pURRA8Δ linearized with *I-SceI* did not perform CO and NHEJ, and it showed an increase in GC (Figure 14A). In the absence of sequence homology, breaks were repaired by all three possible pathways, with an increase in NHEJc (Figure 14B). CO levels were 1.7 times more frequent than in Y0 strain during the repair of inverted repeat substrate (Figure 14C). MMS sensitivity was high (Figure 17A).

5.1.4 HNS plasmid system applications. By using he HNS system, we can generate, either *in vivo* or *in vitro,* DSBs on the plasmid substrate and after that analyse the repair profile under different genetic backgrounds. The studies presented in this thesis demonstrate that the use of the *in vitro*-induced DSB substrate allows the DSBR profile characterization in a quantitative way, as each transformant obtained represents a repair event. It is important to consider that in this case, the DNA ends are more prone to be modified during the transformation process, and that the repair profile obtained can be diverse from the one obtained when the DSB is induced *in vivo*. The major use of the *in vitro* system is to generate

DSBR profiles from mutants that can be compared simultaneously with those obtained from wild-type strain. This analysis can be used as a tool to understand the role of certain proteins in the DSBR process.

The induction of the DSB *in vivo* is based on the use of the inducible expression of *I-SceI* endonuclease. In our case, we could not be sure that 100% of the colonies obtained after induction had a DNA break that was then, eventually repaired. The *in vivo* DSB induction and repair system is going to be used as a screen for mutants defective in DSBR. All the selected mutants will be characterized using the *in vitro-*induce DSB repair system.

5.2. TRANSPOSON MUTAGENESIS.

5.2.1 Transposon mutagenesis I. Transposon mutagenesis I was performed in the Y strain carring pURRA plasmid. A 3360 single transformants were tested for their ability to grow in SC-Ura, SC-His or YPD plates after *in vivo* DSB induction. Mutants that were unable to perform HR (Circled Ura $^-$ in Figure 15C), or that were not able to repair the break and maintain the plasmid (Trp $^-$ red circles in Figure 15A and 15B) were selected.

From the first round of selection, 150 mutants were chosen. Using these 150 mutants, a second round of more restricted selection was performed, to obtain finally 20 mutants.

Plasmid loss was induced in those mutants, and transformation with the *I-Sce* I and *Kpn* I linearized plasmid was performed to obtain a DSBR profile of each transposon mutant (Tn-mutant Table 8).
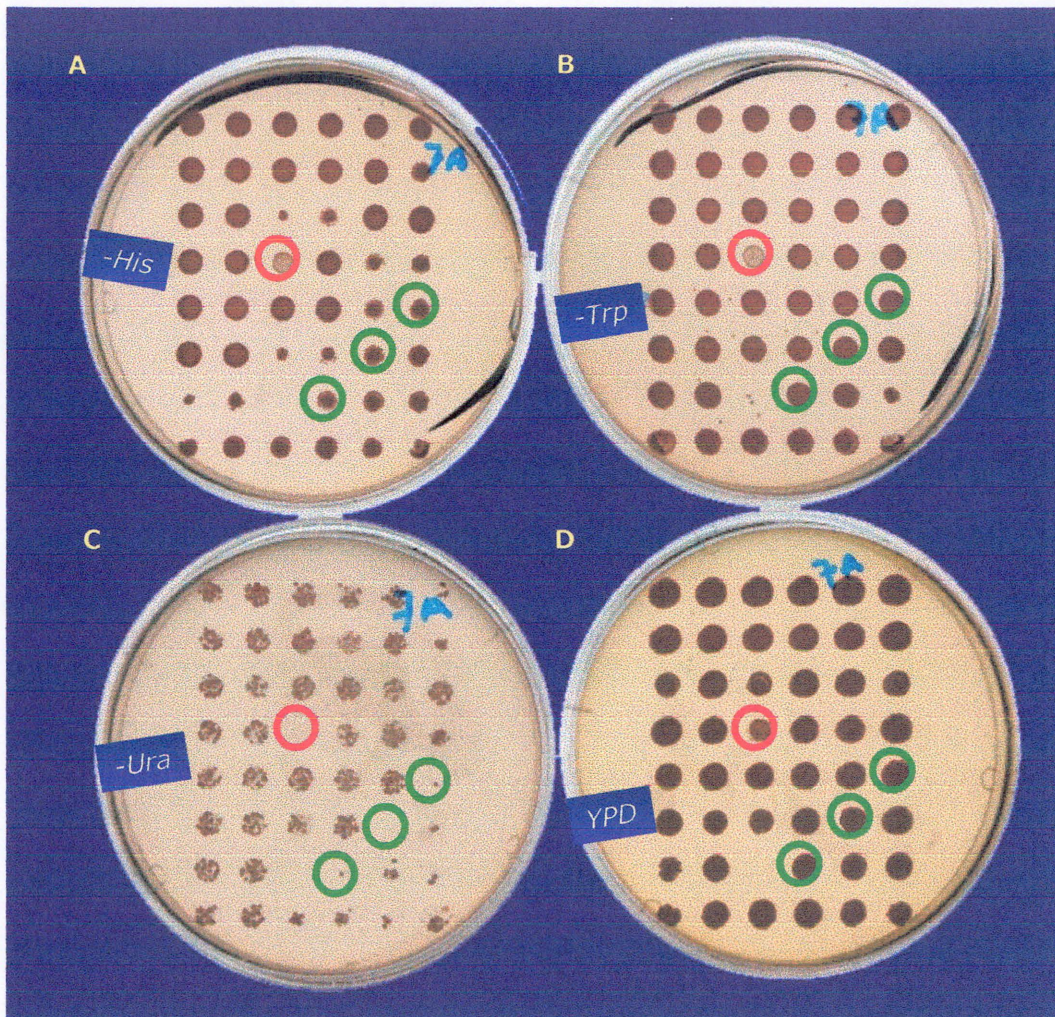
FIGURE 15. Example of transposon mutants screen in the first transposon mutagenesis. Cells were replica-plated after *in vivo* DSB induction, in **A**. SC-His plates, **B**. SC-Trp plates, **C**. SC-Ura plates and **D**. YPD plates. Circles indicate the selected colonies with deficient growth. Green Ura⁻ mutants and red Trp ⁻ or His ⁻.

TABLE 8. Transposon mutagenesis I: Identification of transposon insertion location.

MUTANT	GENE	FUNCTION	%(CO+SSA)-SSA
A	NUP188	Structural Nuclear Protein (Npc)	28-84
B	SIN4	RNA Pol II Transcription Mediator	49-108
C	SPE3	Met Transferase	53-140
D	BUD 16	Unknown-Random Budding	54-81
E	ATF1 YOR378w	Alcohol Dehydrogenase Unknown	42-92
F	ITR1	Sugars Transport	49-116
H	YRB2 ARC15 YIL064c	Nuclear-Cytoplasm Transport Structural Cell Growth Unknown	33-153
I	RSC2	Unknown/Chromatin Remodeling	42-45
K	SWR1	Chromatin Remodeling (Putative Helicase)	47-83
L	YLRO89c	Unknown Alanin Amino Transferase	19-6
M	MCH2 FRE2	Unknown/Monocarbohydrato Permease Iron Homeostasis	12-96
O	YPR071 NOT5 MED1	Unknown Transcription Subunit I of the Mediator Complex	14-69
R	SLI15	Unknown/Mitotic Spindle Involved Kinase	49-66
T	PEP12 SHE4	Golgi Vacuolar Transport Unknown/HO Expression. Meiotic Segregation	66-7

%(CO+SSA)= (CO+SSA) mutant/ (CO+SSA) WT, %SSA= SSA mutant/SSA WT
%(CO+SSA)rad52 null mutant= 66, %SSA rad52 null mutant = 16, %SSA hdf1 null mutant= 123

87

5.2.1.1 Position of Transposon Insertion. Vectorette-PCR (RILEY *et al.* 1990) was used (see 4.8.2.1) to identify the place of the transposon insertion. In Table 8, 14 gene(s) disrupted by the transposon are listed. Based on the information collected in Table 8, 7 genes were chosen to be further studied (shown in bold letters in Table 8). The *SIN4, BUD16, NUP188* and *YLR089* genes were found to be mutagenized more than once in this screening.

5.2.1.2 Gene Knockout. After a gene knockout of the selected genes a new DSBR profiling (Figure 16) as well as MMS sensitivity tests was performed for each null mutant (Figure 17B).

5.2.1.3 Null mutant phenotypes.

5.2.1.3.1 *SIN4.* *YNL236w* gene encodes an RNA polymerase II holoenzyme mediator, involved in positive and negative regulation of transcription including *MATa* cell-specific genes, possibly via changes in chromatin accessibility (MACATEE *et al.* 1997). It has been found as a component of two subcomplexes Rgr1 and Med6. It has been reported that its expression is not changed by γ irradiation treatment (MERCIER *et al.* 2001).

Null mutant in Y0 background flocculates. The DSBR profiling showed a decrease in CO events, SSA was almost not affected, while NHEJc decreased. MMS sensitivity was medium.
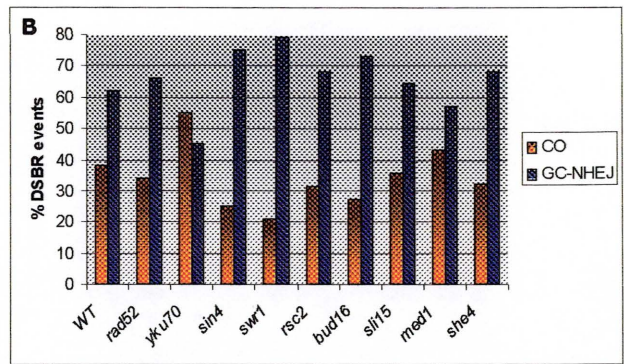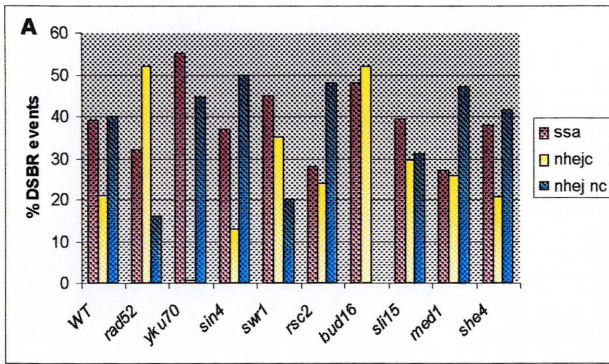
FIGURE 16. **DSBR profile off Y0 (*WT*), rad52, hdf1(yku70), sin4, swr1, rsc2, bud16, sli15, med1, and she4 strains**. Cells were transformed with linear plasmids and selected on SC-Trp plates. Relative frequencies of each event were assessed by phenotype and molecular assay. **A**. Cells transformed with pURRA8Δ linearized with *Bam*HI. **B**. Cells transformed with pRURA8 linearized with *I-Sce*I. CO: crossing-over, GC: gene conversion, NHEJ: non homologous end-joining (C: conservative, NC: non conservative), and SSA: single strand annealing.
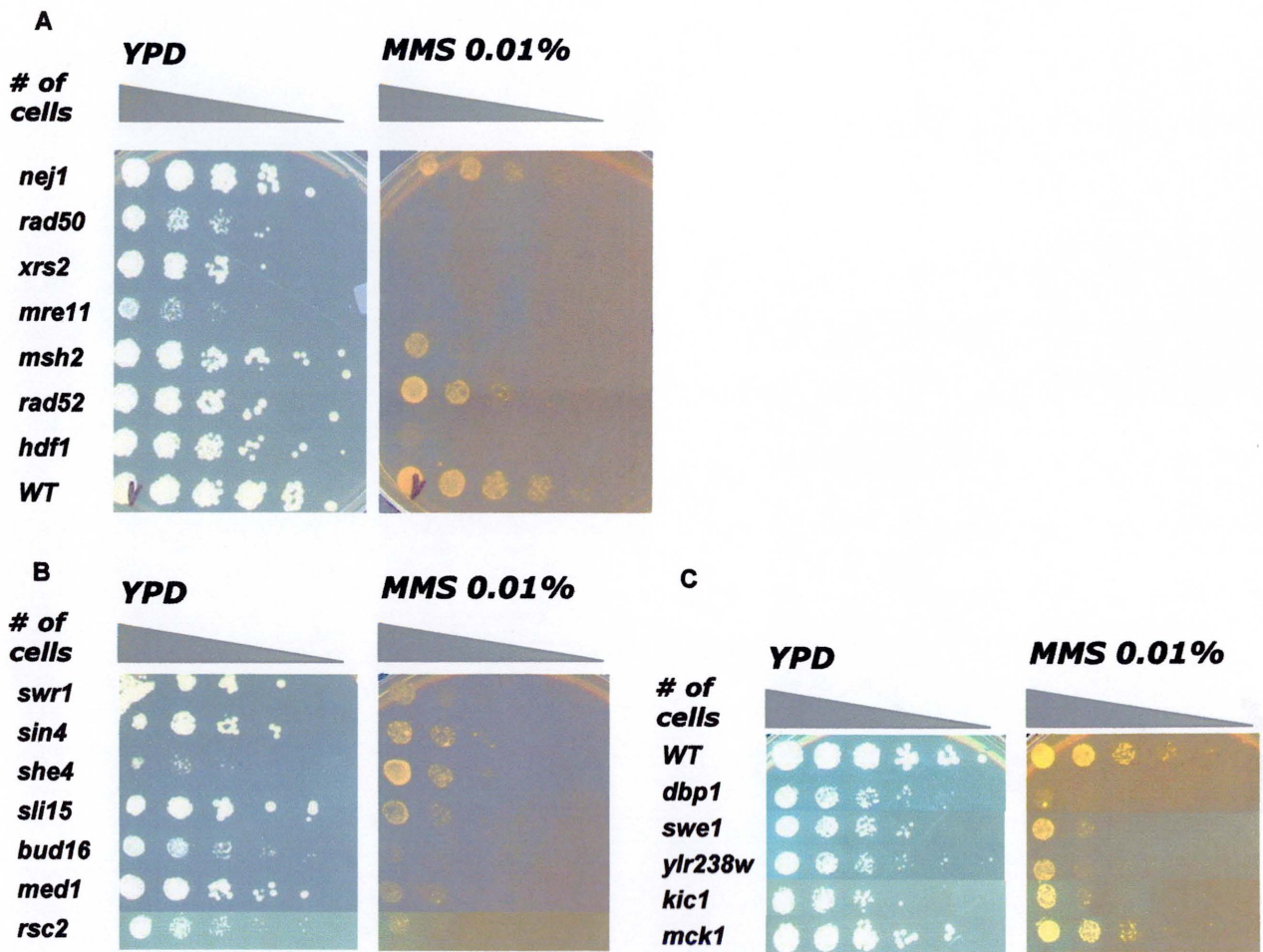
FIGURE 17. MMS sensitivity. Cells were spotted in serial dilutions on YPD and YPD containing 0.01% MMS plates, and let grown at 30°C for three days. **A**. DSBR genes null mutants. **B**. Null mutants from Transposon mutagensis I. **C**. Null mutants and Tn-mutants from transposon mutagenesis II. Y0 strain (WT).

5.2.1.3.2 *SWR1*. *YDR334w* gene encodes a DEAD-box protein, a putative RNA helicase, with an SNF2-related domain. BLAST analysis shows high similarity with *INO80* and *SNF2* (10^{-102} and 10^{-95} score respectively), *RAD54* and *RDH54* (10^{-42}). GRID protein interaction analysis shows interaction with Rad1p.

Null mutant DSBR profiling showed a decrease in CO events and non-conservative NHEJ, while SSA events increased. MMS sensitivity was high.

5.2.1.3.3 *RSC2*. *YLR357w* gene encodes a member of the RSC (remodeling the structure of the chromatin) complex, which remodels the structure of chromatin. Protein domain analysis showed a BAH domain, 2 bromo domains and an AT-hook. Rsc2p has been shown to be essential for 2μ plasmid maternal inheritance bias (WONG *et al.* 2002).

GRID protein interaction analysis shows interaction with Mud1p and Crt1p (transcription repressor factor, that is activated after DNA damage).

Null mutant exhibited slow growth. The DSBR profiling showed a decrease in CO and SSA events, but an increase in non conservative NHEJ. MMS sensitivity was high, even after 5 days of incubation at 30°C.

5.2.1.3.4 *BUD16*. *YEL029c* gene encodes a protein with unknown function; the null mutant shows random budding in diploids and slow growth. The DSBR profiling showed a decrease in CO and no repair events by NHEJnc. MMS sensitivity was high, even after 5 days of incubation at 30°C.

5.2.1.3.5 *SLI15*. *YBR156c* encodes a protein kinase activator, involved in mitotic chromosome segregation. GRID protein interaction analysis shows interaction with Ipl1p (protein involved in regulation of yeast chromosome segregation).

Null mutant DSBR profiling showed a small decrease in CO events, and NHEJnc. MMS sensitivity was high.

5.2.1.3.6 *MED1*. *YPR070w* gene encodes a subunit 1 of the mediator complex essential for transcription regulation. Null mutant DSBR profiling showed a small increase in CO, while SSA decreases. MMS sensitivity was medium-high.

5.2.1.3.7 *SHE4*. *YOR035c* gene encodes an unknown protein required for mother cell-specific gene expression of HO. Null mutant DSBR profiling showed a decrease in CO, while SSA is not affected. MMS sensitivity was low.

5.2.2 Transposon mutagenesis II. The second transposon mutagenesis was performed in the Y0 and Y0-*hdf1* strains caring pRURA8 plasmid. A 3800 single transformants were tested for their ability to grow in SC-Ura and YPD plates after DSB induction. Mutants that were not able to perform repair by HR (Ura ⁻), or that were not able to repair the break (Trp ⁻) were selected. From the first round of selection, 65 mutants were chosen. Using these mutants, a second round of more restricted selection was performed, to choose at the end 13 mutants. Qualitative MMS sensitivity test was performed (Table 9).

5.2.2.1 Position of Transposon Insertion. Vectorette-PCR (RILEY *et al.* 1990) was used to identify the locus of transposon insertion. In Table 9, gene(s) disrupted by the transposon are listed. Five genes were chosen to be knocked-out (shown in bold letters in Table 9). Transformations with the *I-SceI* and *Bam*HI linearized plasmids were performed to obtain a DSBR profile of null mutants and Tn-mutant.

RAD50 was found as one of the selected mutants disrupted by the transposon insertion. The *rad50* null mutant was already tested and its characteristics have been presented previously.

5.2.2.2 Gene Knockout. Gene knockout of *YLR238w, SWE1* and *DBP1* genes was performed. It was not possible to obtain the knockouts of *MCK1* and *KIC1*. All the data presented are referring to *MCK1* and *KIC1* transposon mutants and not to the null mutants. A new DSBR profile was obtained for each null mutant (Figure 18), and a MMS sensitivity tests were performed (Figure 17C).

5.2.2.3 Null Mutant Phenotypes

5.2.2.3.1 *YLR238w* gene encodes an unknown function protein that possesses a FHA (Forkhead-associated domain) and BZIP (transcription factor domain). GRID analysis shows an interaction with Rox3p (RNA pol II transcription mediator), Rpc40p (RNA pol III C subunit) and Ydr200p (unknown protein that contains a FHA domain).

Null mutant DSBR profiling showed an increase in CO when an inverted-repeat substrates were used. MMS sensitivity was medium.
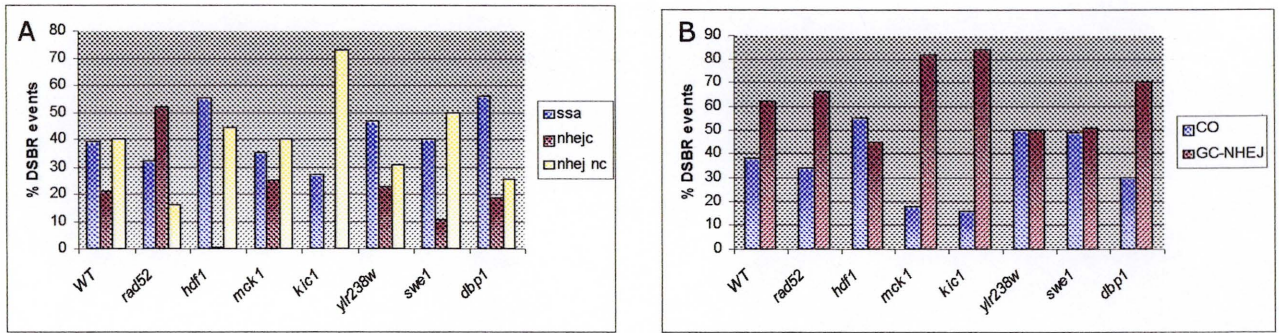
FIGURE 18. DSBR profile off Y0 (*WT*), rad52, hdf1(yku70), ylr238w, swe1, dbp1 null mutants and mck1, kic1 Tn-mutants. Cells were transformed with linear plasmids and selected on SC-Trp plates. Relative frequencies of each event were assessed by phenotype and molecular assay. **A**. Cells transformed with pURRA8Δ-linearized with *Bam*HI. **B**. Cells transformed with pRURA8-linearized with *I-Sce*I. CO: crossing-over, GC: gene conversion, NHEJ: non homologous end-joining (C: conservative, NC: non conservative), and SSA: single strand annealing.

TABLE 9. Transposon mutagenesis II: Identification of transposon insertion location

MUTANT	GENE	FUNCTION	MMS Sensitivity	% CO-SSA
Y01	MCK1	Tyr Kinase	++	35-80
Y02	*YMR278w* *YMR279c*	Unknown Glucose Transport	+	
Y03	*LTR*		+	
Y04	*PMR1*	Ca2 $^+$Pump-ATPase	+	
YK1	*LHS1*	Hsp70 Family Required For Efficient Translocation of Protein Precursor Across the ER Membrane.	++	
YK2	*RAD50*	DNA Repair	++++	
YK3	*RRN9*	RNA Polymerase Transcription Factor	++	
YK4	*KIC1*	Kinase	++	31-57
YK5	*DAN1*	Unknown, Putative Cell Wall Manoprotein	+++	19-18
YK6	*NPY1*	NAD $^+$ Pyrophosfatase	++	
YK7	*YLR238w*	Unknown	++	23-36
YK8	*SWE1*	Tyr Kinase	+++	-38
YK9	*DBP1*	RNA Helicase	+++	80-31

%CO= CO mutant/ CO WT in pRURA8Δ, %SSA= SSA mutant/SSA WT, %CO *rad52* null mutant= 69, %CO *hdf1* null mutant= 80, %SSA *rad52* null mutant = 66, %SSA *hdf1null* mutant= 22
+ sensitivity.

2.2.3.2 SWE1. *YJL187c* gene encodes for a Ser/Tyr kinase, that inhibits G2/M transition when morphogenesis is perturbed. Fission yeast (wee1), Xenopus (Xwee1) and human (Wee1Hu) homologs have been identified. GRID protein analysis show interaction with Sgs1p (ATP dependent DNA helicase), Clb2p (cyclin-dependent protein kinase regulator).

Null mutant DSBR profiling showed an increase in CO and non-conservative NHEJ repair pathways. MMS sensitivity was medium-high.

5.2.2.3.3 DBP1. *YPL119c* gene encodes a putative ATP-dependent RNA helicase, DEAD box protein. Null mutant DSBR profiling showed an increase in SSA and a decrease in CO. MMS sensitivity was high.

5.2.2.3.4 MCK1. *YNL307c* gene encodes a protein Thr/Tyr kinase, required for chromosome segregation. GRID protein analysis shows interaction with Cdc19p (requiered for START A in the cell cycle and sporulation) and with some CBF proteins (centromere/microtubule binding proteins). It has been reported that its expression is not changed by γ irradiation treatment (MERCIER *et al.* 2001). Wilson shows that *MCK1* promotes fully efficient NHEJ by a regulatory mechanism activated in postdiauxic stationary phase that is distinct and separable from the action of *NEJ1* (WILSON 2002). Transposon mutant DSBR profiling showed, a decrease in CO repair pathway, while conservative NHEJ increased. MMS sensitivity was medium.

5.2.2.3.5 *KIC1*. *YHR102w* gene encodes a kinase, involved in regulation of cell shape and cell size. GRID analysis shows that it interacts with Cdc31p (structural component of the cytoskeleton), Ctf19p (Chromosome transmission fidelity protein). Transposon mutant DSBR profiling showed a decrease in CO repair events. Conservative NHEJ repair events were not detected. MMS sensitivity was medium-high.

5.3. Selected Genes Preliminary Characterization

Both rounds of mutagenesis yielded a of groups of protein with different functions. Between them we could distiguish, 4 kinases, 2 nuclear-cytoplasmic transporters or nuclear pore proteins, 9 metabolic proteins, 3 proteins involved in transcription, 5 involved in chromatin structure and segragation, 1 involved in repair and 3 unknown function proteins. Out of the mutants obtained we decided to further study 2 gene products. The reasons why we chose these are because they show particular features that are going to be listed at the beginig of each section (5.3.1 and 5.3.2).

5.3.1 *RSC2*

From the first mutagenesis we chose *RSC2*. The null mutant had a particular decrease in CO and greater impairment in SSA. Moreover it was one of the null mutants that showed strong sensitivity to MMS. Its role in chromatin remodeling and its interaction with Crt1p (transcription factor involved in the DNA damage response), made this protein even more interesting.

A very recent report involved Rsc2p in 2μ plasmid stability (WONG *et al.* 2002). Therefore we wanted to test if the DSBR profile of the *rsc2* null mutant was due to decreased plasmid stability or to some deficiency in the recombinational-repair process.

5.3.1.1 Plasmid loss. We performed a plasmid stability assay to compare the stability of the different HNS plasmids, and 2μ-based plasmid pRAP-Trp in WT and *rsc2* null mutants strains (Figure 19).

Wong et al reported the role of Rsc2p in chromatin structure linked with 2μ segregation. In our experiments, *rsc2* null mutant maintained as stably as the WT strain the 2μ-based plasmid (pRAP-Trp), but showed much lower stability of the CEN-based plasmid pYAC3 that is completely lost after 5 hours (data not shown) when grown in non-selective media. Interestingly, we observed a difference in stability between pURRA8 and pRURA8. These two plasmids differ only in the orientation of the URR fragment. When the direct repeat plasmid is present in the null mutant its maintenance is only 21% compared with the WT strain. When inverted repeats are present the maintenance is 69% compared with the WT strain. This suggests that the chromatin assembly of the secondary DNA structure is different between the two plasmids and may affect the stability of the molecule. The diverse secondary DNA structure between plasmids studied by Wong et al could explain their results.
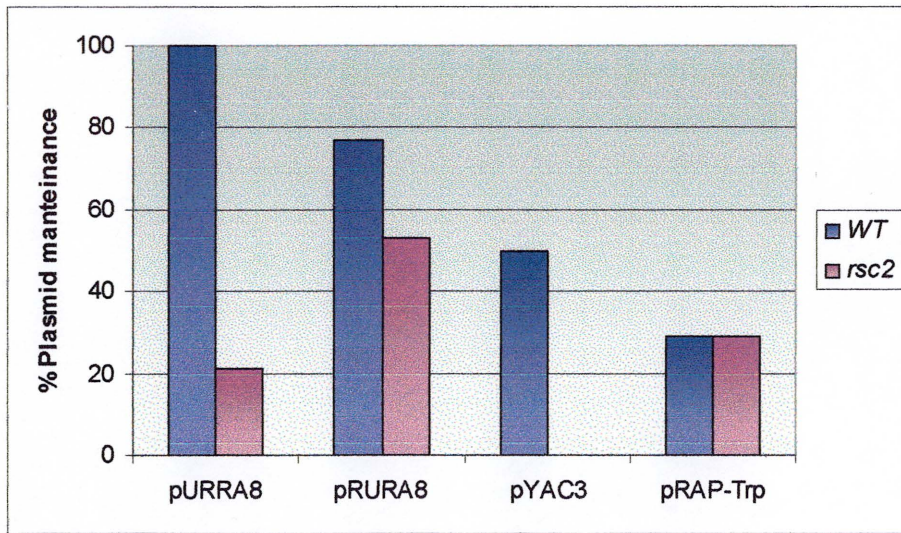
FIGURE 19. Plasmid manteinance. Y0 (*WT*) and *rsc2* mutant carring different plasmid were let grow in not selective YPD liquid medium per 9 hours. Appropiate dilution were plated on SC-Trp and YPD. PUrra8, pRURA8 and pYAC3 are CEN plasmids. PRAP-Trp is a 2µ-based plasmid.

5.3.2 *YLR238w*

From the second round of mutagenesis we chose *YLR238w*. Protein interaction analysis showed interaction with RNA pol II and III subunits (Rpc40p and Rox3p) and Ydr200p (unknown protein that contains a FHA domain).

Protein domain analysis showed the presence of FHA and B-ZIP domains. FHA domain is a phosphopeptide recognition domain found in many regulatory proteins, present also in some DNA repair proteins (Rad53p, Dun1p, and Xrs2p). To determine if Ylr238p is involved in DNA damage repair, we performed epistatic analysis with *ylr238w* null mutant and mutants of key repair proteins of both HR and NHEJ pathways (*rad52* and *hdf1* null mutants). Epistatic analysis was also performed in the double mutant *ylr238w* and it putative partner *ydr200c*. Double mutant *ydr200c/hdf1* was not possible to obtain. Both single and double mutants were analysed by several tests.

5.3.2.1 MMS survival. MMS was added to logarithmic-phase cultures to a final concentration of 0.1%. After 2 hrs incubation at 30°C appropriate dilutions were plated on YPD. The number of viable colonies was compared between treated and untreated cells (Figure 20A). The 88% of *ylr238w* mutant survived while only a 47% of the *ydr200c* null mutant survived after treatment. The 49% survival of double mutant *ylr238w/ydr200c* indicated that there is no synergistic sensitivity of these two genes to MMS.

The *rad52* and *hdf1* null mutants showed 29 and 31% of survival after the treatment. Double mutant *ylr238w/hdf1* showed the same level of WT survival (94%), while

double mutant *ylr238w/rad52* showed intermediate survival if compared with the two single null mutants (72%). Both results suggest a rescue of the MMS sensitivity of the *hdf1* and *rad52* null mutant when the *YLR238w* gene is absent. The double mutant *ydr200c/rad52* showed also a protection effect. This double mutant is much more resistant to MMS than both single mutants (71%).

5.3.2.2 Sensitivity to MMS. Serial dilutions of exponential cells were spotted in YPD plates containing different concentrations of MMS (Figure 20B).

The single mutant *ylr238w* showed slight sensitivity to MMS, *ydr200c* null mutant was slightly more sensitive, and their double mutant was not very sensitive. The single null mutants *hdf1* and *rad52*, showed high and medium sensitivity to MMS respectively (Figure 17A and 20B). Interestingly, the double mutant *ylr238w/rad52* and especially with *ylr238w/hdf1*, showed less sensitivity to MMS if compared with the single *rad52* or *hdf1* single mutants, indicating again a protection effect due to the absence of *YLR238w* gene. The same but less marked effect was observed in double mutant *ydr200c/rad52* (Figure 20B).

5.3.2.3 DSBR *in vivo*. When DSB is induced *in vivo* in pRURA8Δ, the CO repair can be followed by the reconstitution of functional *URA3* gene (Figure 21A). Considering that Y0 has the ability to perform 100% of the CO repair, *ylr238w* null mutant was able to perform only 46%, which represented a big decrease if compared with the 60% or 112% of *rad52* and *ydr200c* mutants, respectively. The double mutant *ylr238/ydr200* showed only 38% of CO ability, while both *rad52* and *hdf1* double
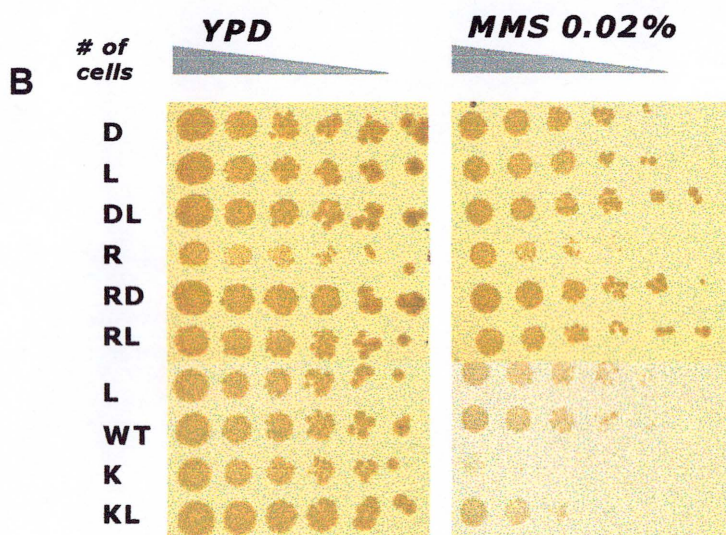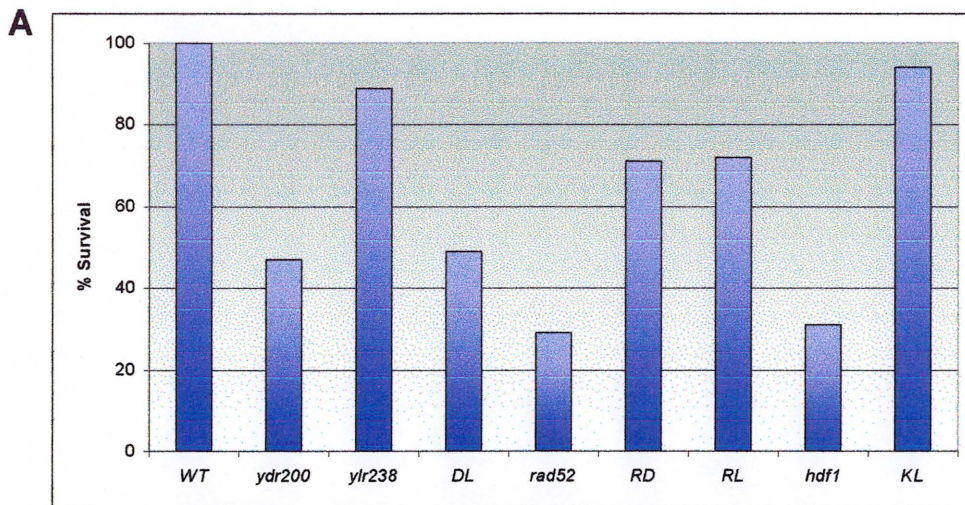
FIGURE 20. MMS sensitivity and survival. A. Cells were incubated in YPD with or without MMS 0.1 % for 2 hours. Appropiate dilutions were plated in YPD. Proportion of cells with/without treatment are shown taking as a 100 % the survaival showed by Y0 (WT) strain. **B**. MMS sensitivity, serial dilutions of cells are spotted in YPD and YPD + MMS 0.02%. WT: Y0 strain, R: *rad52* null mutant, K: *hdf1* null mutant, L: *ylr238w* null mutant, D: *ydr200c* null mutant.

mutants with *ylr238w* showed very low or undetectable growth after galactose-DSB induction. Double mutant *ydr200c/rad52* showed CO events in the same range as *rad52* single mutant. In all the last three double mutants the number

of cells after galactose incubation decreased, indicating that in this background cells are not surviving after the induction of a DSB or that they are not able to grow in media containing galactose as the unique carbon source.

5.3.2.4 Spontaneous pRURA8Δ Inversion. The cells carrying pRURA8Δ plasmid were performing spontaneous CO by inversion and becoming Ura⁺. Taking as a 100% the CO repair events that YO strain is performing, y*lr238w* null mutant performed it only 32.5%, while *ylr200* does it in 110% of the cases. As *ydr200c* null mutant, *ylr238/hdf1* double mutant showed increased effect (Figure 21B).

5.3.2.5 DSBR *in vitro*. Both *ylr238w* and *ydr200c* null mutants showed similar percentage of CO as the WT when transformed with pRURA8Δ-linearized with *I-SceI* (Figure 21C). Double mutant *ylr238w/ydr200c,* showed the same CO frequency as WT strain. Both *rad52* double mutants (*ylr238w or ydr200c*) showed decreased CO events, even lower than the frequency of CO of *rad52* single null mutant. The double mutant *ylr238/hdf1* showed few cells after transformation, precluding a quantification of the CO repair events.

5.3.2.6 DNA damage response. DNA damage results in Mec1-dependent phosphorylation of Rad9p, leading the recruitment of Rad53p to phosphorylated Rad9p through Rad53p FHA domains (SCHWARTZ *et al.* 2002), inducing the phosphorylation of the transducer protein kinase Rad53p at any stage of the cell cycle (PELLICIOLI *et al.* 1999). If the phenotype observed in the double mutants is due
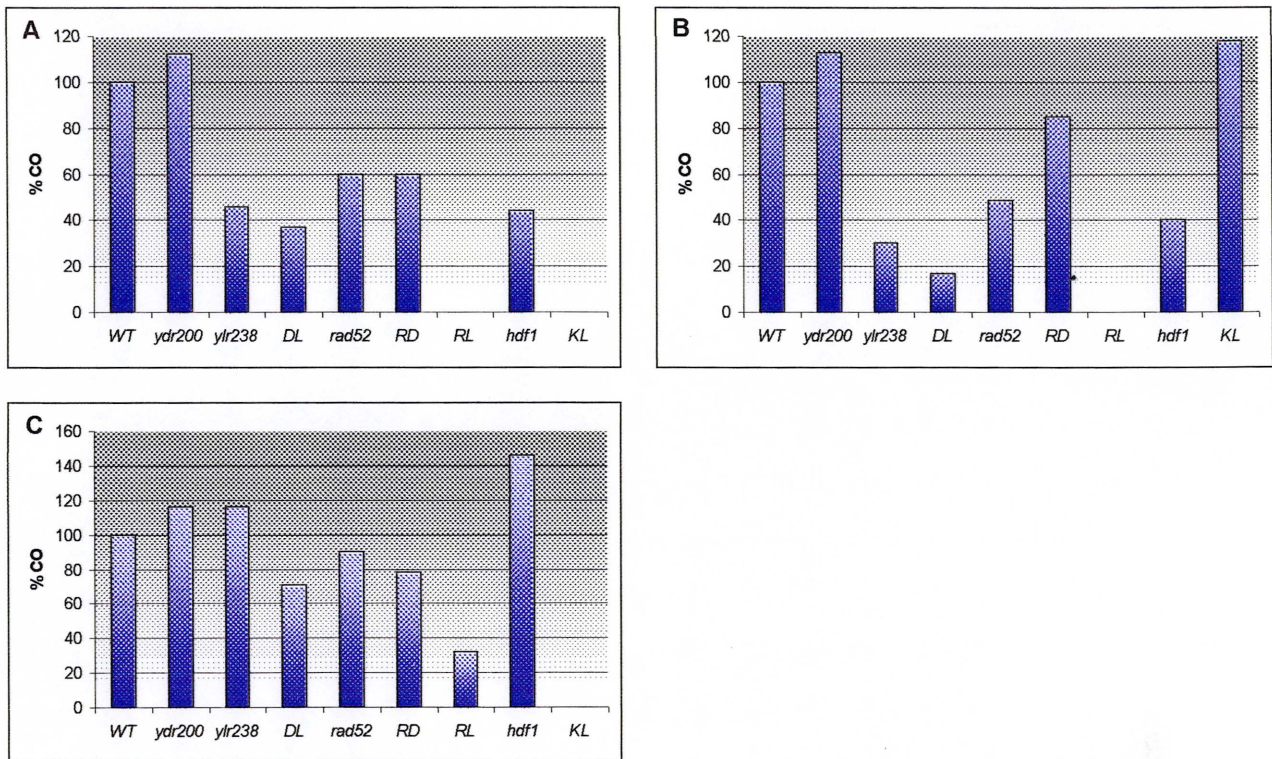
FIGURE 21. DSBR profile of double mutants. **A**. Percentage of repair by CO, when DSB is induced *in vivo* in pRURA8Δ plasmid, taking as 100% the CO performed by Y0 (WT) strain. **B**. Percentage off not induced CO by invertion events in cells carring pRURA8Δ plasmid, taking as 100% the CO performed by Y0 (WT) strain. **C**. Percentage of of repair by CO take from the distribution of repair events of pRURA8Δ-linearized *in vitro* by I-*Sce* I, taking as 100% the CO performed by Y0 (WT) strain. R: *rad52* null mutant, K: *hdf1* null mutant L: *ylr238w* null mutant, D: *ydr200c* null mutant.

to the defects in the DSB detection or in the DSB signaling pathway, the phosphorylation induction of both Rad9p and/or Rad53p can be altered in the null mutants. Using *ylr238w, ydr200c* and their double mutant, we performed western-blot analysis of both Rad9p and Rad53p phosphorylation response upon MMS treatment (Figure 22). From the shift in the protein migration, we can determine that in both single mutants *ylr238w, ydr200c* and in the double mutant, the MMS mediated phosphorylation is normal for both Rad9p (Figure22A) and Rad53p (Figure 22B). From these results we can assume that the signaling and the detection of the of DNA damage is normal in both null mutants as well as in the double mutant.

5.3.2.7 DNA-damage transcription activation. Little is known about the checkpoint effectors operating downstream from Rad53p and controlling the cell cycle arrest, the transcription activation of DNA damage repair genes and DNA repair. Genes known to be transcribed in response to DNA damage include *RAD54, RNR2, RAD51, DUN1, CRT1* and others. Some of them function directly in the repair of the DNA damage (*RAD54, RAD51*), and others function at the regulation of the transcription (*CRT1*). We performed a RT-PCR in the presence and in the absence of DNA damage. The RT-PCR analysis of the transcription activation of *RNR2*, showed that in normal conditions the transcripts were not detectable (Figure 23A lane 1), while upon MMS treatment there is an induction in the transcription (Figure 23A lane 2 and 3). In both null backgrounds *RNR2* was constitutibly expressed and there was no transcription induction after MMS treatment (Figure 23 lanes 4-8).
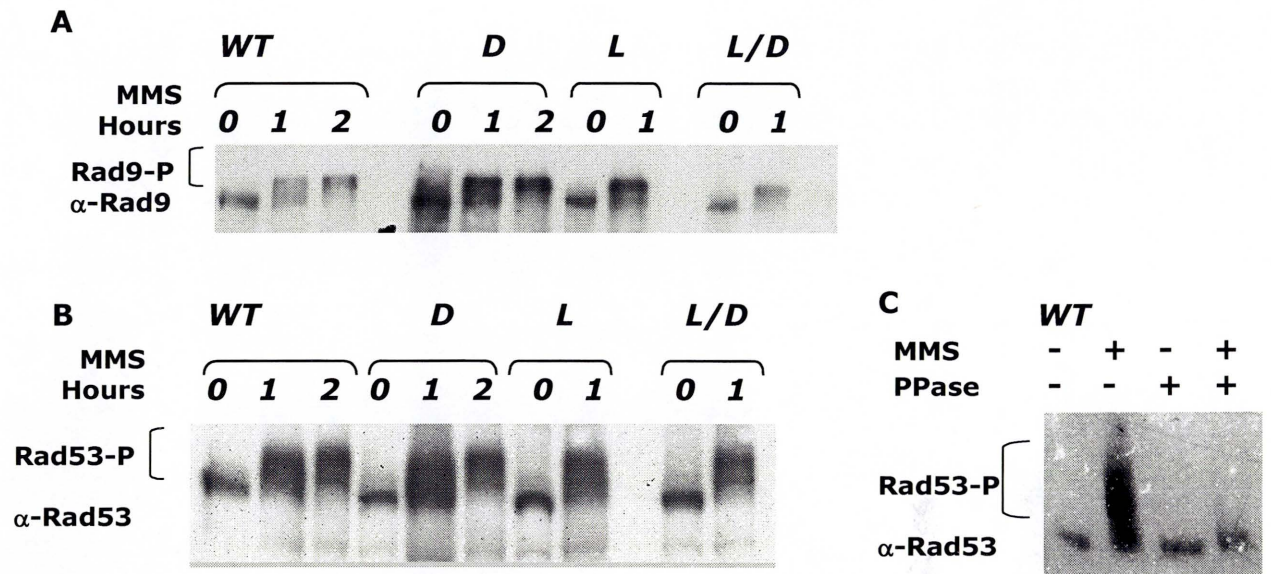
FIGURE 22. DNA damage response. **A** Anti-Rad53 immunoblot analysis. **B**. Anti-Rad9 immunoblot analysis. **C**. Anti-Rad53 immunoblot analysis of protein samples previously treated with calf intestinal phosphatase. Protein extracts in all cases were from Y0, *ydr200c, ylr238w* and *ydr200c/ylr238w* strains. Asynchronous cultures were either mock treated (0) or treated with 0.1% MMS for (1) and in some cases also (2) hours. WT: Y0 strain, L: *ylr238w* null mutant, D: *ydr200c* null mutant.
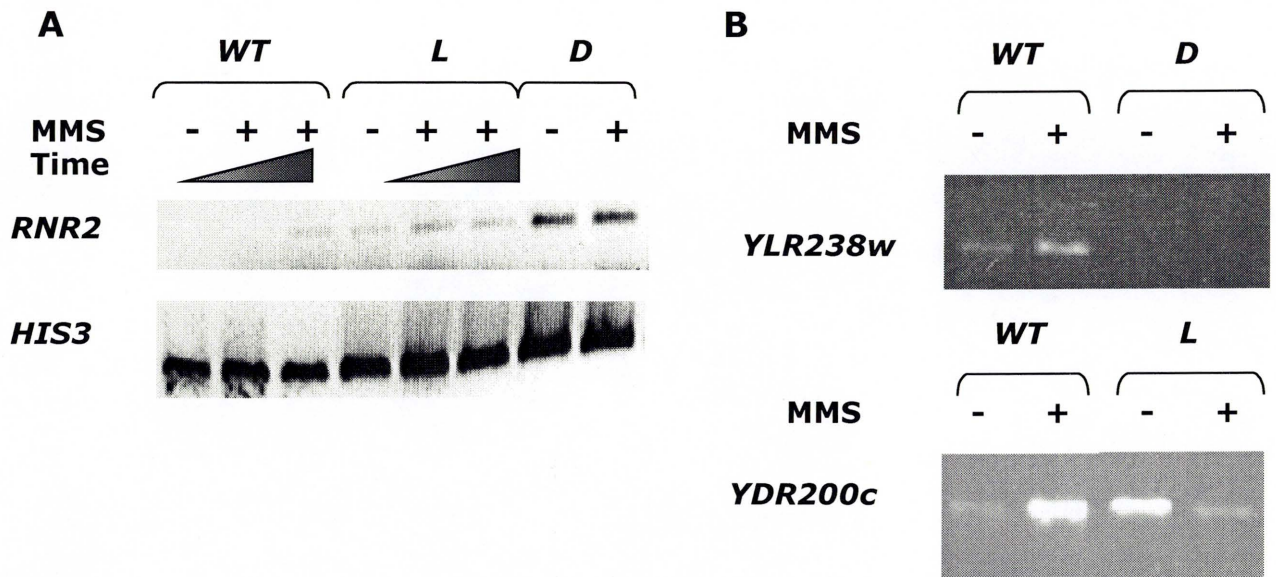
FIGURE 23. Transcription regulation analysis. RT-PCR analysis of wild type (WT), *ylr238w* and *ydr200c* null mutants. **A.** *RNR2* transcripts and **B.** *YLR238w* and *YDR200c* transcripts were analysed in the presence and abscence of DNA damage. WT: Y0 strain, L: *ylr238w* null mutant, D: *ydr200c* null mutant.

Moreover, both genes (*YLR238W* and *YDR200C*) were transcriptional induced upon DNA damage (Figure 23B). This transcriptional control seems to be lost in the absence of the putative partner gene. In this way

YDR200C or *YLR238W* expression in normal conditions in *ylr238w* or *ydr200c* background respectively, showed already a high level of transcription that is not longer induced upon MMS treatment (Data not shown).

From these results we can conclude that the effect of the deletion of *YLR238W* and *YDR200C* is directly involved in the transcription repression-regulation of some DNA damage induced genes (*RNR2, YLR238W* and *YDR200C*).

6. DISCUSSION

6.1. HNS Plasmid System

6.1.1 HNS Plasmid System: Simultaneous Detection of DSB Repair Pathways. Double-stranded DNA ends can be channeled for repair by one of a number of homologous recombination pathways or they can be rejoined via NHEJ with or without further processing. The HNS plasmid system that we have constructed offers a novel way to analyze the dynamic interplay between the DSB repair pathways. The system is composed of two topologically different plasmids containing regions of DNA homology in direct or inverted orientation. In these plasmid constructs a single DSB can be introduced in a region of either homology or heterology. This provides a versatile tool allowing the possibility to study different repair mechanisms: those based on the presence of DNA sequence homology such as CO, GC and (when the break is flanked by directed repeats) SSA, and those in which no sequence homology is required namely, conservative and non-conservative NHEJ. The selection of colonies on SC-Trp medium, after transformation with linearized plasmids, allows the unselected recovery of all repair events, the distribution of which provides a comprehensive overview of the entire DSB repair processes.

We have shown that all the repair pathways known to repair a single linear substrate are detectable. Thus, an overall DSBR profile can be derived by transformation with topologically different plasmids linearized within the region of homology or heterology (Figures 11). Our procedure allows the

interplay between the pathways to be examined. We have also generated plasmids that are highly sensitive at detecting a single pathway, facilitating the study of each individual repair mechanism. For example, the most appropriate plasmid to study CO events is pRURA8Δ linearized with *I-Sce*I (Figures 11C). In contrast, pURRA8Δ linearized with *Bam*HI would be preferred to examine SSA (Figures 11B). The most sensitive studies of NHEJ (conservative and non-conservative) can be carried out by using *Bam*HI-linearized pRURA8Δ. The combination of plasmids and their DSB position and the repair events that can be detected are summarised in Table 7.

6.1.1.1 *rad52* and *hdf1* Mutants Show a Differential Distribution of DSBR Events.

Impairment of one DSB repair mechanism results in the redistribution of repair events among the remaining pathways. For example, in the *rad52* mutant, of all HR pathways (CO, GC, SSA), we mainly observed a decrease of SSA in agreement with previous reports (KARATHANASIS and WILSON 2002; VAN DYCK *et al.* 2001).

It is interesting to note that in all cases, the profile is not extremely different from the wild-type, supporting the idea of the presence of more than one way to repair DNA breaks. Rattray et al. have also shown that the rate of recombination events decreases, but their distribution remains as the WT (RATTRAY *et al.* 2000). In all repair driven by homology, Rad52p is taking part and in some cases there is a partial functional complementation with Rad59p (BAI *et al.* 1999; DAVIS and SYMINGTON 2001). It is evident that SSA is the most affected pathway, perhaps because it is a Rad51p independent event.

In addition, in the *hdf1* mutant, we observed a decrease of total NHEJ repair events in general and, in particular almost a complete absence of NHEJc, as expected (CLIKEMAN *et al.* 2001; VAN DYCK *et al.* 2001).

6.1.2 Repair Pathways Are Subject to Mating-Type Control. It has been shown that *Mat* heterozygosity enhance DSB repair by HR (CLIKEMAN *et al.* 2001). Results from our laboratory are in concordance with this previous report. In diploid strains we were not able to detect NHEJ when HR pathways were available (data not shown). If the break can be repaired only by NHEJ, diploid cells repair them in a non-conservative way. Moreover, in the cases in which more than one HR pathway is available, GC is preferred over SSA and CO (data not shown).

The type of repair depends not only on the ploidy or background status of the cell but also on the nature of the substrate-undergoing repair. This fact is evident in the case of the CO repair pathway.

Haploid Y0 strain performs CO by resolution (pURRA8Δ linearized with *I-Sce*I) independently of Rad52p (2-3% of the repair events), while diploids show dependence on Rad52p and Hdf1p. When diploids perform CO by resolution, they do it 10 times more frequently than haploids. In the case of inversion (pRURA8Δ linearized with *I-Sce*I), haploids perform CO five times more frequently than diploids (wild type and *rad52*). The backup mechanism dependent on *Mat* heterozygosity that repairs these kind of breaks by BIR-SSA has been proposed (KANG and SYMINGTON 2000; MALAGON and AGUILERA 2001; SYMINGTON 2002).

Differences in the repair of the two plasmids linearized at the same site (*I-SceI*) could be explained by the differences that these two substrates have at the structural level. It may be caused by resolution vs. inversion process performed to obtain CO products, or perhaps because of the differential stability of the intermediates, or perhaps simply because they are two different processes. We have seen that these two plasmids show a differential stability in Y0 strain, even if the only difference between them is the orientation of the URR fragment (Figure 19).

Very recently Marcand and collaborators showed that breaks induced *in vitro* are repaired by NHEJ less frequently than breaks produced *in vivo*. The instability of the end will make DNA ends more prone to be repaired by HR processes (FRANK-VAILLANT and MARCAND 2002). It is possible then, that in the case in which NHEJ was not detected, the strain degraded 3' single strand ends more efficiently, hence explaining why it was not possible to detect NHEJ events in this cellular background.

6.1.3 *NEJ1* Null Mutant. In diploid cells, DSB repair uses mainly the error-free HR pathway, as NHEJ is down regulated throughout the cell cycle. Nej1p has been proposed to be a ploidy-dependent regulator of NHEJ (VALENCIA et al. 2001). Strikingly, in our study, the haploid *nej1* mutant also shows impairment in NHEJ similarly to that observed in a diploid strain or a haploid *hdf1* mutant (Figure 13 and 14, B and D). When CO events were analyzed, we noticed that *nej1* has a behavior similar to the *hdf1* haploid when the pURRA8Δ was used (Figure 14A), but it showed a different behavior with pRURA8Δ (Figure 14C).

Moreover, when DSBs are induced *in vivo* in the inverted repeat substrate, the *nej1* mutant shows 4 times lower rates of CO in comparison with WT, and around two times lower rates of CO than the *hdf1* null mutant (Figure 21A). It is important to notice that in all cases analyzed, CO events are performed less frequently than in WT strain. This fact can be partially explain by the impairment of NHEJ, leaving only the CO and GC pathways available, and that the *nej1* null mutant in particular is more efficient in repairing DNA breaks by GC than by CO. This suggests that this protein may be involved in more than one aspect of DSB repair regulation, including topology-dependent CO.

More work has to be done on this aspect but it is obvious that NHEJ regulation is not the only role of Nej1p. The study of the protein-protein interaction of Nej1p after DNA damage in haploid and diploid cells, and its expression through the cell cycle could be an interesting subject to study in order to understand its role in DNA damage repair. These results show that Nej1p has another role in regulation that is still unknown.

The above considerations show that even the genes like *NEJ1*, that are not directly involved in the repair processes themselves can be successfully studied with the HNS system, and subtle differences in the repair profile can be detected.

6.1.4 MRX Complex. MRX complex genes are required for telomere maintenance, cell cycle checkpoint signaling, meiotic recombination and efficient repair of DSBs by HR and NHEJ (JACKSON 2002). Recent studies support the hypothesis that the primary function of the Mre11-Rad50 complex is structural and serves to bridge

sister chromatids and/or DNA ends. The nuclease activity of Mre11p is required to remove covalent adducts from DNA ends and to process unusual DNA structures, but it may not play a significant role in resection of ends to produce long 3' single-stranded tails. Xrs2p/Nbs1p is restricted to eukaryotes and may function to recruit other factors for specialized functions within the cell, such as the Dnl4p-Lif1p complex for end joining. Given that the trio of Rad50p, Mre11p, and Xrs2p are involved in both homologous recombination and non homologous end-joining, it is interesting to understand whether they are executing the cellular command to conduct repair process either via recombination through HR or NHEJ.

In an attempt to answer to this question we did a DSBR profiling of these null mutants using HNS system. Complete abolishment of NHEJ in all three mutants was even more drastic than in the *hdf1* null mutant (Figure 14D). It is possible that somehow the ends are i) not protected from resection, or there is a loss of resection regulation, or ii) the efficiency of the conservative NHEJ is affected because the other components of the NHEJ machinery are not localized to the site of the DNA damage (remember the interaction between Xrs2p and Dnl4p), leaving the break to be repaired by the HR pathways when possible.

For all three mutants a hyper-recombination phenotype was described (FREEDMAN and JINKS-ROBERTSON 2002). In our case, the GC and SSA pathways seems to increase, and *xrs2* null mutant repaires *in vivo* introduced DSBs in a hyper-recombination manner (Data not shown).

Different DSBR profile was obtained in all three cases when the null mutants were transformed with plasmids in which the DSB was introduce in the homology region

(Figure 14 A and C). The *mre11* null mutant shows a particular repair event when transformed with *I-Sce*I -linearized pURRA8. More than 30 % of the transformants were white Ura⁻ colonies (a phenotype we did not predict). This phenotype could have arise from a large resection, that could have extended within the *ADE8* gene, or from the failiture in finding the microhomology giving rise to either inefficient SSA, or increased non-conservative NHEJ.

The DSBR profile of the *xrs2* null mutant is very similar to that of *hdf1* null mutant. The interaction between Xrs2p and Lig4p (CHEN *et al.* 2001), could be the way in which MRX and NHEJ protein complexes interacts, and channel the DSB to be repaired by NHEJ. This hypothesis is supported by the fact that the first step in the DNA damage signaling pathway is the phosphorylation induction of both Xrs2p and Mre11p in Tel1p dependent manner (D'AMOURS and JACKSON 2001). The presence of an FHA domain in Xrs2p makes this protein the sensor for some other phosphoprotein (Mre11p, H2Ap, others ?), and at the same time it is able to be phosphorylated in order to continue with the DNA damage signaling pathway. How the phosphorylation affects the Xrs2p-Dnl4p interaction and what proteins are recognized and are recognizing phosphorylated Xrs2p, are still unsolved questions. All three components of this complex are required for Rad53p activation, indicating their key role in the DSB detection and signaling (GRENON *et al.* 2001).

To understand the temporary order of events and the regulatory role of this protein complex, epistatic studies with mutants of various sensing and repair pathways can be used.

6.1.5 MSH2 Null Mutant. The mismatch repair proteins, Msh2p and Msh3p, are required to remove nonhomologous DNA ends during both the initiation of GC and the resolution of SSA, only when the ends are not perfectly matched to their donor template. In SSA, Msh2p and Msh3p become less important as the length of the flanking region increases. In addition, they act to facilitate Rad1/Rad10 dependent removal of nonhomologous DNA (SUGAWARA *et al.* 1997). On the other hand, the Msh2p-Msh6p complex functions in the recognition of recombination intermediates and may also have roles in their resolution (MARSISCHKY *et al.* 1999).

In our DSBR profiling, the *msh2* mutant does not perform CO in the direct repeat substrate, but increases the CO frequency when the substrate is an inverted repeat plasmid. This maybe due to the different stabilities of the intermediates, or to differences in the mechanisms of repair (MALAGON and AGUILERA 2001).

In contrast to *in vitro* observations, *in vivo* studies reveal that CO induction in inverted repeat substrate is less efficient in *msh2* null mutant than in the WT strain. This is consistent with the idea that this inversion-CO process is actually BIR-SSA process that is dependent on Msh2p (KANG and SYMINGTON 2000; MALAGON and AGUILERA 2001).

All of the null mutants of the known repair genes tested by HNS system show a specific DSBR profile. Molecular analysis of the final repair products will give more information on the repair at the molecular level in every single situation.

6.1.6 DSB Repair Regulation. The regulation of the DSB repair process is still not well understood. Previous models admit that more than one pathway can repair a single DSB. The competition and interference model propose that the repair pathway chosen is determined by which protein arrives first at the site of the break. Thus, the NHEJ pathway will prevail when Ku binds DSB first, while HR will be preferred when Rad52p acts instead. In other models, the ploidy and the mating-type of the cell have priority in the decision of the DSB repair pathway to be used (CLIKEMAN *et al.* 2001; LEE *et al.* 1999). The competition can be governed by the kind of DNA break, the stage of the cell cycle and the presence of DNA sequence homology (TAKATA *et al.* 1998).

More recently, a new view of the process proposes cooperation of different repair pathways rather than a passive competition (KARATHANASIS and WILSON 2002; WILSON 2002). Yet another view of the regulation of the process takes into account the stability of the DNA ends (FRANK-VAILLANT and MARCAND 2002). In this model, the repair pathways are ordered by the initial timing of DNA processing. When cohesive ends are intact NHEJ precedes HR, whilst HR functions when the break has been left unrepaired by NHEJ. Recently, a new molecular system has been described to follow NHEJ and SSA (KARATHANASIS and WILSON 2002). Moreover, Wilson has reported a genomic-based screen for mutants altered in the ratio of SSA/end-joining (WILSON 2002).

6.1.7 Conclusion I. In our work, we demonstrated that all possible repair processes could occur at the same time in a balance between pathways. In this

model, it is conceivable that the co-ordination of all the pathways is subjected to a fine regulation that allows competition, so that the most favored one for error-free repair is able to perform most of the repair events. However, when this pathway is impaired, other competitive mechanisms take over in a hierarchic order. This hierarchy is determined by the following factors affecting the preference of pathways utilized, in particular:

i)   the genetic background - especially in cells defective in a second component of the repair pathways;

ii)   the influence of mating type control and ploidy;

iii)   the nature of the DSB substrate, including the presence of homology, the protection vs. degradation of the DNA free ends, and the topology of the homologous sequences.

Our HNS system is able to derive the overall profile of utilization of all the DSB repair pathways (CO, GC, SSA and NHEJ). In addition, it can be used to monitor a specific DSB repair process. Thus, the HNS system is a useful tool to test new genes involved in all DSB repair pathways as well as genes involved in their regulation, and to define their involvement in the entire process. Since the DSB is introduced (*in vivo* and *in vitro*), in a well-defined region, it makes it easy to study the accuracy of the repair at the DNA sequence level, a feature especially important to define NHEJ products.

6.2 Transposon Mutagenesis.

To date, there are still some unidentified DSBR proteins, and, as discussed in the introduction, several methodologies are currently being applied to look for new recombination-repair genes. In some cases (OOI *et al.* 2001), the screen was direct to detect mutants affected in a single DSB repair pathway. Some of the already known NHEJ proteins, but also a NHEJ regulatory protein (Nej1p) were found in this case. In other cases a more general approach, such as screening for mutants sensitive to certain DNA damaging agents (MMS, γirradiation, UV irradiation) was used (BIRRELL *et al.* 2001; CHANG *et al.* 2002; GASCH *et al.* 2001). In these approaches the initial challenge was not directed to a single DSBR mechanism, but to all the broad mechanisms that allow the cell to survive upon treatment by damaging agents.

Recently Wilson searched for mutants with altered ratios between two DNA repair pathways (SSA/NHEJ). He was able to detect not only genes involved in both of the pathways, but also some that seem to be involved in a regulatory step, more precisely in the decision between the repair pathway that is going to be used (WILSON 2002).

Our approach was based on *in vivo* induction of a single and localized DSB break, and on the possibility to detect mutants that have lost the ability to repair it.

6.2.1 Transposon Mutagenesis I

The first mutagenesis we performed was in the WT cell background, and using the pURRA plasmid as a tester.

Almost all the null mutants of the selected genes are sensitive to MMS, giving an indication of their general role in DNA damage response. Analyzing the 22 selected mutants, we were not able to detect any already known REC gene. This fact was not encouraging, but we can explain it in several ways:

i)   Redundant pathways. As discussed in the section (6.1.1), the *I-Sce* I DSB introduced in this plasmid can be repaired by 4 different pathways, with SSA and GC being the most favored (by *in vitro* DSBR analysis). When one repair pathway is impaired (by a mutation in a key gene like *RAD52*), some other pathway can perform the repair. Studies in which HO endonuclease is used to create a DSB between repeats support this idea. Ivanov et al demonstrate that SSA occurs efficiently and with normal kinetics in *rad51, rad54, rad55,* and *rad57* mutants (IVANOV *et al.* 1996).

ii)   Redundant protein function. As shown by some previous studies, the redundancy of certain functions in the cell can mask a specific gene deficiency. Some null mutations show no striking decreases of recombination, even if only one repair pathway is available. An example of this fact is seen in *rad52* background, where SSA can still be performed by Rad59p (BAI *et al.* 1999; DAVIS and SYMINGTON 2001).

iii) Lethality. If the gene disrupted by the transposon is essential, or important for the normal cell growth ratio, we would not be able to see them using this transposon mutagenesis and screening system.

The genes we identified as responsible for the transposon mutant phenotypes are more likely to be involved in some initial step of the DSBR process, i.e. like detection and signaling, much more than with the DSB repair itself. Chromatin structure modification and transcription are known to influence the DSB repair efficiency (see section 3.5 and 3.6). In this screen we detected genes involved in transcription regulation, chromatin structure, maintenance and DNA accessibility (*SIN4*, *RSC2*, *SWR1* and *MED1*).

Some other genes are part of the nuclear transport-import machinery (*NUP188* and *YRB2*) that may be involved in the DSBR regulation based on the delocalization of the proteins from the site of action, in the same way in which Nej1p is regulating NHEJ (VALENCIA *et al.* 2001).

The selection of some metabolic genes can be explained by the deficiencies in growth rates under the restricted conditions we used. An interesting gene that was found twice is *YLR089* (putative alanine amino transferase), which shows a strong decrease in CO and SSA in our DSBR profile.

From this group of genes *SWR1* and *RSC2,* showed the most interesting phenotypes. The *swr1* null mutant shows a larger defect in CO when repairing inverted repeat substrate, a particular decrease of non-conservative NHEJ and a high sensitivity to MMS. This behavior can be due to the resection deficiency, since Swr1p can acts as the anchor or regulator of Rad1p/10p nuclease. The Rad1/10 heterodimer is a structure-specific nuclease that cleaves 3' tails from branched intermediates. A deficiency on Rad1/10 function could explain the *swr1* phenotype.

To date the specific role of Swr1p in recombination repair has not been studied, but our result suggests a role in DNA damage response.

As a summary of the first mutagenesis we conclude that most of the genes selected seem to be involved somehow in the initial steps of DNA break repair or in the regulation of the repair.

6.2.2 Transposon Mutagenesis II

Taking into consideration that in the first transposon mutagenesis the targeted genes were not the REC genes (as discussed in section 6.2.1), in this case we wanted to restrict the screening. For this reason this mutagenesis was performed in WT cell background Y0, and *hdf1*-Y0 null mutant, using the pRURA8 plasmid as a tester. As discussed in the results (5.1.1.1), the DSB induced in this plasmid can be repaired by CO (inversion), GC or NHEJ. In the *hdf1*-Y0 mutant background the NHEJ pathway is impaired, the pathways remaining to repair the break are GC and CO. Using this conditions, we wanted to target the genes involved in the HR (CO or GC) pathways.

The *RAD50* gene was detected as one of the 13 selected mutants listed in Table 9. This fact was encouraging because of the central role of Rad50p in all the repair pathways, and its putative role in the channeling of the type of repair that will take place.

We detect 3 kinases that may be involved in some signaling-regulation pathway. A good example is Swe1p kinase. The Xenopus and fission yeast homolog (wee1 and Xwee1) are known to be involved in G2/M transition in response to DNA damage

(MICHAEL and NEWPORT 1998; RHIND and RUSSELL 2001), but in *Sc* they seems to be involved only in morphogenesis checkpoint activation (SIA *et al.* 1998). Our results suggest the involvement in repair processes, because the *swe1* null mutant shows an increase in CO events, and moreover it is sensitive to MMS. Supporting this idea, some reports show its interaction with Sgs1p, an ATP-dependent helicase involved in chromosomal stability and processing of recombination intermediates as well as in the prevention of recombination repair during chromosomal DNA replication (AJIMA *et al.* 2002).

An other interesting mutant is *DAN1*. This gene encodes a cell wall manoprotein that does not seem to have any logical involvement in DNA damage response, but the transposon mutant shows high sensitivity to MMS and low level of CO and SSA. Additional studies should be performed, to establish the role of these proteins in DNA damage response.

In the screen we managed to select the *RRN9* gene that is supposed to be essential as it codifies for a RNA polymerase transcription factor. As we have already discussed, transcription is known to be linked with recombination repair processes (AGUILERA 2002).

6.3 Selected Genes Characterization

6.3.1 Rsc2p: RSC Complex Component. In eukaryotes, the wrapping of DNA around histone octamers to form nucleosomes reduces the accesibility of DNA binding factors and/or advancing polymerases, resulting in inhibition of transcription, replication, repair and recombination. The conservation of nucleosome-remodeling

enzymes from yeast to humans indicates the evolutionary importance of their activity. The yeast Snf/Swi and RSC are subsets of these enzymes. Snf/Swi is not essential for viability, whereas loss of RSC is lethal. In addition, RSC is required for cell cycle progression through mitosis, whereas Snf/Swi is not (NG *et al.* 2002). While the involvement of Snf/Swi in transcriptional control has been extensively studied both genetically and biochemical, the precise role of RSC in transcription or in other chromatin-related processes *in-vivo* has not yet been established (NG *et al.* 2002).

RSC (Remodels the structure of Chromatin) is an abundant 15 protein complex that uses ATP hydrolysis to reposition nucleosomes. RSC has been isolated in distinct forms, containing either Rsc1p or Rsc2p, with or without Rsc3/Rsc30. The Rsc1p and Rsc2p isoforms associate with the same genes, RSC is generally targeted to Pol III promoters, and is specially recruited to specific Pol II promoters in response to transcriptional activation or repression (NG *et al.* 2002).

Moreover, the RSC component Sfh1p is phosphorylated specifically during G1 (CAO *et al.* 1997). These results suggest that chromatin remodeling by RSC is regulated at compositional and posttranscriptional levels. In fact, Rsc9p revealed genome-wide re-localization occupancy after stress (DAMELIN *et al.* 2002).

Koyama and collaborators showed that the bromo-domain of an other RSC component, Nps1p plays an important role in the maintenance of the integrity of RSC. They proposed that the reduced interaction between Nps1p and Sfh1p results in the release of Rsc2p from the complex and the deterioration of functional RSC causes enhanced sensitivity to DNA-damaging agents (KOYAMA *et al.* 2002).

Abundant RSC could facilitate the rapid response of the cell to overcome DNA damage by transcriptional activation or repression, and/or by assisting the access of repair enzymes to damage site (KOYAMA *et al.* 2002).

Our results show that in *rsc2* null mutants SSA and CO repair events are impaired, and the MMS sensitivity observed was the highest. These evidences suggest a role of Rsc2p in DNA damage response pathways, that was also very recently proposed by Koyama et al (KOYAMA *et al.* 2002). The GRID analysis shows a interesting interaction between Rsc2p and Crt1p-a transcription factor associated with DNA repair response. It could be interesting to study if this interaction changes upon DNA damage, affecting the Crt1p-DNA interaction. It would also be interesting to study how the Rsc2p protein-protein interactions and chromatin structure change in response to DNA damage.

Other studies propose a role of RSC in 2μm plasmid stability. In yeast, the stable maintenance of the 2μm multicopy circle plasmid depends on its ability to overcome intrinsic maternal inheritance bias (MIB). The 2 μm plasmid encodes for 4 proteins Flp, Rep1p, Rep2p and Rfap. To overcome MIB, 2μ plasmid requires functions of both Rep1p and Rep2p, as well as the presence of STB locus in cis. There is evidence that specific chromatin structures are important in 2μm functions, since the STB region has been reported to be relatively free of nucleosomes, and changes occur in nucleosome positioning in the presence of 2μm gene products. Loss of *RSC2* function results in a failure to overcome MIB effectively, and hence cells are defective in 2μm plasmid maintenance. This plasmid instability correlates with

significant changes in the chromatin structure at the *STB* locus and a loss of the normal localization of the Rep proteins (WONG *et al.* 2002).

The structure of 2µm-derived plasmid constructs has long been suspected to be important in influencing their stability. For example, (FUTCHER and COX 1983) found that plasmids that carry very similar regions of the 2µm genome, but in different DNA contexts, can differ substantially in stability.

On the other hand, our plasmid loss studies showed, in fact, that the *rsc2* null mutant has a great defect in plasmid maintenance that seems to be associated with the chromatin structure or accessibility of particular regions (direct repeats) more than with the kind of plasmid (2µ or CEN based). Further studies should be done, to determine if the chromatin structure in the different plasmids is affected by the absence of Rsc2p and if this affects their stability. Does this chromatin structure correlate with the possibility to have some secondary structure, and/or recombination intermediate structures that are not properly processed or maintained is still to be revealed. To address this functional characterization, it could be useful to address the following points:

Is Rsc2p regulated in response to DNA damage? does Rsc2p reveal genome re-localization after DNA damage?, does Rsc2p reveal any posttranscriptional modification (phosphorylation or acetylation)? Are those modifications changing the integrity of the RSC complex?

Does Rsc2p regulate transcription of specific DNA repair genes (*RNR* genes, *CRT1*, or *RAD54*)? There is some physiological meaning to the Rsc2p-Crt1p interaction? Is this interaction stable or can it be induced?

Is the RSC complex DNA binding specific to 'particular' chromatin regions or structures, such as centromeres, secondary structure adducts (repeats), DNA break regions, or to specific chromatin modifications, such as histone acetylation and/or phosphorylation? Is there a meaning for the lost of plasmid stability due to the presence of direct repeats in the *rsc2* mutant cellular background?

6.3.2 *YLR238w.* The *ylr238w* null mutant DSBR profile shows increased levels of CO (by inversion) when DSB are induced *in vitro*, and low sensitivity to MMS. Repair of the breaks introduced *in vivo* (Figure 21A), strongly reduces CO repair events. It is interesting to note that these two tests give opposite results for the same strain. In almost all the other knockout backgrounds (*rad52, mre11, xrs2, nej1, msh2* but *ydr200c* and *hdf1*) the general repair distribution is the same both *in vivo* and *in vitro*. We observed that impairment in CO repair events is more stressed if the DSB is induced *in vivo*. This is obvious in *rad52* null mutant, in which the decrease of CO events is stronger when the DSB is induced *in vivo*. As discussed in the system validation section (5.1.2), these differences may be due to the differences in the stability of the substrate DNA given to the cell to be repaired (FRANK-VAILLANT and MARCAND 2001). When transformed with linear DNA, ends are less protected and the chromatin structure may not be the same as that present *in vivo*. From this initial general analysis, the Ylr238p role in DNA damage response is not clear. Epistatic mutations were performed in order to understand the Ylr238p function. Two key repair genes (*RAD52* and *HDF1*) were deleted in *ylr238w* background as well as the gene of its putative partner Ydr200p.

ylr238w null mutant shows mild decrease in MMS survival and sensitivity. The *rad52* and *hdf1* single mutants are more sensitive to MMS. Interestingly double mutants with *ylr238w* (RL and KL) are less sensitive to MMS, suggesting a "protective effect" due to the absence of Ylr238p (shown also from the plates on figure 20B). From these results we conclude that Ylr238p is acting:

I   At the initial level of DSB detection and/or repair induction (no DSB detection no cell cycle arrest)

II   General damage response (induction/repression of some DNA damage genes involved in both HR and NHEJ repair pathways). De-repression of some repair genes, leaving the cell permanently on alert status.

III   Checkpoint regulation, in a way that even in conditions in which the cell cycle should arrest (upon MMS damage), cell losses checkpoint control and continue to grow when repair mechanisms are not proficient as they should be.

On the other hand the *ydr200* null mutant shows stronger lethality than *ylr238* under MMS treatment. The double mutant *ydr200/rad52* shows also an increased survival in comparison with both single mutants. The double mutant *ylr238/ydr200* showed an epistatic effect in MMS treatment. The double mutant *ydr200/hdf1* could not be obtained by direct knockout techniques in haploid cells as all the other double mutants, perhaps indicating synthetic lethality. Knockout experiments in the diploid *hdf1/hdf1* strain followed by sporulation analysis could confirm this hypothesis.

DSBs induced *in vivo* are less frequently repaired by CO by inversion in *ylr238w* null mutant than in WT strain. Double mutants *ylr238w/rad52* and *ylr238w/hdf1* are not able to grow after DSB induction; it seems that the break cannot be repaired and after several cellular divisions the plasmid is lost inducing cell death in SC-Trp selective conditions.

Interestingly, in both *in vivo* and *in vitro* experiments, *ydr200c* shows a increased CO frequency of events. While double mutant *ydr200c/rad52,* shows the same level of CO as *rad52* single mutant. Protein sequence analysis, and protein-protein interaction analysis provide some important information that may be useful to explain our results and propose some function-role to Ylr238p.

Ylr238p   FHA domain, B-ZIP domain

Interaction with: Ydr200p, Rox3p (transcription from pol II promoter) and Rpc40p (transcription from pol I, and III promoters)

Ydr200p   FHA domain, involucrin repeats

Interaction with Ylr238p, Far3p (cell cycle arrest) and membrane proteins.

FHA (forkhead-associated) domain is a small protein module (65-100 aa) recently shown to recognize phosphotreonine epitopes on proteins. It is present in a diverse range of proteins in eukaryotic cells, such a kinases, phosphatases, kinesins, transcription factors, RNA-binding proteins, and metabolic enzymes, and is also found in bacterial proteins. This suggests that FHA domain-mediated phospho-

dependent assembly of protein complexes involved in intracellular signaling and regulatory mechanisms (DUROCHER and JACKSON 2002). B-ZIP (Basic-leucine zipper transcription factor) domain is a basic region that mediate sequence-specific DNA-binding followed by a leucine zipper region required for dimerization (FUJII *et al.* 2000; HURST 1995). Some interesting questions are raised:

- Are these proteins "responding" to DNA damage?

    By transcriptional induction, post transcriptional modification, or cell relocalization.

- Does Ylr238p bind DNA? Is this interaction sequence-specific or is an inducible interaction?

- Are there protein-protein interactions patterns changing upon DNA damage?

- Are they recognizing some specific phospho-proteins that get activated after DNA damage?

Our results suggest a dual role of Ylr238p in HR and NHEJ repair pathways, raising the possibility that it could be a mediator protein involved in transducing the DNA damage signal (STEWART *et al.* 2003).

6.3.2.1 DSB recognition and signalling pathway. The normal phosphorylation pattern induced after MMS treatment of Rad9p and Rad53p suggest that the DSB recognition, and signaling pathway activation is performed properly in both single and double null mutants.

6.3.2.2 DNA damage effectors. Signaling pathways in response to DNA DSB involve molecular cascades of sensors, transducers and effectors proteins that activate cell cycle checkpoint and recruit repair machinery proteins. DNA damage checkpoints regulate a number of physiological responses after DNA damage. The transcriptional level of many genes is specifically induced. The DNA damage-induced transcription of *RAD54* and *RNR2* (ribonucleotide reductase gene) are regulated in different way. *RNR2* transcription is controlled by Rad9p, Ddc1p, Dun1p, Crt1p and Mbp1p, but *RAD54* DNA damage induction must depend on other factors (BASHKIROV *et al.* 2003; WALSH *et al.* 2002; ZHU and XIAO 2001). From our RT-PCR analysis, we were able to determine that the transcription of both *YDR200C* and *YLR238W* genes are induced after MMS treatment. Moreover DNA damage specific gene (*RNR2*) is miss-regulated in *ylr238w* and *ydr200c* background. It could be very informative to analyze the general transcription activation or miss-regulation in the *ylr238w* background, in this way we could be certain of it involvement in the transcription regulation of other genes.

6.3.3 Ylr238p role in DNA Damage Repair. From *in vivo* DSB induction experiments we postulate a role for Ylr238p in DNA damage response. Moreover, the MMS protection effect showed in *ylr238w/rad52* and *ylr238w/hdf1* double mutants could indicate an indirect involvement of Ylr238p in DSB repair that affects both HR and NHEJ. We proposed a role as a mediator protein involved in transducing the DNA damage signal at the transcription repression-regulation level.

7. CONCLUSIONS

7.1 HNS Plasmid System

- The HNS molecular plasmid system has been shown to be a tool that allows the study of all the repair pathways at the same time or separately, as preferred, and allows performing the DSBR profiling of different genetic background strains *in vivo* and *in vitro*.

- DSBR profile depends on mating-type and *Mat* heterozygosity, as well as the genetic background of the strain and the site of the DNA break.

- CO process by plasmid inversion seems to be a different process than CO by plasmid resolution, as different regulation and enzymatic activities are involved. It will be interesting to determine the major components that define and take part on each repair event.

- Using the HNS system, we confirmed that the role of Nej1p in NHEJ is regulatory, moreover we had evidence for a second role in CO regulation (inversion vs. resolution). Epistatic studies, induced protein interaction in DNA damage conditions, as well as cellular localization after DNA damage could give some indication of its function at this novel regulation level.

- All MRX complex single mutants are as defective in NHEJ repair as *hdf1* null mutant. The distribution of the HR repair events is differently affected; showing an increase in SSA based repair process.

Mre11p defective strain shows particular repair events, that were not predicted to happen and that have to be further characterized. The molecular characterization of these repair products could shed light into the Mre11p role in the DSB repair process.

- The *msh2* null mutant showed a decrease in CO (by resolution) and increase of CO (by inversion), supporting the idea that these two processes are different and independently regulated.

7.2 Transposon Mutagenesis

- From the first transposon mutagenesis we selected mutants that were not directly involved in any repair pathway in particular but in some initial step of the DNA damage response. The second transposon mutagenesis was performed under conditions in which more restricted repair events were possible (CO or GC). In this case we were able to detect the central repair gene *RAD50*.

- A different approach to detect NHEJ repair genes can be performed using the same transposon mutagenesis strategy but using cells carring a plasmid with the inducible DSB site in the region of non-homology.

7.3 Selected Genes: Preliminary Characterization.

7.3.1 Rsc2p Role in DNA Damage Repair. Both the enhanced sensitivity to MMS and the lower frequency of CO events in the *rsc2* null mutant suggest that Rsc2p has a role in chromatin related processes such as DNA repair. Its precise role has not yet been established.

7.3.2 Ylr238p Role in DNA Damage Repair

From *in vivo* DSB induction experiments we postulate a role for Ylr238p in DNA damage response. Moreover, the MMS protection effect showed in *ylr238w/rad52* and *ylr238w/hdf1* double mutants could indicate an indirect involvement of Ylr238p in DSB repair that affects both HR and NHEJ. When DSBs are induced *in vivo* the *ylr238w/ydr200c* double mutant shows that there is synergism for the MMS survival. Interestingly, the CO events of both single mutants showed the opposite behavior when compared to the WT strain; *ydr200c* increases while *ylr238w* decreases CO. The results presented allow us to proposed a role for Ylr238w as a mediator protein involved in transducing the DNA damage signal at the transcription repression-regulation level.

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136

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